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Ocena wpływu lamiwudyny i zydowudyny oraz kwasu palmitynowego na komórki mikrogleju i neuronów dopaminergicznych jako potencjalnych czynników neurodegeneracyjnych

Evaluation of the effects of lamivudine and zidovudine and palmitic acid on microglia cells and dopaminergic neurons as potential inducers of neurodegeneration

Rozprawa doktorska na stopień doktora
w dziedzinie nauk medycznych i nauk o zdrowiu
w dyscyplinie nauki farmaceutyczne

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Serdecznie dziękuję mojej Pani Promotor prof. dr hab. Agnieszce Piwowar
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Spis treści

1. Wykaz publikacji wchodzących w skład cyklu stanowiącego rozprawę doktorską

Na monotematyczny cykl publikacji, stanowiący podstawę ubiegania się o nadanie stopnia doktora nauk farmaceutycznych, składają się w sumie trzy artykuły: dwie prace oryginalne oraz jeden artykuł przeglądowy. Wyniki uzyskane na podstawie badań własnych zawarte są w dwóch pracach oryginalnych (oznaczone jako nr 2 i nr 3). Artykuły opublikowane zostały w czasopismach naukowych z listy A, o zasięgu międzynarodowym, posiadające współczynnik IF, są to:.

1. **Lipke K**, Kubis-Kubiak A, Piwowar A. Molecular mechanism of lipotoxicity as an interesting aspect in the development of pathological states - current view of knowledge. *Cells*. **2022**, vol. 11, nr 5, s. art.844 [34 s.], doi:10.3390/cells11050844
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3. Wykaz stosowanych skrótów

Skrót	Termin anglojęzyczny	Termin polskojęzyczny
3TC	*lamivudine*	lamiwudyna
AD	*Alzheimer's disease*	choroba Alzheimera
Akt/PKB	*protein kinase B*	kinaza białkowa B
ATP	*adenosine triphosphate*	adenozyno-5'-trifosforan
AZT	*zidovudine*	zydowudyna
BSA	*bovine serum albumin*	albumina surowicy bydlęcej
CART	*combined antiretroviral therapy*	skojarzona terapia antyretrowirusowa
DAPI	*4',6-diamidino-2-phenylindole*	diamidino-2-fenyloindol
DHA	*docosahexaenoic acid*	kwas dokozaheksaenowy
ER	*endoplasmic reticulum*	retikulum śródplazmatyczne
ERK	*extracellular signal-regulated kinase*	kinaza regulowaną sygnałem zewnątrzkomórkowym
ETC	*electron transport chain*	łańcuch transportu elektronów
FABP	*fatty acid binding protein*	białka wiążące kwasy tłuszczowe
FABP4	*fatty acid-binding protein 4*	białko wiążące kwasy tłuszczowe 4
FABP5	*fatty acid-binding protein 5*	białko wiążące kwasy tłuszczowe 4
FAT/CD36	*fatty acid translocase, cluster of differentiation 36*	translokaza kwasów tłuszczowych
FATP	*fatty acid transport protein*	białka transportujące kwasy tłuszczowe
FBS	*fetal bovine serum*	płodowa surowica bydlęca

FFA	*free fatty acids*	wolne kwasy tłuszczowe
HAART	*highly active antiretroviral therapy*	wysoce aktywna terapia antyretrowirusowa
HAD	*HIV-associated dementia*	demencja związana z HIV
HAND	*HIV-associated neurocognitive disorders*	zaburzenia neuropoznawcze związane z HIV
HDL	*high-density lipoprotein*	lipoproteina o wysokiej gęstości
HIV	*Human Immunodeficiency Virus*	ludzki wirus niedoboru odporności
HSL	*hormone-sensitive lipase*	lipaza wrażliwa na hormony
InsR	*insulin receptor*	receptor insuliny
InsRS	*insulin receptor substrate*	substrat receptora insuliny
InsRS1	*insulin receptor substrate 1*	substrat receptora insuliny 1
IR	*insulin resistance*	insulinooporność
JNK	*c-Jun N-terminal kinase*	N-końcowa kinaza czynnika transkrypcyjnego c-Jun
LDL	*low-density lipoprotein*	lipoproteina o niskiej gęstości
MAPK	*mitogen-activated protein kinases*	kinazy białkowe aktywowanych mitogenem
mtDNA	*mitochondrial DNA*	mitochondrialne DNA
NAFLD	*non-alcoholic fatty liver disease*	niealkoholowe stłuszczenie wątroby
NF-κB	*nuclear factor κ-light-chain-enhancer of activated B cells*	jądrowy czynnik transkrypcyjny κB
NRTI	*nucleoside reverse transcriptase inhibitors*	nukleozydowe inhibitory odwrotnej transkryptazy
OS	*oxidative stress*	stres oksydacyjny
OUN	*central nervous system*	ośrodkowy układ nerwowy

PA	*palmitic acid*	kwas palmitynowy
PBS	*phosphate buffered saline*	sól fizjologiczna buforowana fosforanami
PD	*Parkinson's disease*	choroba Parkinsona
PI3K	*phosphoinositide 3-kinase*	kinaza 3'-fosfatydyloinozytolu
PKC	*protein kinase C*	kinaza białkowa C
ROS	*reactive oxygen species*	reaktywna formy tlenu

4. Streszczenie

Zakażenie ludzkim wirusem niedoboru odporności (HIV, *ang. Human Immunodeficiency Virus*) stanowi globalny problem zdrowotny, dotyczący milionów ludzi na całym świecie. Skuteczność leczenia pacjentów zakażonych wirusem HIV znacznie się poprawiła, a oczekiwana długość życia została istotnie wydłużona dzięki opracowaniu licznych strategii terapeutycznych w ciągu ostatnich kilku dekad, jednak zaobserwowano występowanie chorób współistniejących, które obniżają jakość życia takich osób. Ponadto, pomimo stosowanej terapii, niektóre obszary organizmu, w tym ośrodkowy układ nerwowy (OUN), pozostają podatne na trwającą replikację wirusa, stanowiąc jego rezerwuar i uniemożliwiając kompletną eradykację wirusa.

Nukleozydowe inhibitory odwrotnej transkryptazy (NRTI, *ang. nucleoside reverse transcriptase inhibitors*) pozostają kluczowe w leczeniu HIV, a do najważniejszych leków z tej grupy należą abakawir, lamiwudyna (3TC), zydowudyna (AZT) i tenofowir. Jednak długotrwałe stosowanie NRTI wiąże się z efektami ubocznymi, takimi jak zaburzenia metabolizmu lipidów i rozwój zaburzeń neurokognitywnych związanych z redystrybucją tkanki tłuszczowej, co może prowadzić do wzrostu poziomu wolnych kwasów tłuszczowych (FFA, *ang. free fatty acid*) w osoczu krwi i potencjalnie w płynie mózgowo-rdzeniowym, a także przyczyniać się do rozwoju lipotoksycznych zaburzeń metabolicznych w OUN.

Lipotoksyczność jest określana jako zespół nieprawidłowości metabolicznych wynikających ze zwiększonego stężenia FFA w osoczu krwi i ich nagromadzenia w komórkach tkanek innych niż tłuszczowe. Proces ten najczęściej dotyczy komórek trzustki, wątroby, mięśni szkieletowych, mięśnia sercowego lub nerek. Wśród zaburzeń molekularnych wywoływanych przez nadmierną akumulację FFA wymienia się stres oksydacyjny, stres retikulum endoplazmatycznego, indukcję insulinooporności (IR, *ang. insulin resistance*) czy stan zapalny. Zjawisko lipotoksyczności jest stosunkowo dobrze opisane w komórkach wyżej wymienionych narządów, podczas gdy istnieje bardzo niewiele badań dotyczących wpływu lipotoksyczności na komórki układu nerwowego. Istnieją jedynie nieliczne doniesienia o lipotoksyczności w tkankach nerwowych, astrocytarnych i mikrogleju układu nerwowego, a także na temat potencjalnego udziału lipotoksyczności w patogenezie chorób neurodegeneracyjnych, takich jak choroba Alzheimera (AD, *ang. Alzheimer's disease*) i choroba Parkinsona (PD, *ang. Parkinson's disease*).

Istnieje luka w wiedzy na temat bezpieczeństwa leków antyretrowirusowych i ich wpływu na toksyczność lipidów w OUN oraz indukowanie zaburzeń w OUN w związku ze stosowaną terapią u pacjentów zakażonych HIV. Aby odpowiedzieć na to zapotrzebowanie, celem niniejszej rozprawy doktorskiej było zbadanie wpływu lamiwudyny i zydowudyny, na homeostazę lipidów, funkcję mitochondriów i indukowanie insulinooporności w badaniach modelowych na dwóch liniach komórkowych – linii komórek dopaminergicznych SH-SY5Y i linii komórek mikrogleju HMC3. Komórki inkubowano z 3TC i AZT w stężeniach odpowiadających stężeniom terapeutycznym w osoczu krwi (odpowiednio 10μM i 6μM), a także z dwoma stężeniami kwasu palmitynowego (PA, *ang. palmitic acid*) - 200μM i 500μM, które odzwierciedlają fizjologiczne oraz podwyższone stężenia tego kwasu tłuszczowego w osoczu krwi. Jako kontrolę pozytywną wykorzystano kwas dokozaheksaenowy (DHA, *ang. docosahexaenoic acid*). Wyniki pomiarów odnoszono do komórek kontrolnych, które nie zostały poddane działaniu żadnej z badanych substancji. Uzyskane wyniki poddano analizie statystyczną w celu zweryfikowania istotności statystycznej zaobserwowanych zmian.

Aby zbadać metabolizm lipidów oceniono ekspresję dwóch transporterów kwasów tłuszczowych - białka wiążącego kwasy tłuszczowe 4 i 5 (FABP4, FABP5, *ang. fatty acid-binding protein 4, 5*), a także zawartość lipidów w komórkach. Funkcję mitochondriów określono poprzez ocenę poziomu anionorodnika ponadtlenkowego i adenozyno-5'-trifosforanu (ATP, *ang. adenosine triphosphate*). Rozwój insulinooporności mierzono na drodze oceny ekspresji receptora insuliny (InsR, *ang. insulin receptor*), substratu receptora insuliny 1 (InsRS1, *ang. insulin receptor substrate 1*) oraz poziomów fosforylacji kinazy 3'-fosfatydyloinozytolu (PI3K, *ang. phosphoinositide 3-kinases*). Dodatkowo wykonano pomiar aktywności kinaz białkowych aktywowanych mitogenami, N-końcowej kinazy czynnika transkrypcyjnego c-Jun (JNK, *ang. c-Jun N-terminal kinase*), kinazy regulowanej sygnałem zewnątrzkomórkowym (ERK, *ang. extracellular signal-regulated kinase*) i kinazy p38, kluczowych elementów szlaków sygnałowych komórki w odpowiedzi na czynniki stresorowe. Kinazy te zostały wybrane jako uzupełnienie oceny rozwoju insulinooporności ze względu na fakt, że mają również udział w szlaku sygnałowym insuliny.

Na drodze przeprowadzonych badań wykazano, że 3TC, AZT, a także PA, przyczyniły się do obniżenia wewnątrzkomórkowych stężeń FABP4 i FABP5, zarówno w komórkach mikrogleju, jak i neuronach dopaminergicznych. Zaobserwowano odmienny wpływ badanych NRTI na zawartość lipidów w komórce. Zarówno 3TC, jak i AZT zwiększyły zawartość lipidów w komórkach mikrogleju, podczas gdy w neuronach dopaminergicznych

zaobserwowano ich spadek. Zmniejszenie zawartości lipidów nastąpiło również po inkubacji komórek neuronów dopaminergicznych z oboma stężeniami PA. Dodatkowo, badane NRTI wywołały wzrost poziomu anionorodnika ponadtlenkowego w mitochondriach w obu liniach komórkowych, w przeciwieństwie do PA, który przyczynił się do zmniejszenia poziomu tej reaktywnej formy tlenu (ROS, *ang. reactive oxygen species*). Wykazano również, że inkubacja z AZT powoduje spadek stężenia ATP w mitochondriach w komórkach mikrogleju. Badane leki nasilały również proces fosforylacji kinazy ERK. Zmiany wywołane przez 3TC i AZT w linii komórkowej neuronów dopaminergicznych obejmowały również zwiększenie poziomu fosforylacji PI3K i mniejszą ekspresję receptora insuliny. Efekt wywołany przez PA na InsR również obejmował spadek ekspresji, podczas gdy poziom fosforylacji PI3K uległ zmniejszeniu pod wpływem tego kwasu tłuszczowego.

Uzyskane wyniki mogą wskazywać, że 3TC i AZT nie przyczyniają się w istotny sposób do rozwoju procesów neurodegeneracyjnych ani lipotoksycznych zaburzeń metabolicznych za pośrednictwem transporterów kwasów tłuszczowych ani na drodze rozwoju insulinooporności. Wpływ badanych NRTI na zawartość lipidów w komórkach układu nerwowego różni się w zależności od typu komórki. W świetle uzyskanych wyników można jednak przypuszczać, że dochodzi do rozwoju zaburzeń neurodegeneracyjnych pod wpływem NRTI w komórkach raczej poprzez zaburzanie funkcji mitochondriów i indukcję stresu oksydacyjnego. Co zaskakujące, w obszarze przeprowadzonych badań nie wykazano negatywnego wpływu PA na funkcję komórek mikrogleju i neuronów dopaminergicznych, nie potwierdzając jego właściwości do indukowania zaburzeń neurodegeneracyjnych. Przypuszczalnie komórki wybrane do badań modelowtych charakteryzują się większą odpornością na lipotoksyczne efekty zwiększonych stężeń PA w odniesieniu do innych typów komórek i tkanek. Jednakże koniecznie byłoby wykonanie dodatkowych badań, aby móc potwierdzić takie przypuszczenie. Podsumowując, w oparciu o badania przeprowadzone w ramach niniejszej rozprawy doktorskiej, wykazano możliwy udział NRTI w patogenezie zaburzeń neurodegeneracyjnych w OUN na drodze indukcji zaburzeń funkcji mitochondriów, natomiast nie potwierdzono założenia o neurodegeneracyjnych właściwościach PA.

5. Summary

Human Immunodeficiency Virus (HIV) infection is a global health problem affecting millions of people worldwide. The effectiveness of treatment for HIV-infected patients has improved significantly, and life expectancy has been significantly extended thanks to the development of numerous therapeutic strategies over the past few decades, but comorbidities have been observed that reduce the quality of life of such individuals. In addition, despite therapy, some areas of the body, including the central nervous system (CNS), remain susceptible to ongoing viral replication, acting as a reservoir and preventing complete viral eradication.

Nucleoside reverse transcriptase inhibitors (NRTIs) remain key in the treatment of HIV, and the most important drugs in this group include abacavir, lamivudine (3TC), zidovudine (AZT) and tenofovir. However, long-term use of NRTIs is associated with side effects such as lipid metabolism disorders and the development of neurocognitive impairment associated with fat redistribution, which can lead to increased levels of free fatty acids (FFAs) in plasma and potentially in cerebrospinal fluid, as well as contributing to the development of lipotoxic metabolic disorders in the CNS.

Lipotoxicity is defined as a set of metabolic abnormalities resulting from increased plasma concentrations of FFAs and their accumulation in cells of non-adipose tissues. This process most often involves cells of the pancreas, liver, skeletal muscle, heart muscle or kidney. Among the molecular disorders induced by excessive FFA accumulation are oxidative stress, endoplasmic reticulum stress, induction of insulin resistance (IR) or inflammation. The phenomenon of lipotoxicity is relatively well described in cells of the above-mentioned organs, while the studies on the effects of lipotoxicity on cells of the nervous system are conducted to a much lesser extent. There are only a few reports on lipotoxicity in neural, astrocytic and microglia tissues of the nervous system, as well as on the potential involvement of lipotoxicity in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD).

There is a gap in knowledge about the safety of antiretroviral drugs and their effects on CNS lipid toxicity and induction of CNS abnormalities due to therapy in HIV-infected patients. To address this need, the purpose of this dissertation was to study the effects of lamivudine and zidovudine, on lipid homeostasis, mitochondrial function and induction of insulin resistance in model studies on two cell lines - the SH-SY5Y dopaminergic cell line and the HMC3 microglia

cell line. The cells were incubated with 3TC and AZT at concentrations corresponding to therapeutic plasma concentrations (10μM and 6μM, respectively), as well as with two concentrations of palmitic acid (PA) - 200μM and 500μM, which reflect physiological and elevated plasma concentrations of this fatty acid. Docosahexaenoic acid (DHA) was used as a positive control. The results of the measurements were related to control cells, which were not treated with any of the test substances. The results were subjected to statistical analysis to verify the statistical significance of the observed changes.

To study lipid metabolism, the expression of two fatty acid transporters, fatty acid-binding proteins 4 and 5 (FABP4, FABP5), and cellular lipid content were assessed. Mitochondrial function was determined by assessing the levels of superoxide anion radical and adenosine 5'-triphosphate (ATP). The development of insulin resistance was measured by assessing the expression of insulin receptor (InsR), insulin receptor substrate 1 (InsRS1), and phosphorylation levels of phosphoinositide 3'-phosphatidylinositol kinase (PI3K). In addition, the measurement of the activity of mitogen-activated protein kinases was conducted. Selected proteins included c-Jun transcription factor N-terminal kinase (JNK, or c-Jun N-terminal kinase), extracellular signal-regulated kinase (ERK, or extracellular signal-regulated kinase) and p38 kinase, key components of cell signaling pathways in response to stressors. These kinases were chosen to suplement the assessment of the development of insulin resistance due to the fact that they are also involved in the insulin signaling pathway.

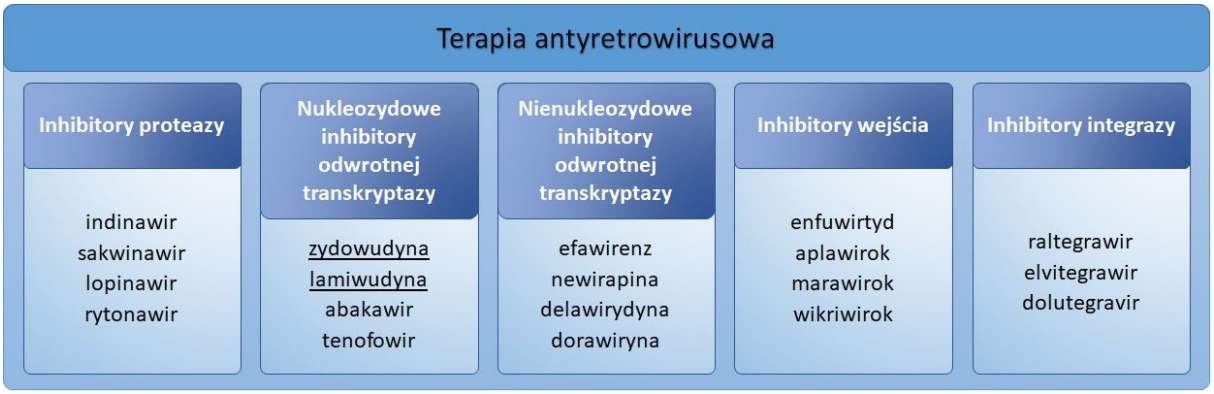
By way of the study, it was shown that 3TC, AZT, as well as PA, contributed to the reduction of intracellular concentrations of FABP4 and FABP5, both in microglia cells and dopaminergic neurons. Different effects of the tested NRTIs on cellular lipid content were observed. Both 3TC and AZT increased lipid content in microglia cells, while a decrease was observed in dopaminergic neurons. A decrease in lipid content also occurred after incubation of dopaminergic neuron cells with both concentrations of PA. In addition, the tested NRTIs induced an increase in the level of superoxide anion radical in mitochondria in both cell lines, in contrast to PA, which contributed to a decrease in the level of this reactive oxygen species (ROS). It was also shown that incubation with AZT causes a decrease in the concentration of ATP in mitochondria in microglia cells. The pharmaceuticals also enhanced the phosphorylation of ERK kinase. The changes induced by 3TC and AZT in the dopaminergic neuron cell line also included an increase in PI3K phosphorylation and a decrease in insulin receptor expression. The PA-induced effect on InsR also included a decrease in expression, while the level of PI3K phosphorylation was reduced under the influence of this fatty acid.

The results may indicate that 3TC and AZT do not significantly contribute to neurodegenerative processes or lipotoxic metabolic disorders via the influence on fatty acid transporters or through the development of insulin resistance. The effect of the studied NRTIs on the lipid content of cells in the nervous system varies depending on the cell type. However, in view of the results obtained, it can be assumed that NRTI-mediated neurodegeneration develops in cells through disruption of mitochondrial function and induction of oxidative stress. Surprisingly, in the area of the conducted studies, PA did not show negative effects on the function of microglia cells and dopaminergic neurons, not allowing the confirmation of its neurodegenerative properties. Presumably, the cells selected for the model studies are characterized by greater resistance to the lipotoxic effects of increased concentrations of PA in relation to other cell types and tissues. However, additional studies should be performed to be able to confirm this supposition. In conclusion, based on the studies conducted within the framework of this dissertation, the possible involvement of NRTIs in the pathogenesis of neurodegenerative disorders in the CNS through the induction of mitochondrial dysfunction has been demonstrated, while the assumption of neurodegenerative properties of PA has not been confirmed.

6. Wprowadzenie teoretyczne

6.1. Leki antyretrowirusowe – działania niepożądane oraz wyzwania związane z terapią zakażeń wirusem HIV

Zakażenie ludzkim wirusem niedoboru odporności (HIV, ang. *Human Immunodeficiency Virus*) wciąż pozostaje globalnym zagrożeniem dla zdrowia, nie tylko ze względu na jego bezpośredni wpływ na układ odpornościowy, ale także poprzez stale rosnące wyzwanie, jakie stanowi optymalna farmakoterapia. Obecnie konwencjonalne podejście terapeutyczne polega na połączeniu 2-3 leków z różnych klas (szczegółowo przedstawione na Rysunku 1) tworząc wysoce aktywną terapię antyretrowirusową (HAART, *ang. highly active antiretroviral therapy*), znaną również jako skojarzona terapia antyretrowirusowa (CART, *ang. combined antiretroviral therapy*). Schemat ten okazał się bardziej skuteczny w hamowaniu progresji choroby w porównaniu z monoterapią, wydłużając przeżycie pacjentów nawet o 7-10 lat. Takie podejście terapeutyczne zaleca się wdrożyć jak najwcześniej u wszystkich osób zakażonych wirusem HIV [1, 2].

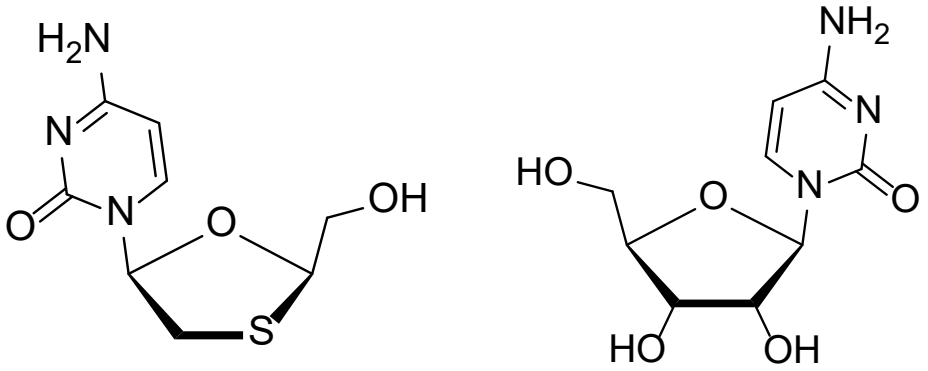


Rysunek 1. Pięć klas leków antyretrowirusowych wraz z przykładami substancji czynnych.

Nukleozydowe inhibitory odwrotnej transkryptazy (NRTI, *ang. nucleoside reverse transcriptase inhibitors*), reprezentowane przez takie leki jak abakawir, lamiwudyna (3TC), zydowudyna (AZT), tenofowir, oddziałują na proces odwrotnej transkrypcji, który jest niezbędny dla cyklu replikacji wirusa. Replikacja wirusa HIV jest zależna od odwrotnej transkryptazy, enzymu, który przepisuje wirusowy genom RNA na DNA. Wirusowe DNA jest następnie integrowane z genomem komórki gospodarza, umożliwiając wirusowi przejęcie kontroli nad procesami komórkowymi gospodarza w celu wytworzenia nowych cząstek wirusowych. NRTI działają poprzez zakłócanie tego kluczowego etapu w cyklu życiowym

HIV, zapobiegając w ten sposób replikacji i rozprzestrzenianiu się wirusa. NRTI są strukturalnie podobne do naturalnych nukleozydów, które służą jako budulec do syntezy DNA. Po aktywacji NRTI konkurują z naturalnymi nukleozydami o włączenie do powstającej nici DNA podczas odwrotnej transkrypcji. Terminacja łańcucha skutecznie zatrzymuje syntezę wirusowego DNA, blokując tym samym replikację wirusa HIV. Selektywność NRTI wobec komórek zakażonych HIV wynika z ich specyficznego oddziaływania na wirusowy enzym odwrotnej transkryptazy, który wykazuje wyższe powinowactwo do wspomnianych analogów w porównaniu z polimerazami DNA komórek gospodarza [3].

Lamiwudyna, inaczej określana jako 20-deoksy-30-tiacytydyna, jest kluczowym składnikiem arsenału terapeutycznego przeciwko zakażeniu wirusem HIV. Jako syntetyczny analog nukleozydu cytydyny (Rysunek 2), lamiwudyna działa na zasadzie podobieństwa do tego niezbędnego budulca DNA. 3TC przenika do ośrodkowego układu nerwowego (OUN) w niedużym stopniu (około 5-10% stężenia w osoczu), gromadzi się głównie w nasieniu i wydzielinach szyjkowo-pochwowych [2, 3, 4]. Jednakże, prowadzone są badania nad nośnikami, które zwiększają stopień przenikania lamiwudny przed barierę krew-mózg [5]. Stężenia 3TC w osoczu krwi pacjentów stosujących ten lek wahają się w granicach 0.02-17.36 μg/ml [6].

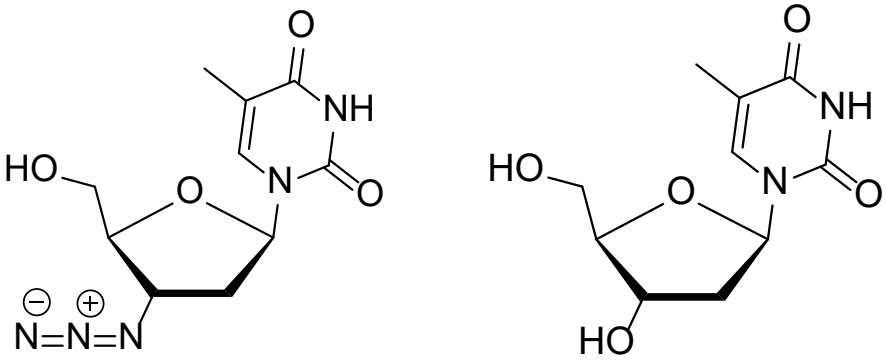


lamiwudyna cytydyna

Rysunek 2. Wzór strukturalny lamiwudyny oraz cytydyny

Zydowudyna, znana również pod nazwą 3-azido-30-deoksytymidyna, przyczyniła się do kluczowych postępów w leczeniu zakażenia ludzkim wirusem niedoboru odporności. Zatwierdzona przez Amerykańską Agencję ds. Żywności i Leków już w 1987 roku, zydowudyna była pierwszym lekiem przeciwretrowirusowym stosowanym w terapii HIV [1, 3]. Jako syntetyczny analog tymidyny (Rysunek 3), zydowudyna wykazuje działanie

przeciwwirusowe poprzez włączanie się do łańcucha DNA wirusa podczas odwrotnej transkrypcji.



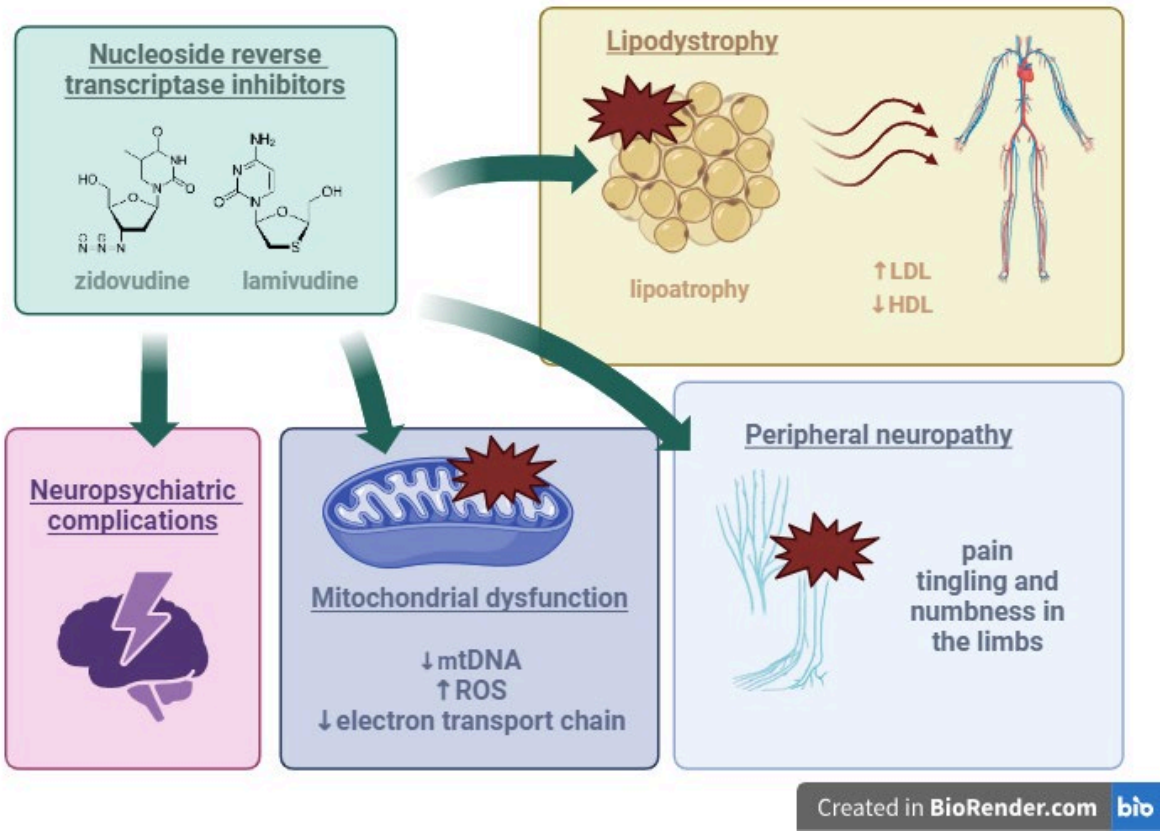
zydowudyna tymidyna

Rysunek 3. Wzór strukturalny zydowudyny i tymidyny

Lek jest łatwo dystrybuowany w organizmie dzięki właściwościom lipofilowym, umożliwiając doskonałą penetrację do OUN, a także wydzielin narządów płciowych. Zydowudyna jest szczególnie skuteczna w zmniejszaniu ryzyka przeniesienia wirusa HIV z matki na dziecko podczas porodu, co czyni ją kluczowym elementem w leczeniu kobiet ciężarnych zakażonych wirusem HIV. Zydowudyna nadal pozostaje fundamentem w leczeniu zakażeń wirusem HIV ze względu na jej udowodnioną skuteczność w zmniejszaniu wiremii i zapobieganiu przenoszeniu wirusa z matki na dziecko [1, 7, 8].

Pomimo ugruntowanej pozycji lamiwudyny i zydowudyny w leczeniu zakażeń wirusem HIV, nadal istnieją wyzwania związane z terapią tymi farmaceutykami. W przypadku monoterapii bądź nieodpowiedniego przestrzegania zaleceń terapeutycznych, leki te mogą doprowadzić do pojawienia się lekoopornych szczepów wirusa. Oporność w wyniku mutacji odwrotnej transkryptazy może rozwinąć się nawet w trzech pierwszy miesiącach monoterapii 3TC [2].

Chociaż NRTI znacząco zmniejszyły śmiertelność związaną z zakażeniem HIV, ich długotrwałe stosowanie wiąże się z szeregiem działań niepożądanych, w tym dotyczących metabolizmu lipidów i układu nerwowego. Na Rysunku 4 przedstawiono schematyczne podsumowanie najbardziej istotnych działań niepożądanych związanych z terapią NRTI.



Rysunek 4. Schematyczne przedstawienie najbardziej istotnych działań niepożądanych NRTI.

Jednym z lepiej udokumentowanych działań niepożądanych terapii NRTI jest zaburzenie metabolizmu lipidów. Pacjenci poddawani długotrwałemu leczeniu NRTI często doświadczają dyslipidemii, charakteryzującej się podwyższonym poziomem trójglicerydów, lipoprotein o niskiej gęstości (LDL, *ang. low-density lipoprotein*) i obniżonym poziomem lipoprotein o wysokiej gęstości (HDL, *ang. high-density lipoprotein*). Ta zmiana w profilach lipidowych jest kluczowym składnikiem zespołu lipodystrofii związanej z HIV, stanu charakteryzującego się nieprawidłową dystrybucją lipidów w organizmie, w tym utratą tłuszczu (lipoatrofią) w obszarach obwodowych i gromadzeniem się tłuszczu (lipohipertrofią) w centralnych regionach ciała. Zespół ten nie tylko wpływa na wygląd fizyczny pacjentów, prowadząc do stygmatyzacji i obniżonej jakości życia, ale także zwiększa ryzyko chorób sercowo-naczyniowych [9]. Rozwój lipoatrofii zaobserwowano przy terapii lekami z grupy NRTI m.in. zydowudyny czy stawudyny [10]. Ponadto, czynniki związane ze stylem życia, takie jak wysokie spożycie kalorii z wynikającym z tego podwyższonym wyjściowym poziomem trójglicerydów, również zostały uznane za czynnik ryzyka lipohipertrofii u pacjentów z HIV [11].

Ponieważ proces replikacji mitochondrialnego DNA wykazuje podobieństwo do transkrypcji wirusowego RNA na DNA, NRTI mogą mieć udział w zaburzeniach tego procesu. Skutkiem jest hamowanie aktywności mitochondrialnej polimerazy DNA-γ, co skutkuje zubożeniem ilości mitochondrialnego DNA (mtDNA, *ang. mitochondrial DNA*). Zmniejszone poziomy mtDNA prowadzą do upośledzenia funkcji mitochondrialnego łańcucha oddechowego, powodując gromadzenie się reaktywnych form tlenu i rozwój stresu oksydacyjnego. Nasilony stres oksydacyjny zaburza prawidłowy metabolizm lipidów, prowadząc do zaburzenia ich homeostazy w komórkach. Działania niepożądane związane ze stosowaniem NRTI obejmują również negatywny wpływ na metabolizm glukozy oraz idącą za tym indukcję insulinooporności (IR, *ang. insulin resistance*) [12, 13].

Oprócz wpływu na metabolizm lipidów i funkcję mitochondriów, działanie NRTI jest również związane z toksycznością względem układu nerwowego (neurotoksycznością), która może objawiać się neuropatią obwodową. Skutki te są szczególnie niepokojące, biorąc pod uwagę długotrwały charakter leczenia HIV, które często wymaga od pacjentów przyjmowania NRTI przez dziesięciolecia. Neuropatia obwodowa charakteryzuje się bólem, mrowieniem i drętwieniem kończyn. Uważa się, że mechanizm neuropatii obwodowej wywołanej przez NRTI obejmuje toksyczność mitochondrialną spowodowaną hamowaniem mitochondrialnej polimerazy DNA-γ w neuronach, prowadzącą do zmniejszonej produkcji energii i zwiększonego stresu oksydacyjnego. Neurony, w szczególności nerwy obwodowe, są wysoce zależne od funkcji mitochondriów w zakresie wytwarzania energii, a zakłócenie tego procesu może prowadzić do uszkodzenia tych komórek i rozwoju neuropatii [12, 13, 14].

Zakażenie wirusem HIV powoduje liczne powikłania neuropsychiatryczne. To sprawia, że w wielu przypadkach trudno jest ustalić, które problemy są związane z CART, a które z samym działaniem wirusa. Terapia NRTI została powiązana z szeregiem zespołów neuropsychiatrycznych, takich jak psychoza, depresja i mania. Inne niepożądane skutki neurologiczne obserwowane podczas stosowania NRTI to bezsenność, bóle mięśni i silne bóle głowy, ale także drgawki, szczególnie w przypadku przedawkowania tych leków. Istnieją również doniesienia o utracie słuchu u pacjentów leczonych NRTI [14, 15]. Przewlekły stan zapalny, często występujący u osób zakażonych wirusem HIV, może dodatkowo nasilać te skutki uboczne, prowadząc do wyraźniejszego pogorszenia funkcji neurokognitywnych [16, 17].

6.2. Kwasy tłuszczowe i lipotoksyczność

Homeostaza i metabolizm kwasów tłuszczowych

Kwasy tłuszczowe odgrywają kluczową rolę w różnorodnych procesach fizjologicznych (takich jak termoregulacja, czy też funkcja budulcowa w błonach komórkowych) oraz stanowią jeden z podstawowych składników energetycznych w organizmie człowieka. Są niezbędnymi składnikami błon komórkowych, a także posiadają właściwości bioaktywnych mediatorów. Kwasy tłuszczowe występują w dwóch głównych postaciach: jako wolne kwasy tłuszczowe (FFA, *ang. free fatty acids*) obecne w krwiobiegu oraz jako triacyloglicerole zmagazynowane w cytoplazmie komórek. Determinuje to ich rolę zarówno w natychmiastowym dostarczaniu energii, jak i długoterminowym jej magazynowaniu. FFA w krwiobiegu są zazwyczaj związane z albuminą, która ułatwia ich transport do tkanek [18, 19].

W obrębie komórki, kwasy tłuszczowe są transportowane przez różne białka transportujące kwasy tłuszczowe (FATP, *ang. fatty acid transport protein*) i białka wiążące kwasy tłuszczowe (FABP, *ang. fatty acid binding protein*). Większość kwasów tłuszczowych jest transportowana przez błonę komórkową za pośrednictwem translokazy kwasów tłuszczowych (FAT/CD36, *ang. fatty acid translocase, cluster of differentiation 36*), FATP i innych transporterów związanych z błoną. Po wniknięciu do wnętrza komórki, kwasy tłuszczowe muszą zostać przetransportowane do różnych organelli w celu dalszego ich przetworzenia. Wewnątrzkomórkowy transport jest możliwy dzięki białkom cytoplazmatycznym - FABP, które wiążą się z kwasami tłuszczowymi i kierują je do miejsc przeznaczenia takich jak mitochondria, gdzie mogą być poddane β-oksydacji w celu wytworzenia energii [20, 21].

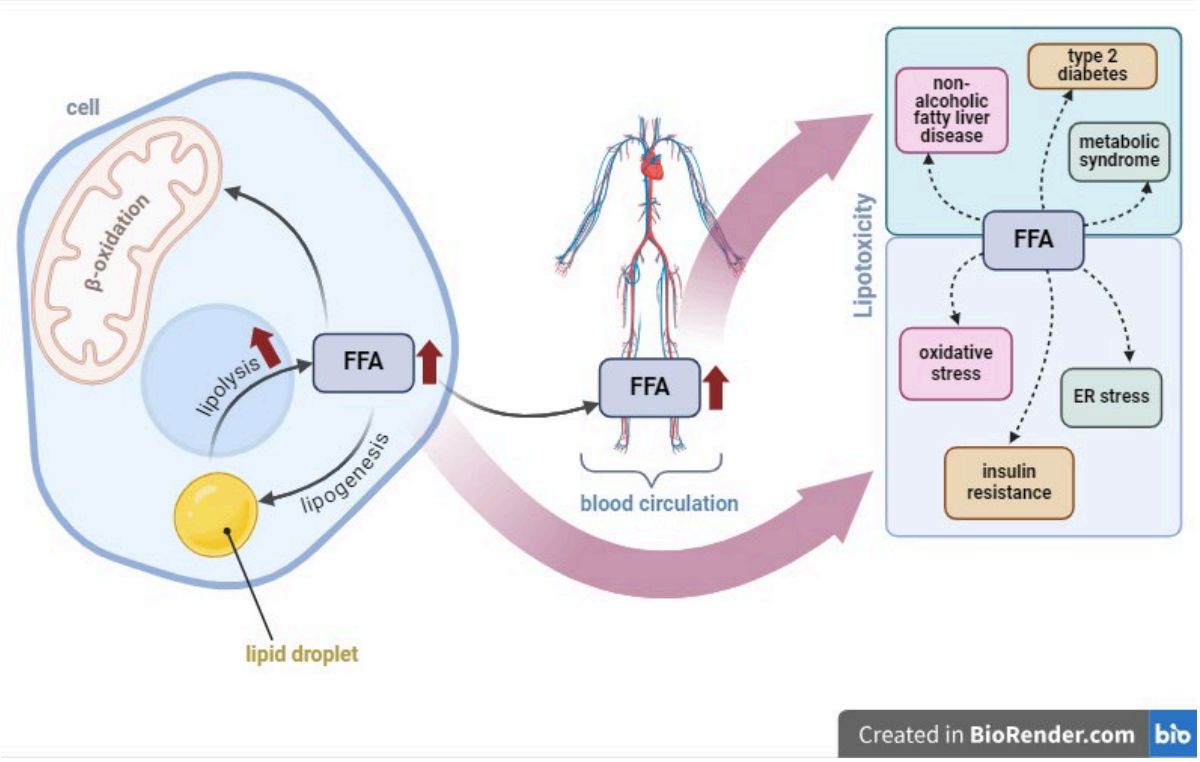
Kwasy tłuszczowe magazynowane są w komórkach głównie w postaci trójglicerydów, przechowywanych w wyspecjalizowanych organellach zwanych kroplami lipidowymi. Krople te są dynamicznymi strukturami, które nie tylko przechowują trójglicerydy, ale także regulują ich uwalnianie i hydrolizę zgodnie z zapotrzebowaniem komórki na energię. Powierzchnię kropli lipidowych pokrywają perilipiny, które regulują dostęp lipaz do przechowywanych trójglicerydów. Dwa procesy regulujące homeostazę kropli lipidowych to lipoliza i lipogeneza, a intensywność tych procesów zależna jest od insuliny, katecholamin lub hormonów tkanki tłuszczowej, takich jak leptyna lub adiponektyna [22, 23].

Lipogeneza, proces odpowiedzialny za magazynowanie kwasów tłuszczowych, rozpoczyna się od syntezy trójglicerydów, które następnie są transportowane do kropli lipidowych, gdzie są przechowywane do czasu zwiększonego zapotrzebowania energetycznego. Insulina odgrywa ważną rolę w tym procesie, stymulując wychwyt FFA przez komórki, aktywując enzymy lipogenne (takie jak karboksylaza acylo-koenzymu A lub syntaza kwasów tłuszczowych) i hamując enzymy lipolityczne, takie jak lipaza wrażliwa na hormony (HSL, *ang. hormone-sensitive lipase*). Aktywacja czynników transkrypcyjnych, takich jak białka wiążące sterolowe elementy regulatorowe lub receptory aktywowane proliferatorami peroksysomów również stymuluje ten proces. Lipogeneza odnosi się również do syntezy *de novo* kwasów tłuszczowych w cytoplazmie komórki [21, 23, 24].

Proces przeciwstawny, czyli lipoliza, skutkuje hydrolizą trójglicerydów i uwalnianiem FFA z kropli lipidowych do cytoplazmy. Czynnikami nasilającymi lipolizę są wspomniane wcześniej HSL, a także leptyna, glikokortykosteroidy, czynnik martwicy nowotworów α i interleukina-6. Uwolnione FFA są aktywowane poprzez wiązanie z koenzymem A, a następnie transportowane do mitochondriów, gdzie ulegają β-oksydacji [21, 23, 24].

Lipotoksyczność

Tkanka tłuszczowa stanowi strukturę wyspecjalizowaną w magazynowaniu lipidów, podczas gdy inne rodzaje tkanek mają ograniczoną zdolność w tym zakresie. Nadmierne gromadzenie się kwasów tłuszczowych w tkankach innych niż tłuszczowe może prowadzić do dysfunkcji komórek i apoptozy. Terminem wspólnym dla zaburzeń metabolicznych związanych z wpływem FFA na komórki i tkanki jest lipotoksyczność. Uproszczony schemat mechanizmu rozwoju lipotoksyczności został zilustrowany na Rysunku 5. Na drodze nasilonej lipolizy i zaburzonej β-oksydacji dochodzi do zwiększonego uwalniania FFA do cytozolu komórki oraz z komórki do krwioobiegu. Uwalnianie FFA do krwioobiegu powoduje większe narażenie na nie komórek i tkanek nietłuszczowych. W komórkach tych dochodzi więc do zaburzeń metabolizmu objawiających się m.in. stresem oksydacyjnym (OS, *ang. oxidative stress*), IR i stresu retikulum śródplazmatycznego (ER, *ang. endoplasmic reticulum*). Zwiększone stężenia FFA w krwioobiegu wskazuje się również jako przyczynę rozwoju chorób takich jak cukrzyca typu 2, niealkoholowe stłuszczenie wątroby (NAFLD, *ang. non-alcoholic fatty liver disease*) czy zespół metaboliczny. Podejrzewa się również udział lipotoksyczności w rozwoju schorzeń o podłożu neurodegeneracyjnym [25, 26, 27].



Rysunek 5. Schematyczne przedstawienie sposobu magazynowania lipidów i ich wpływu na rozwój zaburzeń metabolicznych i lipotoksyczności.

Upośledzenie zdolności do magazynowania lipidów w tkance tłuszczowej, dieta wysokotłuszczowa skutkująca zwiększonym poziomem FFA w osoczu krwi oraz ukierunkowanie metaboliczne na lipolizę dostarczają zwiększoną ilość FFA do cytozolu komórek [28]. Metabolizm komórkowy wymaga skutecznych mechanizmów eliminacji FFA, takich jak estryfikacja do triacyloglicerolu i nasilona β-oksydacja [29, 30, 31]. W przypadku tkanek nietłuszczowych enzymy wymagane do β-oksydacji mogą zostać wyczerpane. Prowadzi to do kumulacji produktów pośrednich metabolizmu lipidów, takich jak diacyloglicerol lub ceramidy, które mogą zakłócać inne szlaki metaboliczne, przyczyniając się do szeroko rozumianego lipotoksycznego stresu komórkowego [32, 33].

Istnieje pozytywna korelacja między zawartością lipidów a odpowiedzią zapalną i insulinoopornością, co sugeruje, że zmagazynowane trójglicerydy są bezpośrednio odpowiedzialne za efekty lipotoksyczne {34, 35]. Jednak to niekontrolowane uwalnianie FFA z kropli lipidowych i tkanki tłuszczowej oraz ich ingerencja w szlaki metaboliczne przyczyniają się do rozwoju lipotoksyczności [36, 37, 38].

Stres oksydacyjny

Stres oksydacyjny to termin odwołujący się do braku równowagi między czynnikami utleniającymi i redukującymi, przesuniętego w kierunku środowiska utleniającego, wynikającym ze wzrostu zawartości reaktywnych form tlenu (ROS, *ang. reactive oxygen species*). Przyczynia się on m.in. do zaburzeń integralności błon mitochondrialnych, upośledzając ich funkcję i prowadząc do utraty potencjału błony mitochondrialnej - elementu krytycznego dla wytwarzania adenozyno-5'-trifosforanu (ATP, *ang. adenosine triphosphate*). W warunkach fizjologicznych FFA są pobierane i transportowane do mitochondriów, gdzie ulegają β-oksydacji. Proces ten przekształca FFA w acetylo-koenzym A, który następnie wchodzi w cykl kwasu cytrynowego w celu wytworzenia ATP, podstawowej jednostki energetycznej komórki. W sytuacji podwyższonego poziomu FFA, konieczne jest utlenianie większej ilości substratów niż pozwalają na to zdolności enzymatyczne komórki. Dlatego też, nadmierny proces utleniania FFA może przyczyniać się do nadprodukcji ROS, a co za tym idzie – rozwoju lipotoksyczności [39, 40].

Insulinooporność

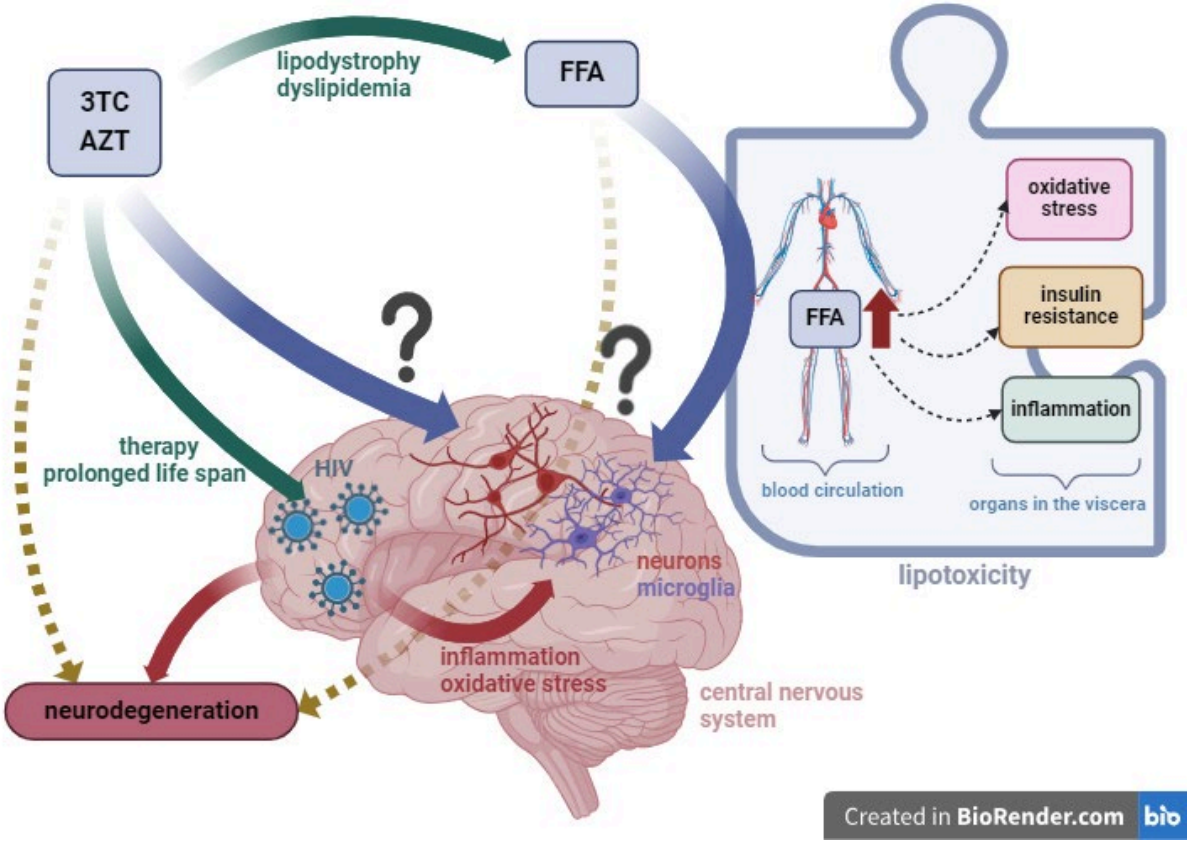
Insulinooporność określa zmniejszoną zdolność komórek do reagowania na działanie insuliny i również zaliczana jest do zaburzeń związanych ze zwiększonym stężeniem FFA w osoczu krwi i transportowania ich do tkanek nietłuszczowych, a co za tym idzie z rozwojem lipotoksyczności. Receptor insulinowy (InsR, *ang. insulin receptor*) ulega autofosforylacji po związaniu z insuliną, co prowadzi do rekrutacji substratów receptora insuliny (InsRS, *ang. insulin receptor substrate*), które następnie również są fosforylowane. Następuje aktywacja dwóch głównych szlaków sygnałowych: szlaku kinazy 3'-fosfatydyloinozytolu (PI3K, *ang. phosphoinositide 3-kinase*) i kinazy białkowej B (Akt/PKB, *ang. protein kinase B*), odpowiedzialnego za większość metabolicznych działań insuliny, oraz szlaku kinaz białkowych aktywowanych mitogenem (MAPK, *ang. mitogen-activated protein kinases*), regulującego ekspresję niektórych genów i współpracującego ze szlakiem PI3K. Fosforylowane białka InsRS służą jako miejsca dokowania dla różnych cząsteczek sygnałowych, w szczególności kinazy PI3K. Aktywowana PI3K katalizuje wytwarzanie fosfatydyloinozytolo-3,4,5-trifosforanu, lipidowego przekaźnika drugorzędowego, który rekrutuje i aktywuje kinazę białkową B [41]. FFA mogą wpływać na szlaki sygnałowe insuliny poprzez zakłócanie prawidłowej fosforylacji białek InsRS [42, 43, 44]. Zaobserwowano, że aktywność różnych elementów szlaków sygnałowych insuliny, takich jak Akt/PKB lub kinaza PI3K, ulega zmianie pod wpływem FFA [45, 46]. Współistniejące zahamowanie ekspresji

genów związanych ze szlakami sygnałowymi insuliny jest również związane z lipotoksycznym działaniem FFA [43, 47]. Inne badanie pokazuje, że ROS ma udział w desensybilizacji szlaków sygnałowych insuliny poprzez aktywację N-końcowej kinazy czynnika transkrypcyjnego c-Jun (JNK, *ang. c-Jun N-terminal kinase*), osłabiając tym samym fosforylację InsRS [48].

6.3. Zależność pomiędzy lekami antyretrowirusowymi, kwasami tłuszczowymi, a zakażeniem wirusem HIV w ośrodkowym układzie nerwowym

Istnieje ogromna trudność w zweryfikowaniu czy neurodegeneracja u pacjentów z HIV wynika z niezależnych zaburzeń gospodarki lipidowej, stosowanej terapii czy samego zakażenia HIV. Z tego względu badanie molekularnych mechanizmów może dostarczyć cennych informacji zarówno na temat mechanizmów działań niepożądanych leków oraz etiologii chorób o podłożu neurodegeneracyjnym u osób żyjących z HIV.

Pomiędzy działaniem leków z grupy NRTI, efektami lipotoksycznymi wywołanymi przez FFA, a także konsekwencjami zakażeń wirusem HIV istnieje sieć powiązań, których punktem zbieżnym jest wpływ na OUN. Propozycję zależności w tej sieci schematycznie zaprezentowano na Rysunku 6.



Rysunek 6. Zależności pomiędzy NRTI, FFA, infekcją HIV a OUN.

Wpływ zakażenia HIV na OUN

Rozwój terapii antyretrowirusowej znacznie poprawił rokowania osób zakażonych wirusem HIV, prowadząc do wydłużenia oczekiwanej długości życia. Pomimo skutecznej supresji wiremii we krwi obwodowej, zakażenie przyczynia się do szeregu powikłań neurologicznych określanych jako zaburzenia neuropoznawcze związane z HIV (HAND, *ang. HIV-associated neurocognitive disorders*). Zaburzenia te mogą przybierać różne formy, od bezobjawowych zaburzeń neuropoznawczych do cięższych przypadków, takich jak demencja związana z HIV (HAD, *ang. HIV-associated dementia*) czy rozwój chorób neurodegeneracyjnych, jak choroba Alzheimera (AD, *ang. Alzheimer's disease*) czy choroba Parkinsona (PD, *ang. Parkinson's disease*) [49, 50, 51, 52].

Na wczesnym etapie zakażenia może dojść do przeniknięcia wirusa HIV do OUN, powodując wytworzenie rezerwuaru wirusa, który jest trudno dostępny zarówno dla układu odpornościowego, jak i dla leków antyretrowirusowych. To siedlisko w OUN pozwala wirusowi HIV pozostawać w organizmie, przyczyniając się do ciągłego stanu zapalnego i uszkodzenia neuronów, a także trudności w skutecznej eradykacji [53].

Cechą znamienną dla neurodegeneracji wywołanej wirusem HIV jest przewlekły stan zapalny układu nerwowego. Zainfekowany mikroglej i makrofagi uwalniają kaskadę cytokin prozapalnych, chemokin i ROS, które przyczyniają się do uszkodzenia i śmierci neuronów. Przewlekły stan zapalny jest nie tylko szkodliwy dla neuronów, ale także przyczynia się do zaburzeń funkcji innych komórek OUN, takich jak astrocyty i oligodendrocyty, dodatkowo nasilając procesy neurodegeneracyjne [54, 55].

Białka HIV, takie jak transaktywator transkrypcji Tat, białko otoczki wirusa gp120, wirusowe białko R i białko Nef mają istotny udział w aktywacji komórek mikrogleju i przyczyniają się tym samym do uwalniania prozapalnych cytokin, chemokin i ROS [55, 56]. Stres oksydacyjny nie tylko bezpośrednio uszkadza neurony, ale także wzmacnia odpowiedź zapalną, tworząc błędne koło neurodegeneracji [57]. Kumulacja uszkodzeń oksydacyjnych może przyczynić się do wystąpienia i progresji chorób neurodegeneracyjnych, takich jak AD i PD u osób zakażonych wirusem HIV. Hipokamp i kora czołowa, regiony mózgu zaangażowane w pamięć i funkcje wykonawcze, są szczególnie podatne na neurotoksyczność związaną z HIV. Utrata neuronów w tych obszarach koreluje z nasileniem pogorszenia funkcji poznawczych u osób zakażonych HIV [58].

Związek między NRTI a OUN i lipotoksycznością

Zaburzenia metaboliczne, takie jak hiperglikemia i dyslipidemia, a w konsekwencji także insulinooporność powszechnie występują u osób żyjących z HIV [9, 11]. Insulina odgrywa kluczową rolę w utrzymaniu prawidłowej funkcji neuronów, w tym reguluje uwalnianie neuroprzekaźników, promuje plastyczność synaptyczną i chroni przed stresem oksydacyjnym. Zakłócenie szlaków sygnałowych insuliny w mózgu potencjalnie przyczynia się do pogorszenia funkcji poznawczych i zwiększenia ryzyka chorób neurodegeneracyjnych [59].

Co więcej, lipodystrofia związana ze stosowaniem NRTI może spowodować zwiększenie stężenia FFA w osoczu krwi. W obliczu zaburzeń gospodarki lipidowej, jest zatem prawdopodobne, że może dojść do rozwoju lipotoksyczności pod wpływem zarówno samej infekcji, jak i zastosowanej terapii. Jak wspomniano w rozdziale 5.1, zydowudyna charakteryzuje się dobrą przenikalnością do OUN, natomiast lamiwudyna jest przedmiotem badań nad zwiększeniem jej przenikalnością za pomocą nośników. Dlatego też ich wpływ na komórki ośrodkowego układu nerwowego stanowi istotny aspekt bezpieczeństwa terapii. Potencjalne negatywne działanie tych leków na neurony i glej implikowałoby ich udział w procesach neurodegeneracyjnych.

Wpływ FFA na OUN

W kontekście układu nerwowego, lipotoksyczność została powiązana z występowaniem chorób neurodegeneracyjnych, takimi jak AD i PD. Nagromadzenie kwasów tłuszczowych i ich toksycznych metabolitów w tkankach nerwowych może prowadzić m.in. do stresu oksydacyjnego, dysfunkcji mitochondriów, insulinooporności i stanu zapalnego, przyczyniając się do degeneracji neuronów i progresji tych chorób [59].

Stres oksydacyjny

Mitochondria są szczególnie wrażliwe na zaburzenia gospodarki lipidowej ze względu na ich rolę w procesie β-oksydacji. W sytuacji podwyższonego poziomu FFA, konieczne jest utlenianie większej ilości substratów niż pozwalają na to zdolności enzymatyczne komórki. Dlatego też, nadmierne procesy utleniania FFA mogą przyczyniać się do nadprodukcji ROS. Badania wskazują jednak na dużę liczbę mechanizmów odpowiedzialnych za rozwój OS związanego z lipotoksycznością. Przykładowo są to: zaburzenia łańcucha transportu elektronów (ETC, *ang. electron transport chain*) i idący za tym spadek produkcji ATP,

zmniejszenie aktywności enzymu fosforylacji oksydacyjnej, zwiększenie aktywności białek rozprzęgających i inne [60, 61, 62, 63]. Konsekwencją długotrwałego stresu oksydacyjnego jest apoptoza komórki [64]. Neurony są wysoce zależne od funkcji mitochondriów w zakresie produkcji energii, a nagromadzenie FFA w tkance nerwowej może potencjalnie zakłócić równowagę biologiczną mitochondriów, prowadząc do apoptozy neuronów, a co za tym idzie do neurodegeneracji. Co więcej, uszkodzenia oksydacyjne spowodowane przez ROS mogą nasilać agregację patologicznych białek, takich jak β-amyloid w AD lub α-synukleina w PD, przyspieszając postęp choroby [59].

Insulinooporność

OUN jest coraz częściej wskazywany jako kluczowy obszar rozwoju insulinooporności indukowanej FFA, co ma istotny wpływ na rozwój chorób neurodegeneracyjnych i pogorszenie funkcji poznawczych. W OUN insulina odgrywa kluczową rolę w regulacji metabolizmu neuronów, plastyczności synaptycznej i prawidłowej funkcji mózgu. Jednak podwyższony poziom krążących FFA o potencjale zakłócającym szlaki sygnałowe insuliny w różnych komórkach mózgu, w tym neuronach, astrocytach i mikrogleju, może doprowadzić do upośledzenia metabolizmu glukozy i zwiększonej podatności na stres oksydacyjny [65].

Stan zapalny jako czynnik łączący OS i IR wywołane przez FFA

Odpowiedź zapalna może zaburzać szlaki sygnałowe insuliny zarówno w mikrogleju, jak i neuronach, nasilając insulinooporność i przyczyniać się do neurozapalenia w tych komórkach. Utrzymująca się aktywacja mikrogleju spowodowana ekspozycją na FFA może skutkować przewlekłym stanem zapalnym układu nerwowego, który jest związany z rozwojem zaburzeń neurodegeneracyjnych. Indukcja stanu zapalnego stanowi również pomost przyczynowo skutkowy pomiędzy zjawiskami OS i IR wywołanymi przez FFA. Badania wykazały, że stres oksydacyjny związany z lipotoksycznością jest również związany z indukcją szlaków zapalnych, przykładowo szlaku jądrowego czynnika transkrypcyjnego κB (NF-κB, *ang. nuclear factor κ-light-chain-enhancer of activated B cells*), a także czynnika transkrypcyjnego 3 [66, 67]. Wykazano, że indukowana przez FFA produkcja ROS i aktywacja szlaku NF-κB jest również odpowiedzialna za wywoływanie IR, co sugeruje wzajemne oddziaływanie na siebie różnych mechanizmów lipotoksyczności [27]. Jednak inne badanie przeczy istnieniu bezpośredniego związku między OS a insulinoopornością w obecności nadmiaru kwasów tłuszczowych [68], dlatego też ten aspekt wymaga dodatkowych badań w celu ustalenia i wyjaśnienia dokładnych mechanizmów. Potencjalny związek między stresem

oksydacyjnym a insulinoopornością, dwoma procesami, które mogą być indukowane przez FFA, sugerowałby wzajemną zależność lipotoksycznych mechanizmów molekularnych wyzwalanych przez FFA.

7. Cel pracy

Głównym celem niniejszej rozprawy doktorskiej była ocena wpływu lamiwudyny, zydowudyny i kwasu palmitynowego na funkcje neuronów i mikrogleju w badaniach modelowych na liniach komórkowych, jako potencjalnych czynników związanych z rozwojem procesów neurodegeneracyjnych.

Dla realizacji tego celu sformułowano trzy cele szczegółowe:

1. Ocena indukcji zaburzeń mitochondriów, zaburzeń transportu i magazynowania lipidów w komórkach mikrogleju pod wpływem lamiwudyny, zydowudyny i kwasu palmitynowego.
2. Ocena indukcji stresu oksydacyjnego, zaburzeń transportu i magazynowania lipidów, a także aktywności wybranych białek szlaku sygnałowego insuliny w komórkach neuronów dopaminergicznych pod wpływem lamiwudyny, zydowudyny i kwasu palmitynowego.
3. Wskazanie podobieństw i różnić w działaniu w oddziaływaniu badanych substancji na obie linie komórkowe: neuronalnej dopaminergicznej oraz mikroglejowej.

Jako hipotezę badawczą przyjęto, że leki z grupy NRTI oraz kwas palmitynowy powodować będą następujące efekty w badanych liniach komórkowych:

- spadek żywotności komórek,
- zmniejszenie produkcji ATP w mitochondriach,
- wzrost produkcji anionorodnika ponadtlenkowego w mitochondriach,
- wzrost stężeń białek transportowych FABP4 i FABP5,
- zwiększenie aktywności kinaz białkowych MAPK,
- zwiększona zawartość lipidów w komórce,
- spadek poziomu fosforylacji białka PI3K,
- zmniejszone stężenia InsR i InsRS1.

8. Materiały i metody

8.1. Hodowla komórkowa

8.1.1. HMC3

Linia komórek adherentnych HMC3, posiadająca cechy odpowiadające pierwotnym komórkom mikrogleju, została pozyskana z American Type Culture Collection (CRL-3304). Komórki te hodowano w kolbach hodowlanych o powierzchni 25 cm^2 lub 75 cm^2 (Nunc™ EasYFlaskTM, 156367, 148576, Thermo Fisher Scientific, Dania) w kontrolowanych warunkach w temperaturze 37°C, z 5% CO^2 w pożywce Eagle's Minimum Essential Medium (ATCC 30-2003). Pożywkę wzbogacano 2 mM L-glutaminą, 1 mM pirogronianem sodu, 1500 mg/l wodorowęglanem sodu, 10% płodową surowicą bydlęcą (FBS, *ang. fetal bovine serum*, 26140079, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) i penicyliną-streptomycyną (Penicillin-Streptomycin, 15070063, Gibco, Thermo Fisher Scientific, Waltham, MA, USA).

8.1.2. SH-SY5Y

Linia komórkowa SH-SY5Y jest ludzką linią komórkową neuroblastoma powszechnie stosowaną w badaniach naukowych ze względu na cechy charakterystyczne dla neuronów dopaminergicznych. Linia komórkowa SH-SY5Y została pozyskana z ECACC (European Collection of Authenticated Cell Cultures, numer 94030304, Salisbury, Wielka Brytania). Komórki były hodowane w naczyniach hodowlanych o powierzchni 25 cm^2 lub 75 cm^2. Warunki hodowli komórek były zgodne ze standardem i obejmowały inkubację w temperaturze 37°C z 5% CO^2. Komórki hodowano w pożywce Ham's F12 (234700712, Sartorius, Izrael): EMEM (820100a, CLS, Eppelheim, Niemcy) w stosunku 1: 1 z dodatkiem 2mM glutaminy (Glutamax, 35050061, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 1% NEAA (Non-essential amino acids, M7145-100ML, Sigma-Aldrich, UK), 15% v/v FBS oraz antybiotykami - streptomycyną i penicyliną.

Linia komórkowa była poddawana procesowi różnicowania w celu uzyskania zarówno fizjologicznych, jak i fenotypowych właściwości dla dojrzałych neuronów dopaminergicznych. W procesie tym komórki zostały poddane działaniu kwasu retinowego (R2625-1G, Sigma-Aldrich, St. Louis, MO, USA) w dawce 10 µM przez okres pięciu dni. W celu poddania komórek działaniu czynnika różnicującego, podłoże hodowlane zmieniono na Neurobasal™ Medium (21103049, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) z dodatkiem

suplementu B-27 (A35828-01, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 3% v/v FBS i 2mM glutaminy.

8.2. Badane substancje

Stężenia lamiwudyny i zydowudyny stosowane w eksperymentach odpowiadają stężeniom terapeutycznym obserwowanym w surowicy krwi podczas leczenia farmakologicznego, czyli 10 μM dla lamiwudyny (PHR1365-1G, Sigma-Aldrich, St. Louis, MO, USA) i 6 μM dla zydowudyny (PHR1292-1G, Sigma-Aldrich, St. Louis, MO, USA) Stężenia te zostały wybrane w opraciu o obowiązują literaturę naukową [69, 70, 71, 72]. Roztwory podstawowe 3TC i AZT przygotowano przez rozpuszczenie odważonej ilości związków w soli fizjologicznej buforowanej fosforanami (PBS, *ang. phosphate buffered saline*, 10010023, Gibco, Thermo Fisher Scientific, Waltham, MA, USA).

W badaniach wykorzystano stężenia kwasu palmitynowego (P0500-25G, Sigma, Malezja) wynoszące odpowiednio 200 μM i 500 μM, odpowiadające odpowiednio fizjologicznemu i podwyższonemu stężeniu PA występującemu w surowicy krwi wskazywanemu jako potencjalnie lipotoksyczne, zgodnie z literaturą naukową [73, 74]. Zastosowano trzyetapowy proces koniugacji kwasu palmitynowego z albuminą surowicy, w celu wprowadzenia do wodnego środowiska hodowli komórkowej. Pierwszym krokiem było odważenie 1 g proszku PA i rozpuszczenie go w 7,8 ml 99% etanolu (32294-1L, HoneyWell, Francja) w temperaturze 35-40°C, tworząc roztwór podstawowy o stężeniu 500 mM. Roztwór przefiltrowano przy użyciu filtra 0,2 mm (99722, Genos, Polska). Następnie 1,5 g wolnej od kwasów tłuszczowych albuminy surowicy bydlęcej (BSA, *ang. bovine serum albumin*, A7030, Sigma-Aldrich Saint Louis, MO, USA) rozpuszczono w 15 ml medium hodowlanym bez dodatku FBS (Ham's F12 : EMEM w stosunku 1:1) w temperaturze 37°C. Wreszcie, roztwór PA-BSA o stężeniu 5 mM został uzyskany poprzez zmieszanie dwóch przygotowanych roztworów w stosunku 100:1 (BSA:PA) [75].

Kwas dokozaheksaenowy (DHA, ang. *docosahexanoid acid*, D2534, Sigma-Aldrich, St. Louis, MO, USA) został wybrany jako wskaźnik kontroli pozytywnej do testów i został skoniungowany z BSA przy użyciu tego samego protokołu przygotowania co PA. Stężenie DHA stosowane w badaniu było równoważne stężeniu PA.

W celu zbadania wpływu badanych substancji na komórki neuronalne oraz mikroglejowe, zaprojektowano osiem warunków inkubacji w zależności od dodawanej do

medium hodowlanego substancji i jej stężenia. Zestawienie poszczególnych warunków inkubacji przedstawia tabela 1.

Tabela 1. Podsumowanie warunków inkubacji komórek.

nr	nazwa	substancja badana	stężenie
1	Kontrola	brak – kontrola komórkowa	-
2	BSA	brak – kontrola z albuminą bydlęcą	-
3	PA 200	kwas palmitynowy	200 µM
4	PA 500	kwas palmitynowy	500 µM
5	DHA 200	kwas DHA	200 µM
6	DHA 500	kwas DHA	500 µM
7	3TC	lamiwudyna	10 µM
8	AZT	zydowudyna	6 µM

Po dodaniu odpowiednich składników i odczynników do zawiesiny komórek, inkubowano je przez 24 godziny w temperaturze 37°C z 5% CO^2 i 95% wilgotności. Oznaczenia wybranych parametrów wykonano po przeprowadzeniu inkubacji z badanymi substancjami i zakończeniu eksperymentów.

8.3. Test żywotności XTT

Żywotność komórek określono za pomocą kolorymetrycznego testu XTT (Roche Basel, Szwajcaria, 11465015001). Pomiary przeprowadzono na spektrofotometrze skaningowym Synergy (STAT FAX 2100, Awareness Technology, Inc, Palm City, FL, USA). Test został przeprowadzony zgodnie z protokołem producenta.

8.4. Oznaczanie anionorodnika ponadtlenkowego testem Mitosox™

Pomiar zawartości ponadtlenku mitochondrialnego oceniono za pomocą testu Mitosox™ Red Assay (Thermo Fisher Scientific Waltham, MA, USA, M36008) zgodnie z protokołem producenta. Do pomiaru wykorzystano wielomodowy czytnik mikropłytek SparkR (Tecan, Mannedorf, Szwajcaria). W celu obrazowania fluorescencyjnego, komórki były dodatkowo barwione diamidino-2-fenyloindolem (DAPI, *ang. 4',6-diamidino-2-phenylindole*), a mitochondrialna zawartości ponadtlenku była obserwowana przy użyciu mikroskopu fluorescencyjnego (CKX53, Olympus, Hamburg, Niemcy).

8.5. Testy immunoenzymatyczne (FABP4, FABP5, MAPK, PI3K, InsRS1, InsR)

Testy ELISA wykorzystano do oznaczenia obecności i stężenia następujących białek: FABP4 (*ang. fatty acid-binding protein 4*), FABP5 (*ang. fatty acid-binding protein 5*), kinazy białkowe z grupy MAPK (ERK, p38, JNK), PI3K, InsRS1 oraz receptora insulinowego.

Użyto następujących testów: The Human FABP4 ELISA Kit (ab234565, Abcam, Cambridge, UK), the Human FABP5 ELISA Kit (E1399Hu, Bioassay Technology Laboratory, Birmingham, UK), The InstantOne ELISA™ Kit (IOAP96, ThermoFisher Scientific, Waltham, MA, USA), Phospho-PI 3 kinase p85 + Total In-cell ELISA Kit (ab207484, Abcam, Cambridge, UK), the Human IRS1 SimpleStep ELISAR Kit (ab289646, Abcam, Cambridge, UK), oraz Human InsR (Insulin Receptor) ELISA kit (HUFI00828, AssayGenie, Dublin, Ireland). Testy wykonane zostały zgodnie z instrukcjami dołączonymi przez producentów. Absorbancję mierzono przy użyciu spektrofotometru skaningowego Synergy.

Wyniki otrzymane dla FABP4, FABP5 oraz InsR poddano standaryzacji na zawartość białka całkowitego za pomocą testu Pierce™ BCA Protein Assay Kit (nr cat. 23225, Thermo Fisher Scientific, Waltham, MA, USA). Standaryzację wyników dla PI3K wykonano z użyciem barwnika fioletu krystalicznego dołaczonego do testu.

8.6. Oznaczanie ATP metodą bioluminescencji

Wewnątrzkomórkowe poziomy ATP oznaczono ilościowo przy użyciu zestawu ATP Bioluminescence Assay Kit HS II (Roche, 11699709001) zgodnie z protokołem producenta. Luminescencję zmierzono przy użyciu luminometru (czytnik mikropłytek Spark®, Tecan, Männedorf, Szwajcaria).

8.7. Ocena zawartości lipidów w komórce metodą barwienia odczynnikiem Red Oil O

Aby ocenić poziom gromadzenia się lipidów, przeprowadzono barwienie cytoplazmatycznych kropli lipidowych za pomocą Red Oil O (00625-25G, Sigma Aldrich, Saint Louis, MO, USA). Roztwór roboczy Red Oil O przygotowano zgodnie z protokołem producenta.

Komórki utrwalono 4% paraformaldehydem (22023-20ML, Biotium, Fremont, CA, USA) i inkubowano przez 1 godzinę w temperaturze pokojowej. Następnie komórki inkubowano z 0,5% Tritonem X100 w PBS w temperaturze pokojowej przez 30 minut. Roztwór roboczy Red Oil O dodano do każdego dołka na 2 godziny w temperaturze pokojowej. W kolejnym kroku Red Oil O, został wyekstrahowany z komórek przez dodanie 100 µl izopropanolu do każdego dołka. Każdy ekstrakt został przeniesiony do świeżego dołka, a absorbancja ekstraktów została zmierzona przy użyciu wielodołkowego spektrofotometru skanującego Synergy.

W celu oceny mikroskopowej komórki utrwalono 4% paraformaldehydem na 1 godzinę w temperaturze pokojowej. Po utrwaleniu komórki inkubowano z 0,5% Tritonem X100 w PBS przez 30 minut w temperaturze pokojowej. Następnie komórki poddano dodatkowemu barwieniu za pomocą odczynnika DAPI. Krople lipidowe były obrazowane przy użyciu mikroskopu odwróconego (CKX53 Olympus, Hamburg, Niemcy).

8.8. Analiza statystyczna

Analiza statystyczna została wykonana z użyciem testów parametrycznych, w szczególności ANOVA, wraz z zastosowaniem odpowiednich testów post hoc. Te metody statystyczne zostały wybrane ze względu na normalny rozkład danych i równość wariancji w zbiorze danych. Wyniki zostały przedstawione z uwzględnieniem średniego odchylenia standardowego. Analiza statystyczna została przeprowadzona przy użyciu oprogramowania Statistica 13.1 firmy Dell Software Inc. z Port St. Lucie, FL, USA, a także GraphPad Prism 9 (GraphPad Software, Boston, MA, USA). Dla każdego warunku eksperymentalnego przeprowadzono co najmniej trzy niezależne pomiary w celu zapewnienia wiarygodności wyników. Poziomy istotności oznaczono w następujący sposób: * $p < 0.05$; ** $p < 0,01$; *** $p < 0,001$.

9. Omówienie publikacji wchodzących w skład rozprawy doktorskiej i uzyskanych wyników

9.1. Publikacja 1

Pierwszym artykułem opublikowanym w ramach cyklu publikacji jest praca przeglądowa pt.: *Molecular Mechanism of Lipotoxicity as an Interesting Aspect in the Development of Pathological States—Current View of Knowledge*, opublikowana w czasopiśmie Cells w roku 2022. Jej celem była analiza dostępnej literatury i zbiór aktualnej wiedzy na temat wpływu lipotoksyczności na homeostazę metabolizmu komórkowego. Dodatkowo, poszukiwana była odpowiedź na pytanie, czy nasycone kwasy tłuszczowe wywołują zaburzenia funkcji komórek niezależnie od rodzaju tkanki/narządu. W kontekście zgłębiania wiedzy na temat lipotoksyczności, a także niniejszej rozprawy doktorskiej, poznanie szczegółowych mechanizmów toksycznego działania kwasów tłuszczowych było niezbędne do postawienia dalszych hipotez badawczych i skonstruowała odpowiednich modeli badawczych.

Prace oryginalne cytowane na łamach pracy przeglądowej dotyczą badań na liniach komórkowych pochodzenia ludzkiego, szczurzego oraz mysiego. Mimo znaczących różnic w budowie organizmów przedstawicieli tych gatunków, na poziomie komórkowym zachowane są te same cechy metaboliczne, uwzględniające takie procesy, jak β-oksydacja czy szlaki sygnałowe kinaz białkowych. Transport lipidów do i wewnątrz komórki również nie jest procesem specyficznym dla konkretnego typu komórek. Czołowy transporter kwasów tłuszczowych, FAT/CD36, został pierwotnie zaproponowany jako cel potencjalnych farmaceutyków mających łagodzić objawy chorób metabolicznych. Jednakże, ze względu na fakt, że obecność FAT/CD36 obserwowana jest w komórkach wielu rodzajów tkanek (również tych związanych z hematopoezą), terapia celująca w ten transporter byłaby nieskuteczna [76]. W modelach zakładających atenuację bądź delecję FAT/CD36 dochodziło między innymi do dyslipidemii czy stanu zapalnego [76]. Na przestrzeni ostatnich lat rozpoznano również dwie inne grupy transporterów kwasów tłuszczowych – FABP oraz FATP. Podobnie, jak w przypadku FAT/CD36, ekspresja tych transporterów zachodzi w komórkach wielu rodzajów tkanek, takich jak wątroby, serca, jelita, a także mózgu [77]. FAT/CD36 i FATP są transporterami zlokalizowanymi w błonie komórkowej i odpowiadają za transport kwasów tłuszczowych do komórki. Natomiast FABP mogą brać udział zarówno w transporcie do wnętrza komórki, jak i w jej obrębie. Zarówno FABP jak i FATP charakteryzuje szereg podtypów (FABP1, FABP2, itd.; FATP1, FATP2, itd). FATP5 wykazuje silną ekspresję w

wątrobie, a jego knock-out prowadzi do złagodzenia objawów NAFLD [78]. Występujący w komórkach nabłonka kanalików proksymalnych nerki podtyp FATP2 ma swój udział w rozwoju zwłóknienia wątroby, co czyni go potencjalnym celem terapii tego schorzenia [79]. Jednym z przedstawicieli transporterów odpowiedzialnych za wewnątrzkomórkowy transport FFA jest FABP4 (znany również pod nazwą A-FABP, *ang. adipocyte-FABP*). Jego ekspresja jest powszechna w makrofagach, adipocytach, a także komórkach mikrogleju [80, 81]. Zaobserwowano, że w komórkach makrofagów PA jest w stanie zwiększać ekspresję FABP4, a farmakologiczna inhibicja FABP4 prowadzi do zapobiega apoptozie komórek [80]. Co więcej, badania wskazują na to, że zwiększona transkrypcja CD36 jest związana z gromadzeniem się lipidów w hepatocytach myszy, a nadekspresja FABP typu sercowego w mysich podocytach zaburza metabolizm lipidów prowadząc do rozwoju stanu zapalnego i stresu oksydacyjnego [82, 83]. Szeroka gama izoform transporterów kwasów tłuszczowych, a także ich udział w regulacji różnorodnych procesów komórkowych, czyni je istotnymi punktami uchwytu dla badań nad etiologią chorób związanych z metabolizmem lipidów. Przyczynia się do tego również fakt, że są powszechnie obserwowane w wielu rodzajach komórek i tkanek, często niezależnie od ich funkcjonalności [20, 21].

Niezwykle istotnym procesem patologicznym, który ma wpływ na metabolizm komórkowy, jest stres oksydacyjny. Może on zostać wywołany przez szereg czynników stresorowych, jednakże wysokie stężenia kwasów tłuszczowych są jednymi z kluczowych czynników prowadzących do rozwoju tego zaburzenia i w konsekwencji do lipotoksyczności. Co więcej, stres oksydacyjny leży u podstaw etiologii wielu schorzeń, również chorób cywilizacyjnych, a także neurodegeneracyjnych. Kwerenda bibliograficzna i w tym przypadku wykazała, że proces ten zachodzi w wielu rodzajach komórek i tkanek. Podsumowanie zebranych informacji na temat mechanizmów rozwoju stresu oksydacyjnego związanego z lipotoksycznością, a także rodzaj komórek czy tkanek, w których został zaobserwowany, zostało zaprezentowane w tabeli 2.

Tabela 2. Wybrane mechanizmy lipotoksycznego stresu oksydacyjnego a także miejsca ich potwierdzonego wystąpienia.

Komórkowe mechanizmy stresu oksydacyjnego	**organizm**	**linia komórkowa**	**narząd/rodzaj komórek**	**piśmien-nictwo**
zwiększona produkcja ogólnej ilości ROS	człowiek	wyizolowane chondrocyty	chrząstka	[84]
		linia komórkowa Hep2G	wątroba	[85]
	szczur	wyizolowane miocyty	mięśnie szkieletowe	[86]
		linia komórkowa H9c2	serce	[87]

		linia komórkowa INS-1	trzustka	[88]
	mysz	wyizolowane kardiomiocyty	serce	[87]
		wyizolowane podocyty	nerka	[89]
zwiększona produkcja mitochondrialnych ROS	szczur	linia komórkowa L6	mięśnie szkieletowe	[90]
		linia komórkowa H9c2	serce	[91]
	mysz	wyizolowane kardiomiocyty	serce	[87]
		wyizolowane podocyty	nerka	[92]
aktywacja NOX	człowiek	linia komórkowa Hep2G	wątroba	[85]
		wyizolowane chondrocyty	chrząstka	[84]
	szczur	wyizolowane miocyty	mięśnie szkieletowe	[86]
		linia komórkowa H9c2	serce	[64]
	mysz	wyizolowane kardiomiocyty	serce	[87]
zmniejszenie funkcji ETC	szczur	wyizolowane miocyty	mięśnie szkieletowe	[86]
	mysz	wyizolowane kardiomiocyty	serce	[87]
zmniejszenie MtMP*	człowiek	linia komórkowa HUV-EC-C	śródbłonek	[93]
	szczur	linia komórkowa H9c2	serce	[87]
	mysz	wyizolowane podocyty	nerka	[92]
zmniejszona produkcja ATP	szczur	linia komórkowa H9c2	serce	[91]
		linia komórkowa L6	mięśnie szkieletowe	[90]
	mysz	wyizolowane podocyty	nerka	[92]
toksyczność zależna od żelaza	człowiek	linia komórkowa HUV-EC-C	śródbłonek	[14]
zwiększenie mitochondrialnej ilości Ca^{2+}	szczur	linia komórkowa H9c2	serce	[87]

* MtMP – potencjał błony mitochondrialnej (z ang. mitochondrial membrane potential)

Na podstawie zebranych informacji nasuwają się trzy główne wnioski: 1) indukcja stresu oksydacyjnego nie jest specyficzna dla konkretnego typu komórek i tkanek; 2) stres oksydacyjny może rozwijać się w komórkach w oparciu o wiele mechanizmów uruchamianych równolegle; 3) zwiększona produkcja ROS jest najbardziej powszechnym mechanizmem rozwoju OS w komórce. W publikacji przeglądowej również ujęto szkodliwy wpływ FFA na mitochondrialne DNA. Wpływ na mtDNA wykazują również NRTI, co sugeruje, że te leki mogą wywoływać podobny wpływ na komórki układu nerwowego. Rozwój OS i zmniejszona ilość prawidłowego mtDNA może przyczyniać się do upośledzenia funkcji mitochondriów. Jako że β-oksydacja i dalsze etapy metabolizmu kwasów tłuszczowych w celu ich wykorzystania jako źródła energii zachodzą właśnie w mitochondriach, stan zaburzonej funkcji mitochondriów jest potencjalnym czynnikiem powodującym zwiększanie się ilości lipidów w komórce. Skutkiem może być rozwój lipotoksyczności [14, 18, 19].

Insulinooporność jest zjawiskiem mającym ogromne znaczenie w zgłębianiu mechanizmów lipotoksyczności. Insulinooporność związana z lipotoksycznością objawia się między innymi zaburzeniem szlaku sygnałowego insuliny na różnych jego etapach. FFA wpływają na proces fosforylacji InsR oraz InsRS, powodując nieprawidłowe ich ufosforylowanie [42, 43, 94, 95]. Właściwa fosforylacja w miejscu tyrozynowym zostaje zablokowana, a nieprawidłowo ufosforylowane białka ulegają degradacji. Powoduje to niemożność dalszego przekazania sygnału za pośrednictwem PI3K. PI3K również wykazuje obniżoną aktywność pod wpływem FFA [42, 94]. Następnym elementem w kaskadzie sygnałowej jest Akt/PKB. W wielu badaniach wykazano obniżoną aktywność Akt/PKB pod wpływem zwiększonego poziomu kwasów tłuszczowych. Dane z piśmiennictwa potwierdzają zatem, że FFA oddziałuje na szeroki wachlarz elementów odpowiedzialnych za prawidłową funkcję insuliny [43, 44, 96, 97].

Z powodu szerokiej sieci powiązań szlaków metabolicznych, a także sygnałowych, IR jest również związana z OS i funkcją mitochondriów. Jak wykazano w badaniu przeprowadzonym na komórkach mięśni szkieletowych upośledzony metabolizm glukozy, który może być efektem wpływu FFA, przyczynia się również do rozwoju zaburzeń funkcji mitochondriów [98]. Kluczowym punktem wspólnym dla rozwoju IR oraz OS wywołanych przez FFA jest zwiększona produkcja ROS. Zarówno czynniki przyczyniające się do wzrostu stężeń ROS, jak i wpływ ROS na aktywację szlaków sygnałowych w komórce, przyczyniają się do rozwoju IR. Enzym oksydaza NADPH 2, którego aktywność zwiększa się pod wpływem FFA prowadząc do nasilonej generacji ROS, jest również powiązany z IR. Zahamowanie aktywności oksydazy NADPH 2 przyczyniło się do poprawy reakcji komórek mięśni szkieletowych na insulinę [99]. Wykazano również, że nasilona przez FFA aktywność oksydazy NADPH 3 i idący za tym wzrost poziomu ROS prowadzi do rozwoju IR. Jako mechanizm wyjaśniający badacze zaproponowali zdolność ROS do aktywacji kinazy białkowej JNK [48, 99]. Wzrost fosforylacji JNK prowadzi do nieprawidłowej tyrozynowej fosforylacji białek z grupy InsRS. Następstwem tego zjawiska jest zmniejszona aktywność dalszych kluczowych elementów szlaku sygnałowego insuliny, czyli PI3K oraz Akt/PKB. Innym punktem łączącym IR z OS, jest wywołana przez FFA aktywacja czynnika transkrypcyjnego NF-κB [27]. Poza czynnikami związanymi ze stresem oksydacyjnym, również aktywacja nietypowych izoform kinazy białkowej C (PKC, *ang. protein kinase C*) pod wpływem FFA została powiązana z rozwojem IR. Pośredni produkt metabolizmu lipidów – diacyloglicerol – ma potwierdzoną zdolność aktywacji nietypowych izoform PKC. Podwyższone stężenie FFA

w komórkach, a także infuzje lipidowe i dieta bogata w nasycone kwasy tłuszczone została skorelowana z nasiloną aktywnością izoform PKCε, PKCζ oraz PKCθ. Konsekwencją tego zjawiska jest zaburzenie szlaku sygnałowego insuliny poprzez redukcję aktywności PI3K, Atk/PKB, a także nieprawidłową fosforylację InsRS [94, 95, 96, 97].

Chociaż nasycone kwasy tłuszczowe odgrywają istotną rolę w strukturze organelli, szlakach sygnałowych i zaspokajaniu zapotrzebowania na energię, ich nadmiar w stosunku do zdolności komórki do magazynowania jest szkodliwy dla prawidłowego funkcjonowania komórki. Nadmiar FFA może być konsekwencją otyłości, diety wysokotłuszczowej lub dysfunkcji tkanki tłuszczowej objawiającej się zmniejszoną zdolnością magazynowania FFA i zwiększonym uwalnianiem FFA z adipocytów. Lipotoksyczność jest określana jako szkodliwe procesy spowodowane nadmiarem FFA w tkankach innych niż tłuszczowa, takich jak OS, stres ER, stan zapalny lub insulinooporność. Pomimo wielu prób przypisania konkretnego szlaku metabolicznego do mechanizmu toksycznego działania FFA, niniejszy przegląd pokazuje, że modulują one funkcje komórkowe poprzez różnorodne mechanizmy, powodując wiele równolegle występujących zaburzeń, które mają tendencję do wzajemnego oddziaływania, tworząc błędne koło samonapędzających się procesów lipotoksycznych. W związku z tym sformułowanie skutecznej metody łagodzenia efektów lipotoksyczności wydaje się być niezwykle trudne, ponieważ wyciszenie jednej szkodliwej kaskady sygnalizacyjnej nie gwarantuje, że inne sygnały nie zakłócą funkcji komórki. Co więcej, wspomniana sieć powiązań między procesami indukującymi lipotoksyczność implikuje istnienie wielu czynników, które poprzez zakłócenie równowagi lipidowej, mogą przyczyniać się do upośledzenia funkcji komórki [12].

Szersze poznanie wzajemnych powiązań molekularnych mechanizmów indukujących lipotoksyczność może dostarczyć istotnych informacji na temat rozwoju szeregu chorób. Oprócz najbardziej oczywistych chorób związanych z zaburzoną gospodarką lipidową, takich jak cukrzyca typu 2 czy NAFLD, mechanizmy lipotoksyczności mogą wyjaśnić jeszcze nieznaną etiologię innych chorób, pozornie nie związanych z metabolizmem lipidów, co stanowi dalsze interesujące kierunki badań w tej dziedzinie.

9.2. Publikacja 2

Drugim artykułem opublikowanym w ramach cyklu publikacji jest praca oryginalna pt.: *The influence of nucleoside reverse transcriptase inhibitors on mitochondrial activity, lipid content, and fatty-acid-binding protein levels in microglial HMC3 cells*, opublikowana w czasopiśmie Pharmaceuticals w 2023 roku. Wykorzystując aktualną wiedzę na temat mechanizmów rozwoju lipotoksyczności, opartą na przeglądzie piśmiennictwa dokonanym w publikacji nr 1, postanowiono ocenić wpływ wybranych substancji, tj. 3TC, AZT i PA na funkcję mitochondriów, zawartość lipidów w komórce oraz ekspresję kinaz białkowych z grupy MAPK. W celu weryfikacji postawionych założeń wykonano następujące oznaczenia:

- żywotność komórek,
- zawartość mitochondrialnej frakcji anionorodnika ponadtlenkowego oraz ATP,
- wewnątrzkomórkowe stężenie FABP4 oraz FABP5,
- poziom fosforylacji kinaz białkowych z rodziny MAPK,
- zawartość lipidów w komórce.

Badania przeprowadzono na linii komórkowej HMC3, w warunkach które są szczegółowo opisane w rozdziale 7 niniejszej rozprawy. W tabeli 3 zebrano wyniki dla poszczególnych oznaczeń ww. parametrów przedstawione jako procent w stosunku do kontroli, którą stanowiły komórki nie poddane działaniu badanych substancji (inkubacja bez dodatku substancji badanych).

Tabela 3. Zestawienie wyników pomiarów przedstawionych jako procent zmian w stosunku do kontroli.

Rodzaj badania	**Badana substancja**					
	3TC	AZT	PA200	PA500	DHA200	DHA500
żywotność	100%	100%	72%***	36%***	85%**	90%***
ATP	98%	92%*	97%*	100%	85%**	50%***
$O_2^{\cdot-}$	108%*	115%	101%	90%	125%*	87%
FABP4	61%*	59%*	42%**	40%**	48%***	36%**
FABP5	43%*	46%*	37%*	42%*	40%*	53%*
ERK	116%	119%	107%	147%*	72%	74%
p38	92%	92%	95%	88%	94%	95%
JNK	74%	95%	65%*	74%	120%	65%
zawartość lipidów	113%*	170%***	94%	92%	68%**	64%**

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Zgodnie z wyjściowymi założeniami (opisanymi szczegółowo w rozdziale 6 niniejszej rozprawy), test cytotoksyczności wykazał negatywny wpływ PA na przeżywalność komórek mikrogleju rosnący wraz z zastosowanym stężeniem PA. Nie zaobserwowano natomiast działania cytotoksycznego badanych NRTI. Pomimo, że wyniki te pokrywają się z wynikami uzyskanymi przez Akay et al. [12] po ekspozycji komórek gleju na wybrane NRTI przez 48 godzin, to Hung et al. [49] wykazali, że po dłuższych okresach inkubacji (14 dni) wywołują one cytotoksyczność w neuronach kory mózgu. Dlatego też, nie można jednoznacznie wykluczyć cytotoksycznego wpływu 3TC i AZT na komórki mikrogleju.

Zaobserwowanie obniżonego mitochondrialnego poziomu ATP w komórkach mikrogleju po inkubacji z AZT jest zgodne z założeniami niniejszej rozprawy doktorskiej. Udział NRTI w zaburzeniach funkcji mitochondriów i rozwoju stresu oksydacyjnego jest dobrze poznanym działaniem ubocznym tej grupy farmaceutyków. Na podstawie wykonanych badań na linii komórkowej HMC3 można wnioskować, że zydowudyna przyczynia się do zaburzenia funkcji mitochondriów, czego bezpośrednim efektem byłaby uboższa synteza głównego źródła energii jakim jest ATP. W przypadku 3TC, pomiar stężenia ATP nie wykazał żadnej istotnej zmiany w stosunku do kontroli. Wynik taki stoi w sprzeczności z badaniem przeprowadzonym na hepatocytach w którym wykazano, że lamiwudyna jest w stanie zmniejszyć produkcję ATP [101].

Nie udało się też potwierdzić założenia, że inkubacja z PA spowoduje znaczące obniżenie stężenia ATP w komórkach. Jedynie PA w stężeniu 200 μM wywołało nieznaczne obniżenie, natomiast PA w stężeniu 500 μM nie wywarło żadnego wpływu. Badania przeprowadzone na innych typach komórek (chondrocyty, komórki mięśni szkieletowych) wykazały, że PA wpływa na stężenie ATP w komórce [100, 101] powodując obniżenie jego zawartości. Jednakże Zezina et al. [102] zaobserwowali, że inkubacja makrofagów z PA nie wpłynęła na poziom wewnątrzkomórkowego stężenia ATP.

Na podstawie poczynionych obserwacji można wysunąć przypuszczenie, że wpływ PA na zdolność mitochondriów do produkcji ATP wynika z różnic między komórkami poszczególnych tkanek, co wiąże się ze zróżnicowaną homeostazą lipidów i zdolnością do produkcji energii w tych komórkach.

Dla oceny wpływu wybranych związków na funkcje mitochondriów, zmierzono również zawartość anionorodnika ponadtlenkowego w mitochondriach. Rezultaty potwierdzają negatywny wpływ zarówno zydowudyny, jak i lamiwudyny, skutkujący zwiększeniem ilości

tej formy ROS, co potencjalnie może przyczyniać się do rozwoju OS. W przypadku PA, ponownie nie stwierdzono spodziewanego wyraźnego szkodliwego jego wpływu, które objawiałoby się zwiększeniem ilości wytwarzanego anionorodnika. W opozycji do tych danych stoi badanie przeprowadzone przez Vázquez-Mosquera [100] na chondrocytach, w których zaobserwowano, że PA zwiększa zawartość anionorodnika ponadtlenkowego. Możliwym wyjaśnieniem wyników uzyskanych w publikacji nr 2 jest większa odporność komórek mikrogleju na toksyczny wpływ PA w stosunku do innych rodzajów komórek. Jednakże, żeby to zweryfikować konieczne byłoby wykonanie dodatkowych badań.

FABP4 jest znanym czynnikiem odgrywającym rolę w hamowaniu procesu lipogenezy i promowaniu lipolizy, przez co ma znaczący wpływ na homeostazę FFA w komórce i krwioobiegu [103, 104]. Transporter ten ma również swój udział w rozwoju zaburzeń metabolizmu komórkowego, takich jak stres ER, OS, produkcja ROS oraz stan zapalny. Wzrost stężenia FABP4 może wskazywać na rozwój ww. zaburzeń metabolicznych [20]. Badania nad rolą FABP5 również wskazują, że wzrost jego stężenia obserwuje się przy zbyt dużej zawartości lipidów w organizmie, nieprawidłowym profilu lipidowym, chorobach układu krążenia [105]. Co więcej, FAPB5 wykazuje wysoką ekspresję w rozwijającym się mózgu i dorosłym mózgu, a jego obecność jest obserwowana zarówno w neuronach, jak i komórkach mikrogleju [106]. Wskazuje się również na synergię między FABP4 a FABP5 promującą stan zapalny, zaburzenia metaboliczne, również takie prowadzące do rozwoju miażdżycy [103].

Zmierzone stężenie FAPB4 i FABP5 w komórkach HMC3 po inkubacji ze wszystkimi badanymi związkami okazały się znacznie obniżone. Zatem hipoteza wyjściowa zakładająca negatywny wpływ NRTI i FFA na metabolizm komórki poprzez zwiększenie stężenia FABP4 i FABP5 nie została potwierdzona. W przypadku PA doniesienia literaturowe wykazują na odwotną zależność czyli wzrost stężenia FABP4 [80, 107]. Badania ekspresji genu kodującego FABP4 pod wpływem NRTI wykazały zarówno jej wzrost, jak i spadek [7, 108]. Natomiast zgodnie z moją wiedzą, nie przeprowadzono wcześniej badań wpływu NRTI na stężenia FABP5. Na podstawie niniejszych obserwacji możnaby wysunąć wniosek, że NRTI nie zaburzają funkcji komórek mikrogleju poprzez wpływ na ekspresję FABP4 i FABP5.

Badanie wpływu NRTI na fosforylację kinaz białkowych z grupy MAPK nie wykazało żadnych istotnych zmian. Jedynie w przypadku lamiwudyny zaobserwowano obniżenie poziomu fosforylacji JNK, jednakże bez cech istotności statystycznej, dlatego też, żeby jednoznacznie stwierdzić, czy wybrane NRTI wpływają na aktywację kinaz MAPK w mikrogleju, należałoby wykonać dodatkowe pomiary aktywności wybranych kinaz. Stężenie

PA 500 µM doprowadziło natomiast do znacznego wzrostu fosforylacji kinazy regulowanej sygnałem zewnątrzkomórkowym (ERK, *ang. extracellular signal-regulated kinase*). Nadmierna i długotrwała aktywacja ERK w komórce może wiązać się z nasilonym procesem starzenia się komórki, dlatego wpływ ten może wiązać się z negatywnym wpływem PA na metabolizm mikrogleju. Dodatkowo, zaobserwowano hamujący wpływ PA w stężeniu 200 µM na fosforylację kinazy JNK, przeciwnie do DHA w tym stężeniu, który nasilił aktywność JNK. Jako że JNK jest kinazą ulegającą aktywacji w obecności czynnika stresorowego, wyższy poziom fosforylacji nie jest zjawiskiem pożądanym. Tym bardziej istotne w kontekście niniejszej rozprawy doktorskiej jest przypuszczenie, że aktywacja JNK stanowi podwalinę chorób neurodegeneracyjnych z powodu jej wpływu na apoptozę neuronów [109]. Uzyskane wyniki dotyczące wpływu FFA na aktywację JNK są jednak niejednoznaczne.

Hipoteza wyjściowa zakładała, że wybrane NRTI spowodują wzrost zawartości lipidów w komórkach mikroglejów i taki wpływ udało się potwierdzić, obserwując nieznaczne zwiększenie gromadzenia się lipidów dla lamiwudyny i dużo większy dla zydowudyny. Jednak w obliczu ujemnego wpływu tych leków na ekspresję FABP4 i FABP5 i potencjalnego hamowania procesu lipolizy, sam proces składowania lipidów w kroplach lipidowych może być pozbawiony lipotoksycznego wpływu na metabolizm komórek mikrogleju. W badanych stężeniach i czasie inkubacji nie zaobserowowano wpływu PA na zawartość lipidów w komórce, natomiast DHA doprowadził do znaczacego jej zmniejszenia. W przypadku DHA uzyskany wynik jest zgodny z oczekiwaniami i można wysunąć przypuszczenie, że wpływa on pozytywnie na homeostazę lipidów w mikrogleju.

Tabela 4 przedstawia graficznie w sposób uproszczony wpływ badanych substancji na oznaczane parametry.

Tabela 4. Zestawienie efektów wywołanych w komórkach mikroglejowych przez NRTI i FFA po 24-godzinnej inkubacji. Efekty, dla których uzyskano istotność statystyczną, przedstawiono w pogrubieniu.

Rodzaj badania	**Badana substancja**					
	3TC	**AZT**	**PA200**	**PA500**	**DHA200**	**DHA500**
żywotność	brak efektu	brak efektu	**↓ spadek**	**↓ spadek**	**↓ spadek**	**↓ spadek**
ATP	brak efektu	**↓ spadek**	↓ spadek	brak efektu	**↓ spadek**	**↓ spadek**
$O_2^{\bullet-}$	**↑ wzrost**	↑ wzrost	brak efektu	↓ spadek	**↑ wzrost**	↓ spadek
FABP4	**↓ spadek**	**↓ spadek**	**↓ spadek**	**↓ spadek**	**↓ spadek**	**↓ spadek**
FABP5	**↓ spadek**	**↓ spadek**	**↓ spadek**	**↓ spadek**	**↓ spadek**	**↓ spadek**
ERK	↑ wzrost	↑ wzrost	↑ wzrost	**↑ wzrost**	↓ spadek	↓ spadek

p38	brak efektu	brak efektu	↓ spadek	↓ spadek	↓ spadek	↓ spadek
JNK	↓ spadek	brak efektu	**↓ spadek jak BSA**	brak efektu	**↑ wzrost**	↓ spadek jak BSA
zawartość lipidów	**↑ wzrost**	**↑ wzrost**	brak efektu	brak efektu	**↓ spadek**	**↓ spadek**

Podsumowując, lamiwudyna i zydowudyna nie przyczyniają się do rozwoju zaburzeń w komórkach mikrogleju z udziałem transporterów FABP4, FABP5, ani nie powodują zmniejszonej żywotności komórek mikrogleju. Prowadzą jednak do zwiększonego gromadzenia się lipidów w komórce, co może przyczyniać się do rozwoju lipotoksyczności w przypadku zachwiania innych mechanizmów odpowiedzialnych za homeostazę lipidów. NRTI wpływają również negatywnie na funkcje mitochondriów, powodując spadek stężenia ATP w przypadku AZT, a także wzrost ilości anionorodnika ponadtlenkowego.

Pewnym zaskoczeniem jest obserwacja, że PA nie powoduje zwiększonego gromadzenia się lipidów w komórce, a także obniża wewnątrzkomórkowe stężenie FABP4 i FABP5. Przyczyną takich wyników może być zbyt krótki okres inkubacji komórek bądź zwiększona odporność mikrogleju na toksyczne działanie tego kwasu tłuszczowego.

Odwrotny wpływ FFA i NRTI na stężenie anionorodnika ponadtlenkowego i zawartość lipidów w komórce sugeruje, że te grupy substancji nie działają toksycznie w oparciu o te same mechanizmy. Dalsze wyjaśnienie zależności między tymi grupami substancji w aspekcie wp ływu na metabolizm mikrogleju wymaga przeprowadzenia dalszych badań.

9.3. Publikacja 3

Praca oryginalna pt.: *Investigating the role of nucleoside reverse transcriptase inhibitors in modulating lipotoxicity: effects on lipid dynamics stress pathways, and insulin resistance on the function of dopaminergic neurons*, jest trzecim i ostatnim artykułem opublikowanym w ramach monotematycznego cyklu publikacji. Jej celem była ocena wpływu lamiwudyny, zydowudyny oraz kwasu palmitynowego na funkcje komórek neuronów dopaminergicznych. W pracy tej oznaczane parametry dobrano tak, aby móc zbadać potencjalny szkodliwy wpływ badanych substancji na homeostazę lipidową komórkek, poziom produkcji wolnego rodnika w mitochondriach oraz aktywność szlaku sygnałowego insuliny. Jako model posłużyła zróżnicowana linia komórkowa SH-SY5Y. Aby ocenić wpływ badanych substancji na neurony dopaminergiczne, wykonano następujące oznaczenia:

- żywotność komórek,
- zawartość mitochondrialnej frakcji anionorodnika ponadtlenkowego,
- stężenie FABP4 oraz FABP5,
- poziom fosforylacji kinaz białkowych z rodziny MAPK,
- poziom fosforylacji PI3K,
- stężenie InsRS1,
- ekspresję InsR,
- zawartość lipidów w komórce.

W tabeli 5 zaprezentowano wyniki pomiarów ww. parametrów jako procent w stosunku do kontroli komórkowej, którą stanowiły komórki neuronów dopaminergicznych inkubowane bez dodatku badanych substancji.

Tabela 5. Zestawienie wyników pomiarów przedstawionych jako procent zmian w stosunku do kontroli.

Rodzaj badania	**Badana substancja**					
	3TC	AZT	PA200	PA500	DHA200	DHA500
żywotność	100%	100%	102%	103%	115%*	111%
$O_2^{\bullet-}$	105%	118%***	85%**	83%***	88%**	86%**
FABP4	76%	70%**	70%**	45%***	60%***	37%***
FABP5	92%	97%	69%*	75%*	74%*	67%**
ERK	211%*	105%	99%	73%	95%	75%
p38	131%	127%	101%	95%	141%	197%
JNK	139%	243%	126%	105%	159%	243%
zawartość lipidów	85%*	92%	81%**	71%***	76%***	75%***

PI3K	146%*	118%	96%	56%*	-	-
InsR	78%	81%	70%	73%	81%	66%*
InsRS1	78%	88%	86%	186%	97%	75%

Pierwszą zauważalną obserwacją jest brak wpływu NRTI na żywotność komórek neuronów dopaminergicznych, co jest w opozycji do postawionej hipotezy, że wybrane NRTI będą miały działanie cytotoksyczne. Jednakże, zwraca uwagę fakt, że PA w badanych warunkach eksperymentalnych również nie wykazało takich właściwości, pomimo licznych doniesień literaturowych, które je potwierdzają [110, 111, 112]. Sugerowałoby to, że warunki ekspretymentalne mogły być niewystarczające aby zaobserwować cytotoksyczne działanie PA, dlatego też wynik ten nie będzie poddawany dalszej analizie.

Badanie wpływu NRTI oraz PA na zawartość mitochondrialnej frakcji anionorodnika ponadtlenkowego wykazało, że zarówno lamiwudyna jak i zydowudyna powodują wzrost jego ilości, w opozycji do PA, który doprowadził do spadku jego ilości w neuronach dopaminergicznych. W tym przypadku wyniki uzyskane dla NRTI są zgodne z hipotezą wyjściową, a także badaniem przeprowadzonym przez Kohler et al. [113], zakładającą ich udział w rozwoju zaburzeń mitochondriów poprzez indukcję generacji ROS. Mitochondrialny poziom anionorodnika ponadtlenkowego może być zwiększony z powodu wielu różnych czynników związanych z dysfunkcją mitochondriów i stresem komórkowym. Ponieważ mechanizm działania NRTI obejmuje hamowanie mtDNA, istotną przyczyną zwiększonego poziomu ROS może być gromadzenie się dysfunkcyjnych białek, upośledzone utlenianie kwasów tłuszczowych i upośledzona fosforylacja oksydacyjna. Niespodziewaną obserwacją jest wpływ FFA na poziom ponadtlenku, prowadzący do jego obniżenia. Pomimo wielu doniesień o udziale PA w indukcji stresu komórkowego, niewiele jest badań przeprowadzonych na neuronach dopaminergicznych. Ng et al. [114] wykazali, że PA prowadzi do rozwoju OS w komórkach linii SH-SY5Y, natomiast potwierdzony był jedynie wzrost poziomu H_2O_2. Jest zatem prawdopodobne, że PA jest w istocie w stanie wywołać OS w neuronach dopaminergicznych wpływając na inne frakcje ROS niż ujęte w niniejszym badaniu. Dlatego też, niezbędne byłoby wykonanie dodatkowych oznaczeń innych form ROS, żeby bardziej szczegółowo przeanalizować wpływ PA i uzyskać jednoznaczną odpowiedź. Zmniejszenie generacji ponadtlenku może jednak przyczynić się do łagodzenia toksycznego działania NRTI względem mitochondriów, jednakże aby wysunąć taki wniosek należałoby rozszerzyć badania w tym kierunku.

FABP4, czyli białko wiążące kwasy tłuszczowe adipocytów, wywiera znaczący wpływ na metabolizm lipidów m.in. poprzez osłabienie lipogenezy i nasilenie lipolizy. Co więcej, zmieniające się poziomy tego białka zostały skorelowane ze zwiększonym ryzykiem chorób sercowo-naczyniowych, cukrzycy typu 2 i niealkoholowej stłuszczeniowej choroby wątroby [20, 103, 104]. Zarówno AZT, jak i 3TC wykazały zauważalny spadek stężenia FABP4 w dopaminergicznych komórkach neuronalnych, co jest działaniem przeciwnym do spodziewanego. Może to wskazywać na hamowanie procesu lipolizy, a tym samym zmniejszone uwalnianie FFA do krwiobiegu z komórek. Taki efekt może mieć dwojaki wpływ na homeostazę lipidów. Po pierwsze, może prowadzić do zmniejszonej dostępności FFA do wytwarzania energii, tym samym zaburzając metabolizm energetyczny komórki, szczególnie w tkankach zależnych od kwasów tłuszczowych jako głównego źródła energii. W przypadku komórek OUN jednak, głównym źródłem energii jest glukoza, dlatego też zahamowanie lipolizy może nie przyczyniać się do zaburzeń metabolicznych. Z drugiej strony, zmniejszony rozpad trójglicerydów może potencjalnie obniżyć ryzyko lipotoksyczności i związanych z nią zaburzeń, takich jak insulinooporność czy stan zapalny. Efekt obniżający stężenie FABP4 uznać można za korzystny, nie powodujący rozwoju lipotoksyczności. Należy jednak brać pod uwagę, że uzyskane rezultaty nie odwzierciedlają w wierny sposób homeostazy całego organizmu, co jest szeroko rozpoznaną wadą linii komórkowych jako modelu badawczego. Dodatkowo, inkubacja trwająca 24 godziny może być niewystarczająca, aby zaobserwować długofalowe efekty działania NRTI, co skłania do kontynuacji badań.

W opozycji do postawionej hipotezy, również PA obniża stężenie FABP4, w stopniu znacznie większym niż NRTI. PA ma potwierdzone działanie zwiększające ekspresję transportera FABP4, co wykazano w badaniach na innych liniach komórkowych w tym samym stężeniu i czasie inkubacji [80, 107]. Dlatego też, można przypuszczać, że komórki neuronów dopaminergicznych nie ulegają toksycznemu działaniu PA za pośrednictwem FABP4.

FAPB5 wykazuje wysoką ekspresję w rozwijającym się mózgu i dorosłym mózgu, a jego obecność jest obserwowana zarówno w neuronach, jak i komórkach mikrogleju [115]. Moje badanie wykazało, że 3TC i AZT nie miały zauważalnego wpływu na stężenie FABP5, co sugeruje, że albo nie ma związku między NRTI a badanym białkiem, albo warunki badań wykonanych w ramach tej pracy nie wychwytuje tego związku. Obniżenie stężenia FABP5 pod wpływem inkubacji z PA nie jest zgadne z postawionymi założeniami, a także z doniesieniami literaturowymi wykazującymi wzrost stężenia FABP5 [106, 116]. Jednakże, w związku z niewielką liczbą badań przeprowadzonych na komórkach neuronalnych, można przypuszczać,

że neurony dopaminergiczne nie odpowiadają na PA w ten sam sposób, co komórki innych tkanek.

Spośród zmierzonych ufosforylowanych form kinaz białkowych MAPK, jedynie oddziaływanie 3TC na ERK, powodujące 2,5-krotny wzrost fosforylacji, wykazało istotność statystyczną. W aspekcie etiologii chorób neurodegeneracyjnych, najważniejszą konsekwencją nadmiernej aktywacji ERK jest starzenie się komórki, co przejawia się zahamowaniem wzrostu, a także wydzielaniem czynników prozapalnych. Stan ten jest skorelowany z chorobami wieku podeszłego [117].

Po inkubacji ze wszystkimi badanymi substancjami całkowita zawartość lipidów w neuronach dopaminergicznych była mniejsza w porównaniu z kontrolą. Biorąc pod uwagę, że zarówno stężenie FABP4, jak i zawartość lipidów uległy zmniejszeniu pod wpływem wszystkich badanych związków, można wyciągnąć wniosek, że wybrane NRTI wpływają na komórki dopaminergiczne w podobny sposób jak PA. Odnosząc te wyniki do ogólnego obrazu lipotoksyczności, NRTI mogą odgrywać rolę w jej rozwoju poprzez uwalnianie FFA z komórek (a tym samym wyczerpywanie potencjalnego depozytu energii) i dalszego uwalniania FFA do krwiobiegu. Zwiększone stężenie wolnych FFA w krwiobiegu może przyczyniać się do ektopowego odkładania się lipidów, skutkującego potencjalnym rozwojem chorób związanych z lipotoksycznością.

PI3K jest częścią większej rodziny kinaz lipidowych, które fosforylują 3' grupę hydroksylową fosfoinozytydów, wytwarzając fosfatydyloinozytolo-3,4,5-trifosforan, kluczowy drugorzędowy przekaźnik zaangażowany w wewnątrzkomórkowe szlaki sygnałowe. Jednym z głównych szlaków, na które wpływa PI3K, jest szlak Akt/mTOR. Aktywowana Akt/PKB reguluje szeroki wachlarz substratów zaangażowanych w metabolizm glukozy i lipidów, oraz syntezę białek. Jeśli chodzi o metabolizm lipidów, sygnalizacja PI3K/Akt prowadzi do aktywacji liazy cytrynianowej ATP i karboksylazy acetylo-koenzymu A, które są kluczowe dla syntezy kwasów tłuszczowych *de novo*. Co więcej, sygnalizacja PI3K/Akt promuje ekspresję białek wiążących sterolowe elementy regulatorowe, które są czynnikami transkrypcyjnymi regulującymi ekspresję genów zaangażowanych w biosyntezę lipidów. PI3K jest również kluczowym elementem szlaku sygnałowego insuliny. Zwiększony stosunek formy ufosforylowanej do całkowitej ilości PI3K, Akt/PKB, a także zwiększona ekspresja InsR i InsRS1 zostały powiązane z potencjalną aktywnością przeciwcukrzycową poprzez przywracanie wrażliwości komórek na insulinę [118, 119]. W warunkach przeprowadzonego badania lamiwudyna i zydowudyna doprowadziły do zwiększenia stosunku ufosforylowanej

formy PI3K do całkowitej ilości w stosunku do kontroli. Pobudzenie tego elementu szlaku sygnałowego Akt/PI3K sugeruje, że wybrane NRTI nie mają udziału w indukcji insulinooporności na podstawie tego konkretnego mechanizmu. Co więcej, mogą one wspomagać metabolizm komórkowy poprzez usprawnienie szlaku sygnałowego insuliny, zgodnie z sugestią przedstawioną w pracy Tong et al. [118]. Podobnie, Li i wsp. [120] wykazali, że leczenie 3TC było w stanie zwiększyć aktywność Akt/PKB, która została uprzednio wyciszona farmakologicznie w hipokampie i korze myszy. W opozycji stoi badanie Mohana et al. [121], gdzie zaobserwowano, że 3TC zmniejszała aktywność zarówno PI3K, jak i Akt/PKB w ludzkich komórkach wątrobiaka. Co więcej, Shimizu i wsp. [122] wykazali, że 3TC nie miała wpływu na Akt/PKB w modelach wątrobowokomórkowych. Przeciwstawne wyniki sugerują, że wpływ 3TC na PI3K może różnić się w zależności od typu komórki, warunków eksperymentalnych lub dodatkowych czynników wpływających na regulację PI3K. Zgodnie z założeniami moich badań, PA zmniejszył aktywność PI3K, potencjalnie prowadząc do mniejszej zdolności komórki do radzenia sobie w warunkach nadmiaru glukozy i kwasów tłuszczowych, a co za tym idzie rozwoju insulinooporności.

Ocena wpływu badanych związków na InsR i InsRS1 w neuronach dopaminergicznych wykazała, że inkubacja z NRTI nie wpłynęła zauważalnie na ekspresję InsRS1, wykazano za to niewielki spadek ekspresji InsR. Ponieważ jednak wyniki te nie osiągnęły istotności statystycznej, obserwacja ta może nie odzwierciedlać rzeczywistej zależności pomiędzy NRTI a ekspresją InsR. Dlatego jakikolwiek potencjalny wpływ 3TC i AZT na InsR i InsRS1 w neuronach dopaminergicznych pozostaje przypuszczalny i wymaga dslszych badań.

W tabeli 6 przedstawiono graficznie uproszczone zestawienie efektów wywołanych przez badane związki w komórkach SH-SY5Y.

Tabela 6 Zestawienie efektów wywołanych w komórkach neuronów dopaminergicznych przez NRTI i FFA po 24-godzinnej inkubacji. Efekty, dla których uzyskano istotność statystyczną, przedstawiono w pogrubieniu.

Rodzaj badania	**Badana substancja**					
	3TC	**AZT**	**PA200**	**PA500**	**DHA200**	**DHA500**
żywotność	brak efektu	brak efektu	brak efektu	brak efektu	↑ **wzrost**	↑ wzrost
$O_2^{\bullet-}$	↑ wzrost	↑ **wzrost**	↓ **spadek**	↓ **spadek**	↓ spadek	↓ **spadek**
FABP4	↓ spadek	↓ **spadek**	↓ **spadek**	↓ **spadek**	↓ **spadek**	↓ **spadek**
FABP5	brak efektu /	brak efektu /	↓ **spadek**	↓ **spadek**	↓ **spadek**	↓ **spadek**

	spadek	spadek				
ERK	↑ **wzrost**	↑ wzrost	brak efektu	↓ spadek	brak efektu	↓ spadek
p38	↑ wzrost	↑ wzrost	brak efektu	brak efektu	↑ wzrost	↑ wzrost
JNK	↑ wzrost	↑ wzrost	↑ wzrost	brak efektu	↑ wzrost	↑ wzrost
zawartość lipidów	↓ **spadek**	↓ spadek	↓ **spadek**	↓ **spadek**	↓ **spadek**	↓ **spadek**
PI3K	↑ **wzrost**	↑ wzrost	brak efektu	↓ **spadek**		
InsRS1	brak efektu	brak efektu	brak efektu	↑ wzrost	brak efektu	↓ spadek
InsR	↓ spadek	↓ spadek	↓ spadek	↓ spadek	↓ spadek	↓ **spadek**

Podsumowując, w badanych warunkach lamiwudyna i zydowudyna nie powodują rozwoju zaburzeń metabolicznych o podłożu lipotoksycznym w neuronach dopaminergicznych z udziałem FABP4, PI3K bądź wpływając na zawartość lipidów w komórce. Mogą jednak zakłócać funkcję neuronów poprzez zwiększoną produkcję ROS lub nasilając aktywację ERK. PA również nie powoduje zwiększenia stężenia FABP4, FABP5, a także nie nasila gromadzenia się lipidów, co sugeruje, że w badanych warunkach homeostaza transportu i magazynowania lipidów nie ulega istotnemu zakłóceniu w neuronach dopaminergicznych. PA przyczynił się jednak do obniżenia aktywności PI3K, a także w opozycji do założeń wyjściowych nie zwiększył generacji mitochondrialnego anionorodnika ponadtlenkowego. Wyniki badań wskazują na podobieństwa między działaniem NRTI a PA na neurony dopaminergiczne w zakresie transportu i magazynowania lipidów. Rezultaty stawiają jednak nowe pytania, czyniąc ten kierunek badań wartym dalszego zaangażowania i zgłębienia.

10. Podsumowanie

Zaburzenia funkcji mitochondriów

Badane NRTI - lamiwudyna i zydowudyna, spowodowały wzrost poziomu anionorodnika ponadtlenkowego w obu liniach komórkowych, podczas gdy PA przyczynił się do spadku poziomu tej formy ROS. Nie zaobserwowano w tym przypadku różnic pomiędzy komórkami neuronów dopaminergicznych a mikrogleju pod wpływem tej samej substancji. Widoczny jest jednak przeciwstawny wpływ NRTI i PA. Dodatkową obserwacją był spadek stężenia ATP w komórkach mikroglejowych pod wpływem AZT.

Stężenie FABP4 i FABP5

Oznaczone stężenia wybranych transporterów kwasów tłuszczowych nie uległy zwiększeniu po inkubacji z NRTI i PA zarówno w neuronach dopaminergicznych, jak i mikrogleju. Spodziewanym wynikiem były zwiększone stężenia FABP4 i FABP5 po inkubacji komórek z badanymi substancjami, co wskazywałoby na zaburzenia homeostazy lipidów w komórce i potencjalny rozwój lipotoksyczności. Założenia te nie zostały potwierdzone.

Zawartość lipidów

Pomiar zmian zawartości lipidów w komórkach linii SH-SY5Y pod wpływem badanych substancji wykazał, że ilość lipidów zmniejszyła się po inkubacji z NRTI i PA. Zaobserwowano tu różnicę pomiędzy komórkami neuronalnymi a mikroglejowymi. W linii komórkowej HMC3, 3TC i AZT przyczyniły się do wzrostu zawartości lipidów, podczas gdy PA nie wywał znaczącego efektu. Przyczyniać się do tego mogą różne funkcje pełnione przez neurony i mikroglej w OUN.

Insulinooporność

W komórkach neuronów dopaminergicznych, badane NRTI wykazały wzrost fosforylacji kinazy PI3K, jednego z kluczowych białek w szlaku sygnałowym insuliny, co sugeruje, że nie tylko nie mają one udziału w rozwoju insulinooporności, ale też poprawiają wrażliwość komórek na insulinę. W przypadku receptora InsR zaobserwowano jednak spadek jego ekspresji, natomiast stężenie InsRS1 nie uległo zmianie pod wpływem 3TC i AZT. W świetle uzyskanych wyników, udział badanych leków w rozwoju insulinooporności pozostaje zatem niejednoznaczny. W przypadku PA poziom fosforylacji PI3K uległ zmniejszeniu przy stężeniu 500 μM, podobnie jak poziom ekspresji InsR, co może być przesłanką na temat jego

potencjału zaburzającego szlaki sygnałowe insuliny. Natomiast, stężenie InsRS1 wzrosło po inkubacji z wyższym badanym stężeniem PA. Ze względu na różny wpływ na poszczególne elementy szlaku, wpływ PA również pozostaje niejasny i wymaga dalszych badań.

Jako uzupełnie informacji o wpływie badanych substancji na szlaki sygnałowe w komórce, oznaczono dodatkowo poziomy aktywności kinaz białkowych ERK, JNK i p38. Wyniki pokazały tendencję nasilającą aktywność badanych kinaz po inkubacji komórek z NRTI. Białka te mają swój udział w szlaku sygnałowym insuliny, natomiast nie jest to ich jedyna funkcja. ERK odpowiada za regulowanie wzrostu komórki i syntezy białek, a także za proces starzenia się komórki, natomiast JNK bierze udział w odpowiedzi komórki na czynniki stresorowe. Aktywacja kinaz białkowych z grupy MAPK po inkubacji z 3TC i AZT może nie być związana z ich wpływem na wrażliwość komórek na insulinę. Konieczne byłoby zbadanie innych elementów szlaków sygnałowych, aby móc jednoznacznie określić jakie zmiany powodują badane leki.

Różnice we działaniu NRTI i PA w zakresie prowadzonych badań

Wyniki badań wskazują, że wszystkie badane substancje działanią w podobny sposób na stężenia wybranych transporterów kwasów tłuszczowych w obu badanych liniach, a także na zawartość lipidów w komórkach neuronalnych. W przypadku mikrogleju wpływ ten różnił się – w linii HMC3 inkubacja z NRTI spowodowała wzrost zawartości lipidów, podczas gdy PA nie wykazało znaczących zmian. Niezależnie od badanej linii komórkowej, NRTI różniły się działaniem od PA również w zakresie indukowania zmian poziomu anionorodnika ponadtlenkowego.

3TC i AZT jako potencjalne czynniki neurodegeneracyjne

W zakresie wpływu na homeostazę lipidów w komórce, spodziewano się zaobserwować wzrost stężeń transporterów kwasów tłuszczowych, a także zwiększone gromadzenie się lipidów w komórkach neuronów dopaminergicznych pod wpływem 3TC i AZT. Takie zmiany sugerowałyby wpływ badanych NRTI na potencjalny rozwój lipotoksycznych zmian metabolicznych w komórkach, mogących prowadzić do apoptozy, a tym samym przyczyniać się do neurodegeneracji. Jednakże, uzyskane wyniki nie potwierdziły tej hipotezy. Wzrost zawartości lipidów w badanych warunkach zaobserwowano w komórkach mikroglejowych, dlatego też istnieje przesłanka, aby kontynuować badania w tym zakresie. Przypuszczalnie badane leki mogą przyczyniać się do rozwoju lipotoksyczności na drodze innych mechanizmów.

Efekt zwiększający poziom anionorodnika ponadtlenkowego w mitochondriach jest zgodny z postawionymi założeniami, potwierdzając, że 3TC i AZT mogą stanowić czynniki neurodegeneracyjne na drodze indukcji zaburzeń funkcji mitochondriów. Dodatkowo, AZT wykazała istotny statystycznie spadek stężenia ATP w mitochondriach w komórkach mikrogleju, co również stanowi potwierdzenie ww. założeń.

PA jako potencjalny czynnik neurodegeneracyjny

W przypadku PA, uzyskane wyniki nie pozwoliły na potwierdzenie założeń o jego udziale w rozwoju procesów neurodegeneracyjnych i lipotoksycznych w komórkach neuronów dopaminergicznych i mikroglejowych. Ze względu na dużą liczbę doniesień literaturowych na temat toksycznego działania PA na komórki różnych tkanek, zwłaszcza w podwyższonych stężeniach, wyniki te skłaniają do przypuszczenia, że ten kwas tłuszczowy może wywierać wpływ negatywny na komórki neuronalne i mikroglejowe, albo na drodze innych mechanizmów, albo też po dłuższym okresie inkubacji. Przesłanką do kontynuowania badań w tym zakresie jest również fakt powszechnie występującej u pacjentów zakażonych HIV lipodystrofii.

11. Wnioski

Na podstawie rezultatów uzyskanych z przeprowadzonych badań oceny wpływu lamiwudyny, zydowudyny i PA na komórki neuronów dopaminergicznych i mikrogleju można wyciągnąć następujące wnioski:

1. NRTI mogą przyczyniać się do rozwoju neurodegeneracji na drodze zaburzeń funkcji mitochondriów skutkujących zwiększeniem generacji ROS w komórkach układu nerwowego. Konsekwencją jest potencjalny rozwój stresu oksydacyjnego, co może prowadzić do apoptozy komórek i tym samym zmian neurodegeneracyjnych.
2. NRTI nie przyczyniają się do rozwoju neurodegeneracji ani lipotoksyczności poprzez rozwój zaburzeń transportu lipidów w komórkach układu nerwowego za pośrednictwem badanych transporterów kwasów tłuszczowych. Ich udział w rozwoju insulinooporności również nie został potwierdzony w badaniach na linii SH-SY5Y.
3. Wpływ NRTI na zawartość lipidów w komórce różni się od typu komórki. W komórkach neuronów dopaminergicznych wykazano zmniejszenie zawartości lipidów, podczas gdy w mikrogleju zaobserwowano zwiększenie. Wpływ na rozwój lipotoksyczności i idącej za tym neurodegeneracji jest zatem w tym zakresie niejednoznaczny i może wskazywać na większą podatność mikrogleju na ektopowe odkładanie się lipidów.
4. PA nie przyczynia się do rozwoju neurodegeneracji ani lipotoksyczności na drodze zaburzeń funkcji neuronów dopaminergicznych i mikrogleju w zakresie badanych szlaków metabolicznych. Może to świadczyć o mniejszej podatności tych typów komórek na toksyczne działanie PA w stosunku do innych typów komórek.

Pomimo, iż nie wszystkie założenia dotyczące wpływu wybranych NRTI na komórki układu nerwowego zostały potwierdzone, ich udział w rozwoju procesów neurodegeneracyjnych nie może zostać wykluczony. Rezultaty przeprowadzonych badań nie wskazują natomiast, aby NRTI pełniły istotną rolę w zaburzeniach komórkowej homeostazy lipidów, a co za tym idzie w rozwoju lipotoksyczności.

Co zaskakujące, wyniki uzyskane dla PA sugerują, że nie pełni on znaczącej roli w lipotoksycznych ani neurodegeneracyjnych zaburzeniach funkcji komórek neuronów i mikrogleju poprzez badane szlaki. Taka obserwacja wskazywałaby na to, że nie ma on udziału

w rozwoju procesów neurodegeneracyjnych. Jednakże, ze względu na niejednorodne dane z piśmiennictwa w tym zakresie, uzyskane wyniki skłaniają do kontynuacji badań ze względu na możliwość występowania toksycznych działań badanych substancji w dłuższym okresie inkubacji komórek z badanymi substancjami, a także na drodze innych mechanizmów rozwoju lipotoksyczności i neurodegeneracji.

12. Piśmiennictwo

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13. Załączniki

13.1. Załącznik 1. Publikacje wchodzące w skład rozprawy doktorskiej.

13.2. Załącznik 2. Oświadczenia współautorów publikacji.

13.3. Załącznik 3. Wykaz publikacji i abstraktów potwierdzony przez bibliotekę.

13.1. Załącznik 1. Publikacje wchodzące w skład rozprawy doktorskiej.

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Review

Molecular Mechanism of Lipotoxicity as an Interesting Aspect in the Development of Pathological States—Current View of Knowledge

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Abstract: Free fatty acids (FFAs) play numerous vital roles in the organism, such as contribution to energy generation and reserve, serving as an essential component of the cell membrane, or as ligands for nuclear receptors. However, the disturbance in fatty acid homeostasis, such as inefficient metabolism or intensified release from the site of storage, may result in increased serum FFA levels and eventually result in ectopic fat deposition, which is unfavorable for the organism. The cells are adjusted for the accumulation of FFA to a limited extent and so prolonged exposure to elevated FFA levels results in deleterious effects referred to as lipotoxicity. Lipotoxicity contributes to the development of diseases such as insulin resistance, diabetes, cardiovascular diseases, metabolic syndrome, and inflammation. The nonobvious organs recognized as the main lipotoxic goal of action are the pancreas, liver, skeletal muscles, cardiac muscle, and kidneys. However, lipotoxic effects to a significant extent are not organ-specific but affect fundamental cellular processes occurring in most cells. Therefore, the wider perception of cellular lipotoxic mechanisms and their interrelation may be beneficial for a better understanding of various diseases' pathogenesis and seeking new pharmacological treatment approaches.

Keywords: lipotoxicity; fatty acid; oxidative stress; insulin resistance; inflammation

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1. Introduction

The term "lipotoxicity" was first adopted in 1994 by Lee et al. in the context of elucidating the pathogenesis of obesity-related β-cell alterations both before and at the onset of type 2 diabetes mellitus (T2DM) [1]. This report was the first to associate increased plasma free fatty acids (FFA) with insulin resistance (IR) and β-cell unresponsiveness to hyperglycemia. Currently, due to further research, lipotoxicity is defined as the harmful effect of high concentrations of lipids and lipid derivatives manifested as a set of metabolic disorders in the cells of non-fatty tissues, causing disturbances in their metabolism and/or loss of function or apoptosis. This phenomenon most often affects the cells of the pancreas, liver, skeletal muscles, heart muscle, and kidneys. The cell of non-adipose tissues and organs, such as β-cells, hepatocytes, cardiomyocytes, podocytes, and myocytes, are able to store lipids to a limited extent. The mechanisms involved in lipotoxicity (also referred to as lipotoxic effects) include oxidative stress (OS), the stress of the endoplasmic reticulum (ER), induction of IR, or inflammation. Lipotoxicity is relatively well understood in the organs mentioned above, while there are very few comprehensive descriptions of the effects of lipotoxicity on cell metabolism. Although cell metabolism is based on the same pathways in every cell type, tissue-specific cell differences must be taken into account.

The molecular mechanisms of fatty acids' action vary due to chemical classification as saturated or unsaturated. The research confirms that most deleterious effects are provided by saturated fatty acids (such as palmitic acid, also referred to as palmitate), whereas unsaturated fatty acids relieve cells from lipotoxic effects. Oleic acid, containing one

unsaturated bond in its structure, was shown to be more steatogenic but less damaging than palmitic acid. Furthermore, oleic acid prevents oxidative stress and apoptosis caused by palmitic acid in human hepatocytes and rat and mouse myocytes [2–4]. This review focuses on the deleterious effects of saturated fatty acids on the molecular level; therefore, the term 'FFA' is understood in this article as saturated free fatty acids. Additionally, when mentioning the duration of FFA exposure, the term 'acute' is understood as less than 6 h; however, the term 'chronic' is understood as longer than 10 h.

A review of current knowledge about cellular mechanisms of lipotoxicity was performed. Although there are many existing reviews about lipotoxicity concerning the aforementioned organs, this is, to the best of our knowledge, the first review focusing on the description of the cellular mechanism underlying lipotoxicity.

2. The Role of Homeostasis Disturbances of Fatty Acids in Lipotoxicity

Fatty acids (FAs) occur in the organism in two main forms—free fatty acids (FFAs) circulating in plasma (bound to albumin as a lipoprotein particle) and triacylglycerol (TAG) depot in cytoplasm inside cells. After infiltrating the cell membrane through various transporters, FFAs are either oxidized in the mitochondria to generate adenosine triphosphate (ATP) or esterified in the cytoplasm with a glycerol particle, thus creating TAG. Research distinguishes three main types of fatty acids transporters: fatty acid translocase (FAT, also known as cluster of differentiation 36, CD36, or scavenger receptor), fatty acid binding protein (FABP), and fatty acid transport protein (FATP). Several subtypes of those transporters are specified depending on the main site of expression, e.g., FATP5 in the mouse liver, FATP2 in the mouse kidney, and human adipocyte FABP (A-FABP) [5–7]. The importance of those transporters comes down to regulating fatty acid influx by their increased or decreased expression. There is numerous evidence that lipid accumulation is connected to the expression of fatty acid transporters. Induced transcriptional activation of CD36 is associated with lipid accumulation in mouse hepatocytes, and overexpression of heart-type FABP in mouse podocytes disturbs lipid metabolism and aggravates inflammation and oxidative stress [8,9]. However, the knockout of FATP2 ameliorates mouse kidney fibrosis, and the downregulation of CD36 prevents lipid accumulation in rat cardiomyocytes and mouse macrophages [6,10,11]. FATPs and CD36 have also been recognized as contributing factors to the progression of nonalcoholic fatty liver disease (NAFLD) in a human clinical study [10].

Lipids are accumulated in the cell in the form of lipid droplets—the cellular organelles coated with proteins called perilipins that regulate the rate of exposition of lipids to lipases. There are five members of the perilipin family (perilipin 1–perilipin 5) that are expressed mainly in adipose tissue [11]. However, perilipin 5 is also associated with mitochondria in cells and tissues, such as mouse myocytes or mouse hepatocytes [12,13]. In the state of increased energy demand, TAG can be hydrolyzed back to FFA. The two processes regulating fatty acid homeostasis are lipolysis and lipogenesis, and the intensity of those processes is regulated by insulin, catecholamines, or adipose tissue hormones, such as leptin or adiponectin. Lipogenesis is a term determining the synthesis of TAG from serum FFA or FFA derived from the hydrolysis of lipoproteins catalyzed by lipoprotein lipase (LPL). Insulin plays an important part in the process by stimulating the uptake of FFA by cells, activating lipogenic enzymes (such as acyl-coenzyme A (acyl-CoA) carboxylase, or fatty acid synthase), and inhibiting lipolytic enzymes, such as hormone-sensitive lipase (HSL). Activation of transcription factors, such as sterol regulatory element-binding proteins (SREBP) or peroxisome proliferator-activated receptors (PPAR), also stimulates this process. Lipogenesis refers also to de novo fatty acid synthesis in the cell cytoplasm. The opposite process—lipolysis—results in hydrolysis of TAG and the release of FFA from lipid droplets to the cytoplasm. The factors enhancing lipolysis are aforementioned HSL and also leptin, glucocorticosteroids, tumor necrosis factor α (TNF-α), and interleukin-6 (IL-6). Released FFAs are activated through binding to coenzyme A (CoA) and then transported into mitochondria to undergo β-oxidation. The summary of the homeostasis of FFA has been

shown in Figure 1. FFA metabolism and the function of adipose tissue in FA homeostasis are further discussed in detail in numerous reviews [14–17].

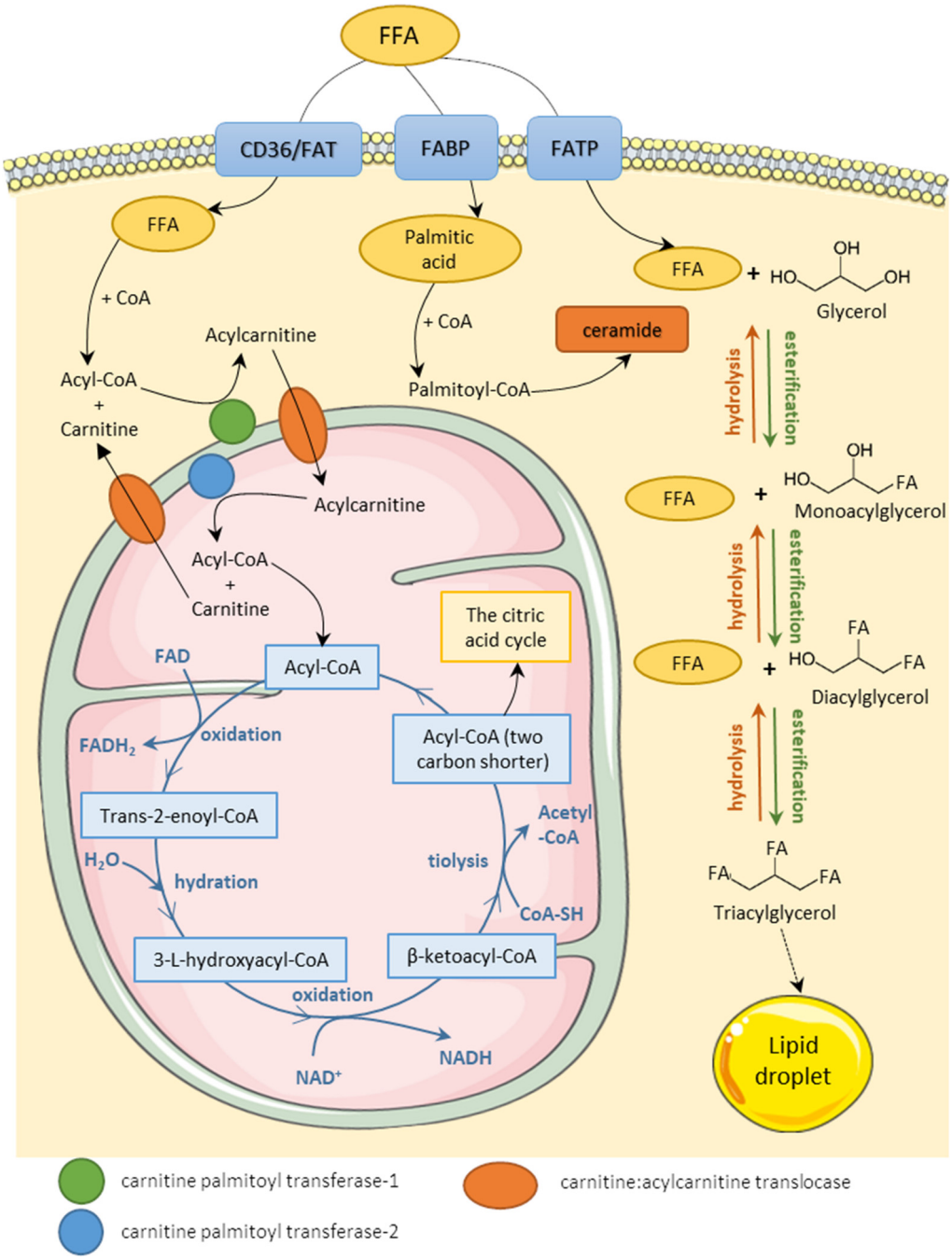

Figure 1. Homeostasis and metabolism of free fatty acids. FFAs circulating in the blood serum are bound to albumin. After the release from the albumin complex, FFAs are transported through the cell membrane by three various transporters: CD36/FAT, FABP, or FATP. Once in the cytoplasm, FFAs undergo esterification to TAG, which may be hydrolyzed to DAG. TAG is stored in lipid droplets. Lipid droplet is surrounded by proteins called perilipins, which regulate TAG exposition to hydrolytic enzymes. FFA may also be activated to acyl-CoA via the reaction with CoA. Palmitic acid is activated to palmitoyl-CoA, which is a substrate to de novo ceramide synthesis. Acyl-CoA is transported via the outer mitochondrial membrane by CPT-1, which also catalyzes acyl-CoA's reaction with L-carnitine to create acyl-carnitine. The form of acyl-carnitine is necessary for the transport through

the innermitochondrial membrane via CACT. There, acyl-carnitine is disassembled via CPT-2 to acyl-CoA and L-carnitine. L-carnitine is transported back through the inner mitochondrial membrane via CACT. Acyl-CoA undergoes β-oxidation by releasing FADH2 and NADH, which serve as a substrate for ETC and acetyl-CoA, which is a substrate for the TCA cycle. Acetyl-CoA is also carboxylated to malonyl-CoA. FFA, free fatty acids. CD36/FAT, a cluster of differentiation 36/fatty acid transporter. FABP, fatty acid binding protein. FATP, fatty acid transport protein. TAG, triglyceride. DAG, diacylglycerol. Acyl-CoA, acyl-coenzyme A. CoA, coenzyme A. CPT-1, carnitine palmitoyl transferase-1. CACT, carnitine: acylcarnitine translocase. CPT-2, carnitine palmitoyl transferase-2. ETC, electron transport chain. Acetyl-CoA, acetyl coenzyme A. TCA cycle, the citric acid cycle [16,18–20].

The accumulation of FFA in non-fatty tissues may result in deleterious effects referred to as lipotoxicity. Dysfunction of adipose tissue fat storage ability, high-fat diet resulting in increased FFA plasma levels, and the shift towards lipolysis of TAG deliver an increased amount of FFA inside cells [21]. The other factor contributing to increased cytoplasmic FFA levels is the disturbance in perilipin expression and activity leading to either tissue steatosis or enhanced lipolysis delivering another load of FFA [14]. Cell metabolism requires efficient disposal mechanisms, such as esterification to TAG and intensified β-oxidation, to handle excess FFA [22–24]. In the case of non-adipose tissue, cell capabilities of FFA storage are limited and β-oxidation-required enzymes may become depleted. This leads to the accumulation of lipid metabolism intermediates, such as diacylglycerol (DAG) or ceramides, which can further disturb other metabolic pathways contributing to widely understood lipotoxic cell stress [25,26].

There is a positive correlation between TAG content and inflammatory responses [27] and insulin resistance [28], therefore suggesting that TAG depot is directly responsible for lipotoxic effects. However, the main cause of lipotoxic effects is the uncontrollable release of FFA from lipid droplets and adipose tissue and their interference with metabolic pathways, which also finds confirmation in studies [29–31].

3. Mechanisms of Lipotoxicity on the Cellular Level

3.1. Fatty Acids Metabolism

FFA are metabolized through a catabolic process referred to as β-oxidation which takes place in the mitochondrial matrix. The FFA intramitochondrial transport requires their activation to acyl-CoA esters in the outer mitochondrial membrane as well as carnitine transporter (carnitine palmitoyltransferase, CPT-1) which enables the transport of long and medium-chain fatty acids through the mitochondrial membrane in the form of acylcarnitines. Short-chain fatty acids do not require CPT-1 transportation. B-oxidation consists of four biochemical reactions (oxidation, hydration, second oxidation, and thiolysis) repeated in cycles. During each cycle, the acyl-CoA ester is shortened by two carbon atoms, and acetyl-CoA, FADH2, and NADH are created. The cycle repeats, further shortening the acyl-CoA ester until the entire carbon chain is cleaved into acetyl-CoA. The produced acetyl-CoAs are fed into the citric acid cycle, whereas FADH2 and NADH are used in the electron transport chain. In brief, β-oxidation is a catabolic transformation of fatty acids into substrates for the creation of the electrochemical proton gradient and the production of ATP. The process of β-oxidation is further explained in other reviews [17,18].

Efficient β-oxidation is the key to maintaining proper FFA homeostasis in cells and preventing the accumulation of FFA in the cell, therefore preventing the lipotoxic effects. The importance of β-oxidation is supported by the observation that enhancing it protects against FFA-induced cytotoxicity. The enhancement of the β-oxidation was performed through the administration of CPT-1 stimulators (such as C75) into the cell culture and through in vivo electrotransfer of purified CPT-1 plasmid into rat tibialis anterior muscle to overexpress the muscle isoform of CPT-1. These actions resulted in the observation that the overexpression of CPT-1 and, therefore, an increased rate of β-oxidation improves insulin sensitivity in high-fat-fed rats and prevents FFA-induced oxidative stress, ER stress, apoptosis, and inflammation in neuronal and β-cell cultures [26]. The disturbance of FFA

disposal mechanisms (inefficient or incomplete β-oxidation and incomplete esterification to TAG) results in abnormal accumulation of lipid metabolism intermediates, such as DAG and ceramides, as well as non-metabolized FFA, which can further interfere with cell signaling pathways, e.g., through the activation of protein kinases and, therefore, inhibiting insulin signal transduction [32]. Furthermore, palmitate (considered the most lipotoxic fatty acid) inhibits β-oxidation, creating a vicious circle [33,34]. There is a negative feedback mechanism, including malonyl-CoA, malonyl-CoA decarboxylase, and CPT-1. Malonyl-CoA is a product of carboxylation of acetyl-CoA and an allosteric inhibitor of CPT-1 resulting in the inhibition of FFA transport to mitochondria and subsequently β-oxidation. Inactivation of malonyl-CoA results in improved lipid oxidation and may prevent lipotoxicity [35], whereas the increase in malonyl-CoA level is inversely correlated to lipid oxidation.

The study was conducted by Asem et al. to determine whether changes in long-chain fatty acid (LCFA) oxidative metabolisms induced by high carbohydrate availability are a result of changes in LCFA mitochondrial transport capacity. The perfusion of 500 μM palmitate and [1-^{14}C]palmitate or [1-^{14}C]octanoate into rat hindquarters as well as with either low (LG) or high (HG) carbohydrate availability was performed. The postperfusion HG group malonyl-CoA levels were significantly higher than the LG group, and the relationships between percent and total palmitate oxidation and postperfusion muscle malonyl-CoA levels were hyperbolic and found to be significant [36]. Palmitate is observed to have an increasing effect on malonyl-CoA levels, which might explain its aforementioned β-oxidation inhibiting properties. Rat cardiomyocytes incubated in 500 μM palmitate showed an increase in malonyl-CoA level after chronic exposure (20 h) [37]. On the other hand, research shows that elevated plasma FFA in high-fat fed rats was associated with the increased expression of malonyl-CoA decarboxylase (MCD), which catalyzes the degradation of malonyl-CoA, creating feedback aimed at lowering FFA levels [38]. A similar situation to malonyl-CoA occurs with CPT-1, the expression and activity of which promotes β-oxidation; therefore, overexpression of CPT-1 enhances rat muscle insulin sensitivity and prevents FFA-induced apoptosis of murine podocyte cell [23,39], while its downregulation should exacerbate lipotoxicity. However, Haffar et al. showed that palmitate alone does not affect CPT-1 while impairing β-oxidation. The study was performed on rat neonatal cardiomyocytes, culture which was treated with 300 μM palmitate and 300 μM oleate for 8 h and the rates of β-oxidation, acetyl-CoA oxidation, citric acid cycle enzyme activities, CPT-1 activity, and DAG levels were measured. The results show that palmitate impairs β-oxidation and citric acid cycle flux, but no CPT-1 activity was observed. The impaired activity of the citric acid cycle enzymes may be due to DAG mediated protein kinase C (PKC) activation [33]. Another key regulator of β-oxidation is the 5′ adenosine monophosphate-activated protein kinase (AMPK), for which its activation triggers ATP production and inactivates an enzyme catalyzing malonyl-CoA formation—acetyl-CoA carboxylase (ACC). The activation of the AMPK pathway stimulates lipid oxidation and reduced murine podocytes' susceptibility to toxic FFA effects by reducing palmitic acid-induced cell death [23]. Hickson-Bick et al. showed that acute 500 μM palmitate exposure causes a reduction in the activity of AMPK and an increase in the malonyl-CoA content; therefore, it decreases the cell's capacity to oxidize fatty acids, resulting in the accumulation of FFA and lipotoxicity [37]. β-oxidation impairment has been observed in various diseases, such as obesity and insulin resistance in rat model, type 2 diabetes in humans, and renal injury in a mouse model, therefore suggesting that this branch of lipotoxic influences might be part of the etiology of those diseases [40–42].

Study shows that DAG, one of the intermediates of lipid metabolism, accumulates in the cell after acute exposure to increased FFA levels (300 μM of palmitate and oleate). According to Akoumi et al., DAG accumulates in the ER, and it is likely the key mechanism for FFA-mediated ER stress, which will be further discussed in Section 3.3 [26]. DAG is also recognized as a more lipotoxic agent than TAG. The research performed by Itani et al., Montell et al., and Chavez et al. on human muscle cultures and muscle cell cultures

shows that the accumulation of DAG is mostly related to blocking insulin signaling and action, resulting in desensitization to the insulin stimulation of glucose uptake and further resulting in a condition referred to as insulin resistance [43–46]. It is hypothesized that the main mechanism of DAG's influence on insulin signaling is due to its ability to activate PKC, a serine-threonine kinase, involved in various signal transduction pathways (such as MAP kinase signaling pathway or PI3K/Akt signaling pathway), for which its translocation to the cell membrane inhibits insulin signaling [47–51]. Due to the wide range of PKC activity, there was a strong premise that its activation is also responsible for FFA-mediated induction of the inflammatory pathways. However, research conducted by Macrae et al. excluded any direct involvement of DAG-sensitive PKC in palmitate-induced proinflammatory signaling [46].

Other intermediates generated by incomplete β-oxidation are acylcarnitines, which is the form of transportation for fatty acids through the mitochondrion membrane. Treatment of murine muscle cell culture with 10 μM of acylcarnitines for 24 h resulted in a 20–30% decrease in insulin response at the level of proteins kinase B (PKB, also known as Akt or Akt/PKB) phosphorylation and a 2–3-fold increase in oxidative stress. Another study shows that endogenous palmitoylcarnitine accumulation might contribute to palmitic-acid-induced insulin resistance in murine muscle cells [47,48].

Ceramides (sphingomyelin derivatives) are considered as potential lipotoxic molecules with the ability to modulate cellular metabolism. Palmitate, the most lipotoxic fatty acid, is activated to palmitoyl-CoA through esterification with CoA and can be transported by CPT-1 to the mitochondrion to undergo β-oxidation. However, palmitoyl-CoA can also serve as a substrate for ceramide synthesis via condensation with L-serine by the reaction catalyzed by an enzyme—serine palmitoyltransferase (SPT). Ceramides are signaling molecules that play a significant role in membrane biology (component of sphingomyelin, a major component of the lipid bilayer) as well as in several physiological events (such as regulating differentiation, proliferation, and programmed cell death). However, abnormal increases in ceramide content can contribute to toxic effects, such as suppressing muscle insulin action or apoptosis [49–51]. Since palmitate is an indirect substrate for ceramide synthesis, its increased levels in the cell have been shown to result in ceramide accumulation. Ceramide accumulation was observed in murine muscle cells after chronic incubation with 750 μM palmitate and in porcine oocytes after chronic incubation with 500 μM palmitate [45,52]. Moreover, the formation of ceramides via sphingosine acylation is observed to be a mechanism of lipotoxic action of palmitate on the cell, such as the induction of IR or apoptosis. Results from the study conducted by Manukyan et al. show the equal contribution of de novo and sphingosine pathways to ceramide generation in murine β-cells after chronic exposure to 250 μM palmitate [25]. Wehinger et al. proposed that the increase in ceramide levels caused by saturated FFA (16 h exposure to 2mM palmitate) induces mitochondrial damage and increases ROS generation and OS in cells, which in turn activates pro-apoptotic pathways in murine β-cells [53]. A study conducted by Chavez et al. presented that endogenously produced ceramides are capable of inhibiting Akt/PKB, a key element of the insulin signaling pathway, and are necessary for the inhibitory effect of FFA on insulin signaling. During the study, murine myotubes were incubated with 750 μM palmitate for 16 h [54]. These results are in accordance with Schmitz-Peiffer et al.'s observations that de novo ceramide synthesis mediates FFA inhibition of the PKB pathway in murine muscle cells [55]. Ceramides are also able to promote atypical isoenzyme PKCζ, which negatively regulates PKB by suppressing its cell–surface recruitment and phosphorylation as was measured after incubating rat myotubes with 750 μM palmitate for 16 h [56]. A novel study conducted by Sergi et al. on murine hypothalamic neuronal cells exposed acutely to 200 μM of palmitate explored the potential role of ceramides in FFA-induced hypothalamic neuronal inflammation. The results demonstrate that palmitic acid increases intracellular accumulation of ceramides together with the upregulation of IL-6 and TNF-α during treatment with the inhibitor of the key enzyme in the ceramide biosynthesis pathway,

proving, at least, the partial involvement of ceramide in the mechanism of FFA-induced inflammation [57].

Palmitoyl-CoA, in addition to serving as a substrate for ceramide synthesis, also plays an important role in the process of protein palmitoylation. Protein palmitoylation is a process of post-translational protein modification in which palmitic acid is attached to the thiol group of specific cysteines. Numerous proteins serve as a target of palmitoylation, such as the following: CD36, G-protein-coupled receptors, ion channels and transporters, and others. Palmitoylation regulates protein localization, stability, and activity and increases protein affinity to the lipid rafts or cholesterol- and sphingolipid-rich membrane microdomains. Palmitic acid is present in the cell in the form of palmitoyl-CoA [58]. There is very little research concerning the process of palmitoylation in molecular mechanisms of lipotoxicity. However, dysregulation of the protein palmitoylation was proposed as one of the mechanisms by which palmitate may induce ER stress. Baldwin et al. showed that a nonmetabolizable palmitate analog that inhibits palmitoylation was able to attenuate palmitate-induced ER stress and cell death in rat β-cells. There was also a suggestion that uncontrolled protein palmitoylation is a foundation of palmitate toxicity [59]. Similar results were obtained in the study conducted on human neuroblastoma cells, where palmitate-induced ER stress, cell apoptosis, and also cell cycle G2/M arrest through protein palmitoylation were observed [60]. These results indicate that protein palmitoylation is a relevant aspect of lipotoxicity, which requires further research to elucidate new underlying mechanisms.

3.2. Oxidative Stress

Oxidative stress is a term referring to an imbalance between oxidizing and reducing agents with a shift towards oxidizing environment resulting from an increase in ROS content. The inability to detoxify ROS results in a free radical attack on proteins, lipids, carbohydrates, and nucleic acids, irreversibly altering them functionally or even destroying them completely. All organelles and compartments of the cells can produce ROS; however, the mitochondrial generation of hydrogen peroxide (H_2O_2) is generally considered to be a major source of oxidants, and additionally, H_2O_2 is highly reactive and acts on the biomolecules in its immediate environment [61].

Any disturbances in mitochondrion function are implications for a potential increase in ROS generation and lipotoxicity. An excess of FFA in the human hepatocytes (chronic exposure to 300 μM palmitate) and rat cardiomyocytes (chronic exposure to 250 μM palmitate up to 1 mM) can impair mitochondrial energetics, resulting in reduced ATP production followed by accelerated mitochondrial and cytosolic reactive oxygen species (ROS) production [62,63]. A few mechanisms of these deleterious FFA actions have been proposed. Szeto et al. proposed morphological changes in mitochondria structures leading to disturbances in electron transport chain (ETC) function and, subsequently, a decrease in ATP production in murine renal tissues [64]. Another mechanism could be a decrease in OXPHOS (oxidative phosphorylation system) enzyme activity, which is responsible for producing the majority of ATP, as was shown on human hepatocytes exposed to 200 μ palmitate for 24 h [65]. Decreased OXPHOS enzyme activity and reduced ATP production are consequences of lipotoxicity due to the uncoupling of these processes. FFA alone have uncoupling properties [66], and their interaction with mitochondrial carriers can also lead to membrane depolarization and the conversion of the carrier into a pore. Uncoupling proteins (UCPs) are members of the mitochondrial carrier family and their function is to regulate the proton's gradient. There are several types of UCPs: UCP1 expressed in brown adipose tissue; ubiquitous UCP2; UCP3 expressed in skeletal muscle, heart, and brown adipose tissue; and others [67]. Research conducted on human hepatocytes shows that the inhibition of UCP2 enhances FFA-induced oxidative stress [68]. However, another research concerning isolated rat islets shows that, after chronic palmitate treatment, UCP2 expression significantly increased [69]. There is also an observed relation between UCP2 upregulation and autophagy in rat hepatocytes after palmitate treatment [70]. Those results suggest that interrelation

between FFA and UCP is complex and reaches beyond mitochondrion function and can impair other branches of cell metabolism.

The progressive increases in mitochondrial DNA (mtDNA) damage after exposure to increasing concentrations of palmitic acid (most likely through the generation of mitochondrial ROS, mtROS) may also contribute to the dysfunctional mitochondria and further compromise the electron chain system, initiating the apoptotic machinery. This effect was observed in rat skeletal muscles after acute exposure to 500–2000 μM palmitate in bovine endothelial cells after chronic exposure to 50 μM palmitate in the presence of glucose and in rat skeletal muscles after chronic exposure to 1 mM palmitate [71–73]. FFA-induced ceramide accumulation (chronic incubation with 500 μM palmitate) in porcine oocytes was also observed to downregulate the AMPK/SIRT3 pathway and cause mitochondrial protein hyperacetylation, resulting in mitochondrial dysfunction [52]. Mitochondrial swelling was observed after chronic incubation of neonatal rat cardiac myocytes with 500 μM palmitate as a result of the opening of the mitochondrial permeability transition pore (MPTP), as manifested by cytochrome c (a component of ETC and apoptosis regulating factor) release to the cytosol [74].

Inhibition or deletion of cathepsin B, a lysosomal cysteine protease, protected against FFA-induced mitochondrial dysfunction in human hepatocytes, suggesting its role in the mechanism of FFA action on mitochondria as well as the role of lysosomes [75]. The role of cathepsin B finds confirmation in a study conducted on human hepatocyte cells, primary hepatocytes isolated from rats, and high-fat-fed rats by Wu et al. where the inhibition of cathepsin B expression and stabilization of lysosomal membrane was presented as major cellular mechanisms that accounted for 18β-glycyrrhetinic acid's protective effects on FFA-induced lipotoxicity (chronic exposure 1 mM palmitate) [76]. However, in our view, the most abundant detrimental action FFA can execute on mitochondria is increased ROS production. This results in oxidative stress and also contributes to apoptosis, further damaging mtDNA and ETC [69,77].

FFAs are known to be one of the factors inducing the production of ROS. The family of NADPH oxidases (NOX) responsible for the transfer of electrons through biological membranes is a crucial factor in the production of ROS. There is evidence that FFAs (acute palmitate exposure) increase superoxide production through the activation of the NADPH oxidase system as well as by mitochondrial ETC in rat myocytes [77]. Another study conducted on human umbilical vein endothelial cells (HUVEC) exposed acutely to 1 mM palmitate suggests the possible mechanisms of FFA-induced NOX subunit gene expression [78] or the activation of NOX through PKC-dependent pathway in bovine aortic smooth muscle cells and endothelial cells treated with 200 μM palmitate for 72 h [79], thus contributing to increased ROS production. The NOX enzymes proven to be involved in lipotoxic ROS production are three isotypes members of the NOX family: NOX2, NOX3, and NOX4. NOX2 induction was observed in murine cardiomyocytes (acute 200 μM palmitate exposure), NOX3 was observed induction in human hepatocytes (chronic 250 μM palmitate exposure), and NOX4 induction was observed in human chondrocytes (chronic palmitate exposure, up to the concentration of 500 μM), suggesting tissue specificities of different isoforms [80–82].

Unresolved OS further damages the cell and can lead to apoptosis, as was shown on human hepatocytes treated with 100–800 μM palmitate for 24 h [83]. Another study conducted on third-order mesenteric arteries isolated from rats and HUVEC and treated with 100 μM palmitate for 24 h shows that lipotoxic OS can mediate vasodilatation via downregulation of potassium channels [84]. Researchers have distinguished various factors contributing to inducing lipotoxic oxidative stress. There is a strong link between oxidative stress and mitochondrial dysfunction since FFAs are involved in mtROS production and also decrease the expression of two major mitochondrial transcription factors, PGC-1α and mitochondrial transcription factor A (TFAM), as shown in a study conducted on rat skeletal myocytes treated with 100–1000 μM palmitate for 24 h [85]. The impairment of mitochondrial energetics was observed to be followed by accelerated mitochondrial and cytosolic

ROS production in embryonic ventricular rat heart-derived cardiomyoblasts exposed to 250–1000 μM palmitate [63]. In high-fat-fed rats, palmitate stimulated oxidative stress in cardiac myoblasts and also altered protein levels involved in mitochondria functions: It increased the protein levels of CPT-1, cytochrome c, and cyclophilin F and induced the appearance of MPTP [86]. Mitochondrial oxygen consumption and ATP production are significantly decreased by FFA, resulting in increased mitochondrial proton leaks [87].

Yao et al. proposed another explanatory mechanism for FFA-induced mitochondrial dysfunction concerning the role of iron, which alone has little effect; however, when in combination with palmitic acid, it forms a complex and is shuttled across the lipid bilayer. Therefore, as observed in HUVEC treated with 300 μM palmitate in combination with 150 μM iron salt for 12 h, fatty acids mediate intracellular iron translocation, leading to iron overload in cytosolic and mitochondrial compartments that contribute to mitochondrial dysfunction, DNA mutation, and oxidative stress [88]. Fatty acids not only directly stimulate the synthesis of ROS but also disturb mitochondrial function, resulting in decreased antioxidant capabilities of the cell or indirect stimulation of ROS production. The aforementioned studies show that fatty acid-induced oxidative stress may be caused by suppressing β-oxidation and disrupting the mitochondrial respiratory chain, resulting in the accumulation of FFA in the cell. Accumulating oxidative damage may affect the efficiency of mitochondria and further promote ROS generation, therefore creating a vicious cycle [80–82].

One of the factors contributing to lipotoxic oxidative stress is the expression of fatty acid transporter CD36. In human kidney tissue, rat kidney tissue, podocyte, and human mesangial cells with increased expression of CD36 as well as increased lipid uptake, ROS production, and apoptosis were observed [89,90]. The novel study shows that perilipin 5 also has a crucial role in the alleviation of oxidative damage through the induction of antioxidant defense, which may suggest that stability of lipid droplets is an important aspect of maintaining redox balance [91]. Therefore, decreases in the activity of perilipin 5 or destabilization of lipid droplets are potential factors contributing to lipotoxicity. There is also a proposed model of FFA-induced cytotoxicity conducted on mouse podocytes, which provides evidence for close interaction between oxidative stress and Ca^{2+} homeostasis in mitochondria and the ER. The model states that FFAs are transported into the cell via CD36/FAT, resulting in an increase in mitochondrial and cytosolic ROS production. After the acute exposure of mouse podocytes to 300 μM palmitate, FFA-induced ROS production results in Ca^{2+} release from ER followed by ER Ca^{2+} depletion, ER stress resulting in podocyte apoptosis, and cytoskeletal derangement. The consequence of both podocyte apoptosis and cytoskeletal derangement is proteinuria. Another branch of the model links FFA-induced ROS production with mitochondrial dysfunction, which also contributes to podocyte apoptosis and proteinuria [92].

Aung et al. show that, in humans, brain microvascular endothelial cells (HBMECs) treated with isolated human triglyceride-rich lipoproteins (TGRL) oxidative stress associated with lipotoxicity are also connected to the induction of inflammatory pathways since the superoxide radical can activate activating transcription factor 3 (ATF3)-mediated inflammatory and apoptotic responses [93]. In a study conducted on cow endometrium epithelial cells treated chronically with 600 μM palmitate, Li et al. revealed that FFAs induce OS-mediated activation of the NK-κB signaling pathway, thereby leading to the release of inflammatory factors, such as IL-8, IL-6, and TNF-α [94]. Another research study conducted on rats infused with lipid emulsions by Barazzoni et al. pointed out FFA-induced ROS production, and the activation of the NF-κB pathway is also responsible for inducing IR, suggesting an interplay of various lipotoxic effects [95]. However, another research study conducted on isolated rat pancreatic islets treated chronically with 500 μM palmitate and other FFAs shows no immediate connection between OS and insulin resistance during the expression of the culture of β-cell islets in the presence of fatty acids [96]; therefore, this aspect requires additional research to determine and explain the relationship. A potential connection between oxidative stress and insulin resistance, two processes that

can be induced by FFA, would suggest another interrelation of FFA-triggered lipotoxic molecular mechanisms.

Table 1 presents the sites of occurrence of lipotoxic oxidative stress molecular mechanisms. The table suggests that cardiomyocytes and myocytes are the most recurring sites of reviewed FFA-induced oxidative stress caused. There is further research and review required to determine whether those kinds of cells are the most prone to oxidative stress and whether this effect applies to more cell types. It also seems that lipotoxic OS is not tied to a specific cell type; however, confirmed lipotoxic OS occurs more often in cell types requiring increased metabolic activity for contraction, insulin secretion, and others. Another question that requires a confirmed answer is whether there are different susceptibilities to OS depending on cell type.

Table 1. Selected molecular mechanisms of lipotoxic oxidative stress and their sites of confirmed occurrence.

Molecular Mechanism of Oxidative Stress	Organism	Cell Culture	Organ/Cell Type	Reference
Increased general ROS production	human	isolated chondrocytes	cartilage	[80]
		cell line Hep2G	liver	[81]
	rat	isolated myocytes	muscle	[77]
		cell line H9c2	heart	[82]
		cell line INS-1	pancreas	[91]
	mouse	isolated cardiomyocytes	heart	[82]
		isolated podocytes	kidney	[89]
Increased mitochondrial ROS production	rat	cell line L6	muscle	[85]
		cell line H9c2	heart	[63]
	mouse	isolated cardiomyocytes	heart	[82]
		isolated podocytes	kidney	[92]
NOX activation	human	cell line Hep2G	liver	[81]
		isolated chondrocytes	cartilage	[80]
	rat	isolated myocytes	muscle	[77]
		cell line H9c2	heart	[83]
	mouse	isolated cardiomyocytes	heart	[82]
Reduction in ETC	rat	isolated myocytes	muscle	[77]
	mouse	isolated cardiomyocytes	heart	[82]
Reduction in MtMP *	human	HUVEC	endothelium	[84]
	rat	cell line H9c2	heart	[82]
	mouse	isolated podocytes	kidney	[92]
Reduced ATP generation	rat	cell line H9c2	heart	[63]
		cell line L6	muscle	[85]
	mouse	isolated podocytes	kidney	[92]
Iron-mediated toxicity	human	HUVEC	endothelium	[88]
increase in mitochondrial Ca^{2+}	rat	cell line H9c2	heart	[82]

* MtMP—mitochondrial membrane potential.

3.3. Endoplasmic Reticulum Stress

The phenomenon of ER stress concerns a large part of biochemical processes taking place in the cell. Therefore, the situation of unresolved ER stress triggers different signaling pathways leading to other disturbances. In the ER, proteins fold into their native conformation and undergo a multitude of post-translational modifications. Any disruption of these processes causes the accumulation of unfolded, aggregated proteins, which activate

the unfolded protein response (UPR), an adaptive and protective mechanism aimed at restoring normal ER function. Three ER transmembrane protein sensors mediate UPR signals: IRE1 (inositol requiring enzyme 1; ERN1—ER to nucleus signaling 1), PERK (protein kinase RNA-like endoplasmic reticulum kinase), and ATF6 (transcription factor activating transcription factor 6) detect unfolded protein loads in the ER lumen and transmit signals to downstream effectors [97,98]. FFAs are one of the agents able to activate UPR. A study conducted by Nivala et al. on rats with administered lipid intravenous infusions shows that an increase in the amount of FFA may provoke ER stress and inflammation in liver and adipose tissues [99]. Another research study concerning mouse hepatocyte and murine podocyte cell cultures revealed that incubation with palmitic acid (chronic 600 μM palmitate treatment in human and rat hepatocytes and chronic 500 μM palmitate treatment in murine podocytes) leads to PERK and eukaryotic initiation factor 2 α (eIF2α) phosphorylation and increased CHOP (CCAAT/-enhancer-binding protein homologous protein, also known as C/EBP) expression that point out that ER stress was induced. It also elucidated the molecular interplay between signaling pathways involved in ER stress, insulin resistance, and apoptosis [100,101]. Unresolved FFA-mediated ER stress is responsible for triggering cell death via the activation of CHOP or caspase-12, or when ER stress exceeds the capacity of UPR in human hepatocytes (acute 1 mM palmitate treatment), in rat mesangial cells (chronic palmitate treatment) and rat myocytes (100 μM, 200 μM and 400 μM palmitate treatment for 12 h) [102–104]. There is also evidence that nuclear protein 1 (Nurp1), a transcriptional regulator downstream of CHOP, plays a critical role in apoptosis, as observed in human chondrocytes after chronic 500 μM palmitate treatment [105]. There is also the assumption that other factors, such as FoxO1, cannabinoid receptor 1 (CB1), or SREBP-1, can have a part in triggering apoptosis related to ER stress. Although the exact mechanisms have yet to be elucidated, there are observations that attenuating FoxO1 (observed in murine β-cells), CB1 (observed in human renal proximal tubular cells), and SREBP-1 (observed in a mouse model) activity prevents FFA-induced cell death [106–108].

The molecular chaperone BiP/GRP78 (binding immunoglobin protein/immunoglobulin heavy-chain binding protein) serves as a UPR regulator and is capable of activating all three transducers, IRE1, PERK, and ATF6, in response to ER stress. Under non-stressed conditions, BiP binds to IRE1, PERK, and ATF6, preventing their activation. When ER is overloaded with newly synthesized unfolded proteins, the pool of free BiP becomes depleted and then IRE1, PERK, and ATF6 are released from BiP, which enables signal transduction for UPR activation [98].

The IRE1 pathway regulates chaperone induction, clearance of unfolded proteins from the ER through upregulation of ER-associated degradation, and the expansion of the ER in response to ER stress to cope with the accumulation of unfolded or misfolded protein in the ER lumen. The activation of IRE1 results in alternative splicing and nuclear translocation of the transcription factor x-box binding protein 1 (XBP-1). Spliced XBP-1 mRNA encodes a potent transcriptional activator (an XBP-1 isoform) for many UPR target genes [97,98]. There is numerous evidence showing that FFAs induce ER stress through the activation of the IRE1 pathway. Although this pathway is meant to resolve ER stress, there is evidence that the activation of the IRE1 signaling pathway is involved in FFA-induced cell death of rat pancreatic β-cells (after chronic 1 mM palmitate treatment), porcine meniscus cells (after chronic 500 μM palmitate treatment), and neonatal rat cardiomyocytes (after acute 200 μM palmitate treatment) [109–111]. This effect seems to be tightly linked to inflammatory responses since the inhibition of FFA-induced activation of IRE1 abolishes IL-1β secretion stimulated by FFA, as observed in isolated primary mouse bone marrow-derived dendritic cells (BMDCs) [112].

Protein kinase mTOR (the mammalian target of rapamycin) complex 1 (mTORC1) (a master regulator in promoting growth and cellular anabolic processes in response to growth factors and nutrients excess) also plays an important role in the IRE1 pathway. Chronic 200 μM and 400 μM palmitate treatment is able to activate mTORC1 in murine hepatocytes; however, mTORC1's inhibition attenuates palmitate-induced activations of IRE1, protecting

cells against apoptosis; therefore, mTORC1 may be crucial for palmitate-induced ER stress-related cell death [113,114]. However, another study conducted on murine muscle cells provides data indicating that the activation of UPR represses the phosphorylation state of mTORC1, eventually resulting in a decrease in protein synthesis in the cell after chronic incubation with palmitate [115]. FFA can also partially induce XBP-1 mRNA splicing in murine preadipocytes (chronic palmitate treatment) and murine macrophages (chronic 400 μM palmitate treatment), causing less robust UPR than thapsigargin (a chemical ER stress inducer, which causes complete XBP-1 mRNA splicing) [116,117]. These observations show that FFAs interfere with the IRE1 pathway in multiple ways; however, further studies are necessary for determining whether the FFA-induced IRE1 pathway activation is dependent on cell type.

After activation by the release of BiP, PERK oligomerizes and phosphorylates substrate proteins, eIF2α, resulting in a reduced frequency of mRNA translation initiation in general. This results in the inhibition of the general protein biosynthesis, which serves as an adaptive mechanism in the accumulation of unfolded proteins [97,98]. Numerous studies conducted on two rat pancreatic β-cell cultures (after chronic 1 mM palmitate treatment in the case of the first cell culture and chronic 250 μM palmitate treatment in the case of the second cell culture) and human liver tissue (after chronic 600 μM palmitate treatment) show that the PERK signaling pathway is activated during FFA-induced ER stress, suggesting that FFAs are able to activate PERK [109,118,119]. Data also indicate that PERK and NF-κB signaling pathways, as well as JNK and PERK-dependent ATF3 activated by FFA, are involved in apoptosis through the regulation of the expression of Bcl-2 family members, as observed in human hepatocytes and human and rat β-cells after chronic treatment with FFA [120,121]. Studies conducted on murine preadipocytes and human islets (after chronic palmitate treatment in both cases) show that palmitate-induced ER stress-related apoptosis is associated with the increase in transcription factor phospho-eiF2α [116,122]. The direct effect of these actions is a decrease in protein synthesis, which also has been observed after chronic FFA exposure in murine muscle cells [115]. Even though the decrease in protein synthesis is meant to rescue ER protein-folding abilities, this mechanism is not sufficient for resolving ER stress induced by FFA, and the final result is often apoptosis as observed in human hepatocytes, rat mesangial cells, and rat myocytes [102–104]. Upon ER stress, phosphorylated eiF2α selectively promotes the translation of activating transcription factor 4 (ATF4) mRNA. ATF4 is required for the expression of genes involved in amino acid import, glutathione biosynthesis, and resistance to oxidative stress. It binds to CHOP, GADD34 (DNA damage-inducible protein), and ATF3. CHOP forms heterodimers with C/EBP family members and controls the expression of genes involved in apoptosis [97,98]. Anusornvongchai et al. and Cao et al. showed that both ATF4 mRNA expression and ATF4 protein expression were increased in human hepatocytes chronically exposed to FFA, therefore suggesting that FFAs are capable of inducing the expression of ATF4 [123,124]; however, it is not certain whether this phenomenon is an independent action or an indirect result of PERK activation. The ability of FFA to influence regulatory elements of UPR refers also to observed increased expressions of CHOP, CHOP gene, and CHOP mRNA under the influence of FFA in rat β-cells, human and mouse neuroblastoma cells, and bovine mammary epithelial cells after chronic exposure to palmitate treatment [125–127].

Different effects concerning FFA-induced ER stress have been observed in studies conducted on murine β-cells after chronic palmitate treatment, such as disrupted ER-to-Golgi protein trafficking resulting in protein overload in contrast to protein-synthesis inhibiting properties [128,129]. These observations are a foundation of the concept that protein overload, rather than disruption of the protein-folding capacity of the ER, is an underlying mechanism of FFA-induced ER stress in β-cells. Another mechanism concerning the depletion of the ER Ca^{2+} reservoir has been proposed as a mechanism of FFA-induced ER stress. According to Cunha et al., chronic saturated FFA treatment decreases ER Ca^{2+} stores in rat insulin-producing β-cell culture and ER Ca^{2+} depletion activates ER stress [130]. The rapid depletion of ER Ca^{2+} in response to FFA was also observed in mouse insulinoma-derived

cells [131]. Zhang et al. suggested that the flux of calcium is required to mediate FFA-induced ER stress, as observed in rat hepatocytes after chronic palmitate exposure [132]. Research data suggest that ER calcium depletion is connected with dysregulated mitochondrial metabolism and that accelerating mitochondrial Ca^{2+} clearance might relieve cytosolic Ca^{2+} overload in rat hepatocytes after chronic treatment with 400 µM palmitate and mouse β-cells after chronic treatment with 500 µM palmitate [133,134]. Ca^{2+} flux is also associated with cell death. In mouse β-cells, Ca^{2+} activated phosphatase calcineurin was suggested to be involved in apoptotic processes via the dephosphorylation of apoptosis-related molecules such as Akt and b-cell lymphoma-2(Bcl-2)-associated death promoter (Bad) [135]. Another molecule with a possible contribution to Ca^{2+}-related apoptosis is calpain-2, a calcium-dependent proapoptotic protease. Calpain-2 was activated in rat insulinoma cells, mouse islets, and human islets treated for 24 h with 500 µM palmitate, suggesting that ER Ca^{2+} depletion can result in the activation of calcium-dependent cell death pathways in the cytoplasm [136].

The ER stress promotes IL-1β synthesis through the stimulation of NF-κB pathways and stimulates inflammasome-dependent responses, therefore contributing to activating inflammatory responses in the cell, as observed in human leukemic monocytes, murine macrophages, and murine adipocytes [137]. Kim et al. demonstrated that FFA-induced ER stress in human vascular endotheliums is mediated through toll-like receptor 4 (TLR4) and that the reduction in ER stress restores vasodilator actions of insulin [138]. There is another visible connection between ER stress and OS. Both these processes contribute to FFA-induced cell death and their signaling pathways might be related, as observed in mouse podocytes, human osteoblast-like cells, and rat renal proximal tubular cells [92,139,140]. There is a probability that both ER stress and OS trigger each other; however, it is also possible that these processes occur simultaneously because both are induced by FFA. More study is needed to elucidate this connection.

Lipotoxicity and subsequent FFA-induced ER stress is also proposed as a mechanism underlying several diseases. Ozcan et al. conducted research on rat hepatocytes and a mouse model and identified lipotoxic ER stress as a molecular link between obesity, the deterioration of insulin action, and the development of type 2 diabetes [141]. The same applies to the development of non-alcoholic fatty liver disease (NAFLD), as observed in rat and mouse in vivo models [142,143]. Another study demonstrated that triggering ER stress might be responsible for lipotoxic rat myocardial injuries [104] and renal proximal tubule injuries in mice [108]. However, due to the differences between experimental FFA administration and pathological disturbances in FFA homeostasis resulting from disease, further studies are needed to confirm lipotoxic mechanisms as mechanisms underlying the mentioned diseases. There also are findings suggesting a connection between FFA-induced ER stress, insulin resistance, and inflammation. Ebersbach-Silva et al. pointed out that FFA-induced reductions in glucose transporter type 4 (GLUT4) expression in rat muscle cells after acute FFA exposure is related to the formation of an IRE1α-TNF-associated factor 2-IκB kinase (IRE1α-TRAF2-IKK) complex and the activation of NF-κB, therefore linking the mechanisms of FFA-induced IR, inflammation, and ER stress [144]. Another study conducted on isolated mouse pancreatic acinar cells indicates that, in FFA-stimulated pancreatic acinar cells, ER stress induces inflammatory responses through the induction of the C/EBP family [145]. Those observations might confirm the vicious cycle of lipotoxicity and interplay between signaling pathways involved in processes induced by FFA, such as ER stress, IR, or inflammation.

Table 2 demonstrates lipotoxic molecular mechanisms of ER stress and their sites of occurrence. The cell types that stand out as the most frequent site of lipotoxic ER stress are hepatocytes and pancreatic β-cells; however, other cell types are also included. It seems that most mechanisms are present subsequently in the mentioned cell types with the exception of mTORC1 activation, which seems to be tied to the liver based on the current review. Moreover, perturbations of protein trafficking and ER Ca^{2+} depletion are mainly observed

in pancreatic β-cells, suggesting the cell-type specificity of those mechanisms, probably due to specific functions of β-cells, such as insulin secretion.

Table 2. Selected molecular mechanisms of lipotoxic ER stress and their sites of confirmed occurrence.

Molecular Mechanism of ER Stress	Organism	Cell Culture	Organ/Cell Type	Reference
Activation/phosphorylation of PERK	human	isolated hepatocytes	liver	[119]
		cell line L02	liver	[124]
		cell line Hep2G	liver	[124]
		cell line SH-SY5Y	neuroblastoma	[125]
	rat	isolated adipocytes	adipose tissue	[99]
		isolated hepatocytes	liver	[99]
		cell line INS-1	pancreas	[110,130]
		isolated neonatal rat cardiomyocytes	heart	[111]
	mouse	cell line RAW 264.7	macrophage	[116,117]
		cell line N2a	neuroblastoma	[125]
		cell line MIN-6	pancreas	[131,134]
Activation/phosphorylation of eIF2α	human	isolated hepatocytes	liver	[100]
		cell line Hep2G	liver	[146]
	rat	isolated adipocytes	adipose tissue	[99]
		isolated hepatocytes	liver	[99]
		cell line INS-1	pancreas	[130]
		cell line BRIN-BD11	pancreas	[118]
	mouse	cell line MIN-6	pancreas	[134]
XBP1 splicing/XBP1s increased expression or activation	human	cell line Hep2G	liver	[123,124]
		isolated β-cells	pancreas	[131]
		cell line SH-SY5Y	neuroblastoma	[125]
	rat	isolated adipocytes	adipose tissue	[99]
		isolated hepatocytes	liver	[99]
		cell line INS-1	pancreas	[110]
	mouse	isolated podocytes	kidney	[101]
		cell line AML12	liver	[113]
		cell line 3T3-L1	preadipocytes	[116]
		cell line RAW 264.7	macrophages	[116,117]
		cell line C2C12	muscle	[115]
		cell line N2a	neuroblastoma	[125]
		cell line MIN-6	β-cell	[131]
CHOP expression	human	isolated hepatocytes	liver	[100]
		cell line Hep2G	liver	[123,124]
		cell line L02	liver	[124]
		cell line SH-SY5Y	neuroblastoma	[125]
		isolated β-cells	pancreas	[131]
	rat	cell line INS-1	pancreas	[110,130]
		cell line BRIN-BD11	pancreas	[118]
		isolated cardiomyocytes	heart	[111]
	mouse	cell line 3T3-L1	preadipocytes	[116]
		cell line RAW 264.7	macrophages	[116,117]
		cell line C2C12	muscle	[115]
		cell line N2a	neuroblastoma	[125]
		cell line MIN-6	pancreas	[131,134]

Table 2. *Cont.*

Molecular Mechanism of ER Stress	Organism	Cell Culture	Organ/Cell Type	Reference
Activation/phosphorylation of IRE1	human	cell line Hep2G	liver	[146]
		isolated hepatocytes	liver	[119]
		cell line SH-SY5Y	neuroblastoma	[125]
	rat	isolated cardiomyocytes	heart	[111]
		cell line INS-1	pancreas	[130]
	mouse	cell line AML12	liver	[113]
		cell line C2C12	muscle	[115]
		cell line N2a	neuroblastoma	[125]
Activation/phosphorylation of ATF4	human	cell line Hep2G	liver	[123,124]
		cell line L02	liver	[124]
		cell line SH-SY5Y	neuroblastoma	[125]
	rat	cell line INS-1	pancreas	[110]
		cell line BRIN-BD11	pancreas	[118]
	mouse	cell line C2C12	muscle	[115]
		cell line N2a	neuroblastoma	[125]
Activation/phosphorylation of ATF3	human	cell line SH-SY5Y	neuroblastoma	[125]
	rat	cell line INS-1	pancreas	[130]
	mouse	cell line N2a	neuroblastoma	[125]
mTORC1 activation	mouse	cell line AML12	liver	[113]
Perturbation of protein trafficking	mouse	cell line MIN-6	pancreas	[128,129]
ER Ca^{2+} depletion	human	isolated β-cells	pancreas	[131]
	rat	cell line INS-1	pancreas	[130]
		cell line H4IIEC3	liver	[133]
	mouse	cell line MIN-6	pancreas	[131,134]

3.4. Inflammatory State

Chronic FFA treatments have been proven to increase cell-autonomous inflammatory responses in various ways, e.g., stimulation of pro-inflammatory gene expression or triggering inflammatory signaling pathways, as observed in human coronary artery smooth muscle cells (HCASMC) and human coronary artery endothelial cells (hCAECs) [147,148]. Another method of activating inflammatory responses is triggering other dysfunctions that further promote inflammation, such as mitochondrial dysfunction, overproduction of ROS, induction of C-reactive protein gene expression, and the following suppression of NO production, as shown in HBMEC treated with isolated human TGRL, rat and human myotubes, and human aortic endothelial cells (HAECs) [93,149,150].

FFA can also induce systemic inflammatory responses tied to adipose tissue through the activation of macrophages or chemotaxis. Macrophages, the primary mediator of the immune response, are present in adipose tissues in lean subjects and their amount is directly proportional to adiposity [151]. We can distinguish two major subpopulations of macrophages: inflammatory "classically activated" M1, which produces proinflammatory cytokines, and anti-inflammatory "alternatively activated" M2, which produces anti-inflammatory cytokines. In lean, healthy subjects, resident macrophages express markers of a subpopulation of M2. Obesity, i.e., excess of white adipose tissue (WAT), imposes a new population of M1-polarized macrophages on resident ones. The balance of M1/M2 macrophages is then shifted towards a more pro-inflammatory environment in adipose tissue, including a high expression of TNF-α, IL-6, and inducible nitric oxide synthase (iNOS) [152,153]. The chemokine system is understood as a chemokine receptor and its ligand (e.g., C-C chemokine receptor type 2 (CCR2) and monocyte chemoattractant protein

MCP-1) plays a crucial role in obesity-induced adipose tissue inflammation via macrophage recruitment, i.e., increased infiltration of the adipose tissue with macrophages [153]. There is evidence that FFAs are able to induce local secretions of chemokines, therefore provoking macrophage infiltration. Induced expressions of MCP-1 was observed in mouse myotubes treated for 24 h with palmitate, and conditioned media from the palmitate treatment mouse myotubes displayed enhanced properties for recruiting mouse macrophages [154]. Similarly, chemotaxis of murine macrophages towards murine β-cells following treatment with conditioned media from murine β-cells treated with palmitate for 24 h was significantly increased [155]. Induced secretions of MCP-1 was also observed in murine adipocytes chronically treated with palmitate [156].

The goal of recruitment of M2 macrophages is to maintain the lipid homeostatic state within WAT by means of β-oxidation of excess FFA. The inability of these macrophages to help relieve excess FFA within WAT contributes to systemic lipid dysregulation and chronic metabolic-related systemic inflammation [157,158], and FFA themselves are proven to activate M2 macrophage polarization, as observed in chronically palmitate-treated murine macrophages [159]. Based on the studies on rat myoblasts and murine macrophages, it is suggested that FFAs are able to induce macrophages to secrete proinflammatory cytokines (TNF-α and IL-6), augmenting the inflammatory response, through c-Jun N-terminal kinase (JNK) and p38 Ras-mitogen-activated protein kinase (MAPK) phosphorylation [160]. Proinflammatory macrophages can worsen or impose the lipotoxic effects on other cells by infiltrating tissues and releasing proinflammatory cytokines in a mouse model [161,162]. However, macrophages can also suffer from an excess FFA in their cells and undergo lipotoxic ER stress or apoptosis, since these processes are not tissue-specific.

TLRs are innate immune cell receptors that include several subtypes (e.g., TLR1, TLR2, TLR3, TLR4, etc.) and it was believed that they can be activated by FFA. TLRs recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), exogenous and endogenous molecules promoting cell-autonomous inflammatory responses, and subsequently activate intracellular signaling pathways via the adaptor molecule myeloid differentiation primary response 88 (MyD88). MyD88-dependent signaling triggers the classical inflammatory cascade, resulting in NF-κB and MAP kinase activation [163,164]. The interaction between TLR and MyD88 results in autophosphorylation of IL-1R-associated kinase (IRAK). Activated IRAK interacts with TNF-associated factor 6 (TRAF6), resulting in the stimulation of JNK and IKK (inhibitor of a κ-B kinase; prevents NF-κB inhibition) signaling pathways, which directly contribute to inducing inflammatory responses [165,166].

There is numerous evidence that FFAs participate in the activation of TLR1, TLR2, and TLR4. TLR1 and TLR2 are activated by receptor dimerization, resulting in the activation of downstream signaling pathways and target gene expression, as stated in the study conducted on murine macrophages and human monocytes. As a result, inflammasome-mediated IL-1β production is induced [167,168]. There are also findings in murine macrophages and rat β-cells that lipotoxic activations of TLR4 are involved in FFA-induced cell death through the activation of the JNK pathway or intersection between TIR-domain-containing adapter-inducing interferon-β (TRIF) signaling and impaired lysosome function [164,166]. In addition to the JNK pathway, the activation of Blc-2-associated X protein (Bax), a pro-apoptotic member of the Blc-2 family, and the engagement of the mitochondrial proapoptotic pathways are observed, as observed in human hepatocytes chronically treated with palmitate and high-fat fed rats [169–171]. The imbalance in proapoptotic (e.g., Bax and Bak) and antiapoptotic (e.g., Bcl-2) protein in the Bcl-2 family was also highlighted by research conducted on human hepatocytes treated for 8 h with 800 μM palmitate and mouse islet chronically treated with 500 μM palmitate by Akazawa et al. and Litwak et al. [172,173]. Proapoptotic proteins of the Bcl-2 family can induce the release of cytochrome c from mitochondria to the cytoplasm, which activates the caspase-dependent apoptotic signaling pathway. There are observations that FFA intervention results in increased expressions of caspases 3 and 9 (key enzymes of the mitochondrial apoptotic pathway) and decreased expressions of

antiapoptotic factor Bcl-2, which carries the potential to alleviate FFA proapoptotic actions. FFA can also significantly induce cytochrome c release in mouse β-cells and human lymphocytes [174,175]. Studies in mouse macrophages and mouse myocytes also provided a link between the activation of TLR2 and TLR4 by FFA and FFA-induced IR by the activation of macrophages via TLR2-, TLR4-, and JNK-dependent inflammatory pathways and the activation of TLR2 in muscle cells [176,177]. As observed in rat β-cells and murine microglial cells, the potential phenomenon of activating TLR gives FFA the ability to stimulate signaling pathways of JNK and NF-κB [166,178], which results in an increased production of pro-inflammatory cytokines and sustained inflammation. Although there is a significant difference between experimental FFA treatment of the cell culture, the activation of TLR is a proposed mechanism of non-alcoholic steatohepatitis (NASH) development from NAFLD in a mouse model [179]. Therefore, this phenomenon might serve as a beneficial beacon for future studies.

The indirect cell-autonomous inflammatory effects of FFA are also related to other lipotoxic actions, such as the aforementioned increased production of ceramides, DAG, or ROS. DAG is an allosteric activator of PKC isoforms. An excess of FFA and following a rise in DAG amount causes the activation of PKC, which can induce OS and activate NF-κB. The activation of the PKC-NF-κB axis and subsequent increase in IL-6 expression has been proposed as an FFA-induced mechanism of insulin resistance based on human studies and studies on mouse myoblasts [43,180]. Increased levels of ceramides are not only an effect of lipotoxicity caused by FFA but are also induced by augmented signaling through TLR4. Ceramides inhibit glucose uptake by blocking the activation of Akt/PKB, which contributes strongly to insulin resistance, as observed in lipid infused mice [181]. ROSs are able to drive ATF3-mediated inflammatory responses in HBMEC treated with isolated human TGRL [93], and FFAs are known for stimulating the synthesis of ROS; thus, the conclusion is that inducing OS by FFA is associated with inducing inflammation. There was also an observation that, in murine macrophages, chronic FFA exposure can induce the expression of cyclooxygenase-2 (COX-2), an important enzyme in prostaglandin synthesis from arachidonic acid, which is expressed in the process of inflammation [182]. Based on studies conducted on rat muscle cells treated acutely with FFA, the formation of the IRE1-TRAF2-IKK complex (with another type of TRAF than mentioned before) and the activation of NF-κB has also been linked to the palmitate's ability to reduce GLUT4 gene expression, therefore indicating the connection between lipotoxic ER stress, inflammation, and insulin resistance [144]. The subsequent presence of lipotoxic inflammation and insulin resistance has been observed in rat myotubes treated chronically with palmitate [149].

Findings confirm that IRE1, the ER stress sensor, can be activated via TRL, leading to the production of pro-inflammatory cytokines in a mouse model and mouse macrophages [183–185]. This phenomenon means that FFA activating IRE1 and TLR simultaneously also triggers TRL-mediated activations of IRE1, leading to the development of both ER stress and inflammation. Moreover, FFA-induced activation of IRE1 can mediate the activation of NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome, an intracellular protein complex that assembles in response to DAMP and PAMP and catalyzes the cleavage and maturation of cytokines IL-β1 and IL-18. Such observations were made in isolated primary mouse BMDC, a mouse model of long-term high-fat fed mice, mouse hepatocytes, and human studies [112,186,187]. FFAs act as a DAMP to activate the NLRP3 inflammasome in the NASH mouse model. NLRP3 inflammasome activation can also be mediated by sensing mitochondrial dysfunction (through the direct binding of NLRP3 with mtDNA) and the decrease in mitochondrial membrane potential, possibly an important prerequisite of mtDNA release from the mitochondria to the cytoplasm (a key factor in NLRP3 inflammasome activation), as observed in Kupffer cells isolated from mouse livers [188]. The study conducted by Weber et al. shows that lysosomes play an important role in the lipotoxic NLRP3 inflammasome in primary mouse macrophages. In the cell treated with both palmitate and lipopolysaccharide (LPS), lysosome destabilization contributed to NLRP3 and caspase-1 activation and subsequent IL-1β release [189]. FFA can also induce

inflammasome-mediated IL-1β production in human monocytes by inducing the activation of TLR2 [168].

Due to the activation of various cell-autonomous inflammatory responses, FFA stimulates the secretion of pro-inflammatory cytokines such as TNF-α, which further induces inflammatory pathways such as IKKβ/NF-κB or PKC/NF-κB cascades. Such observations were made in lipid-infused and high-fat fed mice, in mouse myotubes treated chronically with 500 µM palmitate, and in human hepatocytes treated chronically with 500 µm and 1 mM FFA (2:1 oleate/palmitate) [190–192]. FFA also induce sensitization to TNF-related apoptosis-inducing ligand (TRAIL) mediated apoptosis, as observed in human hepatocytes treated chronically with a mixture of stearic acid and oleic acid with the addition of recombinant human TRAIL [193]. The aforementioned IL-1β subsequently activates IL-1R, which results in IL-1β auto stimulation and the further upregulation of IL-1-dependent chemokines and cytokines, such as IL-8 and IL-6. IL-1R mRNA expression was significantly increased in human islets after chronic exposure to 500 µM of palmitate, stearate, and oleate separately [194].

The means of FFA-associated activation of inflammatory responses are so numerous and intertwined that they resemble a network rather than a straight pathway. The complexity of this network means that FFAs have several means (both direct and indirect) of inducing the same signaling pathways and that FFA-induced inflammation cannot be stopped with the attenuation of one transmitter.

Table 3 presents summarized molecular mechanisms of inflammatory responses induced by FFA. Even though most of those mechanisms concern cells of the immune system, there are examples of inflammatory responses in different types of cells that are not tied to the immune system. Macrophages activated towards inflammation may contribute to systemic inflammation and infiltration of tissues invoking local inflammatory response and worsening further pathological states, such as IR, ER stress, and OS.

Table 3. Selected molecular mechanisms of lipotoxic inflammatory response and their sites of confirmed occurrence.

Molecular Mechanism of Inflammation	Organism	Cell Culture	Organ/Cell Type	Reference
Increased IL-6	human	HCASMC	smooth muscle	[147]
		hCAEC	endothelium	[148]
	rat	cell line L6	muscle	[149]
	mouse	cell line RAW 264.7	macrophage	[155]
		cell line BV-2	microglia	[178]
		cell line C2C12	muscle	[177]
Increased IL-1beta	human	HCASMC	smooth muscle	[147]
		cell line THP-1	monocyte	[168]
	mouse	cell line RAW 264.7	macrophage	[176]
		cell line BV-2	microglia	[178]
		BMDC	dendritic cells	[112]
		isolated Kupffer Cells	macrophages	[188]
Increased IL-8	human	HCASMC	smooth muscle	[147]
		hCAEC	endothelium	[148]
	mouse	cell line RAW 264.7	macrophage	[167]
Increased TNF-alfa	human	HCASMC	smooth muscle	[147]
		cell line Hep2G	liver	[195]
	mouse	cell line RAW 264.7	macrophage	[155]
		cell line 3T3-L1	preadipocyte	[156]
		cell line BV-2	microglia	[178]
		cell line C2C12	muscle	[191]

Table 3. *Cont.*

Molecular Mechanism of Inflammation	Organism	Cell Culture	Organ/Cell Type	Reference
Activation of NF-kB	human	primary HAEC	endothelium	[150]
		cell line THP-1	monocyte	[168]
		cell line Hep2G	liver	[192]
	rat	cell line L6	muscle	[149]
		cell line INS-1	pancreas	[166]
	mouse	cell line C2C12	muscle	[177]
Activation of JNK	human	hCAEC	endothelium	[148]
		cell line THP-1	monocyte	[168]
	rat	cell line INS-1	pancreas	[166]
	mouse	cell line RAW 264.7	macrophage	[176]
		cell line C2C12	muscle	[177]
Involvement of TLRs	human	cell line THP-1	monocyte	[168]
	rat	cell line INS-1	pancreas	[166]
	mouse	cell line RAW 264.7	macrophage	[176]
		cell line BV-2	microglia	[178]
		cell line C2C12	muscle	[177]
Inflammasome activation	mouse	BMDC	dendritic cells	[112]
		cell line Hepa1–6	hepatoma	[186]
		cell line RAW 264.7	macrophage	[186]
		isolated Kupffer Cells	macrophage	[188]
Induced COX-2	mouse	cell line RAW 264.7	macrophage	[182]
Increased MCP-1	human	HCASMC	smooth muscle	[147]
		hCAEC	endothelium	[148]
	mouse	cell line RAW 264.7	macrophage	[167]

3.5. Induction of Insulin Resistance

Insulin resistance alone is considered a disease, but it can also lead to the development of T2DM or metabolic syndrome when untreated. FFAs have been proven to induce insulin resistance and several aspects and mechanisms of this action have been proposed. One of them is the inhibition of glucose transport and phosphorylation with a subsequent reduction in rates of glucose oxidation and glycogen synthesis. Due to defects in β-oxidation, fatty acid metabolites, such as acyl-CoA, are generated, with an ability to induce a defect in glucose transport. Acyl-CoA inhibits the activity of enzymes responsible for the metabolism of glucose-6-phosphate and accumulates glucose-6-phosphate, which inhibits glucose transport by negative feedback [196,197]. FFAs also might inhibit the insulin suppression of endogenous glucose production by interfering with the insulin suppression of glycogenolysis, as observed in human studies [198]. Impaired glucose metabolism is also linked to mitochondrial dysfunction, thus suggesting that FFA-induced mitochondrial dysfunction (e.g., disruption of the mitochondria-associated ER membrane) could be an early event in the development of FFA-induced IR. The reason for mitochondrial dysfunction is most likely an inability to match oxidative capacities to excess required ATP content. The link between impaired metabolism and mitochondrial dysfunction was demonstrated in studies on rat and mouse myotubes treated chronically with 100 μM palmitate and human hepatocytes (insulin resistance induced after chronic 300 μM palmitate treatment and mitochondrial dysfunction induced after acute 300 μM palmitate treatment) [149,199,200]. Therefore, the accelerated transport of FFA to the mitochondria and the improvement of mitochondrial fatty acid β-oxidation seem to protect the cell from FFA-induced IR, as observed in rat skeletal muscle cell treated chronically with 250 μM palmitate and mouse myocytes treated chronically with 500 μM palmitate [201–203].

Another mechanism suggested to be responsible for FFA-induced IR is the inhibition of insulin signaling. In the presence of insulin, the insulin receptor (INSR) phosphorylates the insulin receptor substrate proteins (IRS proteins). The activation of the two main signaling pathways occurs: the phosphatidylinositol 3-kinase (PI3K)-Akt/PKB pathway (responsible for most of the metabolic actions of insulin) and MAPK pathway (regulating the expression of some genes and cooperating with the PI3K pathway) [204]. FFAs can alter insulin signaling through the phosphorylation of IRS proteins, as demonstrated in rat hepatocytes after acute 200 μM palmitate treatment, human podocytes after chronic 750 μM palmitate treatment, and human skeletal muscle cells after chronic 400 μM palmitate treatment [205–207]. The activities of various elements of insulin signaling pathways, such as PKB or PI3K kinase, have been observed to be altered by FFAs in human studies and in a rat model subjected to lipid infusion [208,209]. The coexisting inhibition of insulin signaling-associated gene expression also has been related to the lipotoxic action of chronic FFA treatment of human podocytes and rat hepatocytes [206,210].

Further research on the mechanism of IR caused by FFA resulted in observations that lipotoxic metabolic fatty acid intermediates (DAG, ceramides, and acylcarnitines) disturb insulin action. IR is correlated with the accumulation of DAG, ceramides, and acylcarnitines, which is a result of the inhibition of mitochondrial FFA uptake, incomplete β-oxidation, and others (e.g., de novo ceramide synthesis) [45,47,49]. Some researchers conducted studies on human podocytes and high-fat fed rats and proposed that accumulated FFA intermediates not only correlate with IR but are responsible for its induction [206,211]. The inhibition of PKB signaling was proven to be derived from elevated levels of ceramide by promoting activations of an atypical isozyme of PKC, PKCζ, which negatively regulates PKB [56]. Furthermore, the ability of DAG to activate PKC plays a crucial role in FFA-induced IR. The development of IR in lipotoxic conditions (human studies and rat NAFLD model) is associated with an increase in PKC activity, suggesting that PKC-activating action of DAG is at least in part involved in the mechanism of FFA-mediated IR induction [43,212,213]. This feature resulted in assuming DAG to be a more important determinant of IR than ceramide, as observed in a study on rat skeletal muscle cells after chronic palmitate treatment [214]. In studies on lipid infused rats and rat skeletal muscle cells, the activation of various isoforms of PKC has been linked to disturbed insulin signaling as a result of, e.g., decreased activation of IRS-1-associated PI3 kinase [215–217]. However, there is research conducted on mice by Monetti et al., contradicting the role of DAG in IR, where accumulated lipotoxic intermediates did not cause IR, which resulted in an assumption that other mechanisms and metabolic pathways, such as inflammation, may be required for the development of IR [218].

There are data from studies on rat myoblasts and murine macrophages, as well as human hepatocytes and murine macrophages, indicating that IR is associated with inflammation and suggesting that FFA-induced local secretion of MCP-1 participates in the recruitment of macrophages. Moreover, FFAs mediate M1 macrophage polarization, therefore creating an inflammatory environment through the release of proinflammatory cytokines [160,219]. The linkage between FFA, inflammation, and IR is also demonstrated through the activation of a master regulatory signaling pathway of inflammation—NF-κB or decreased IκB, an inhibitor of NF-κB, as observed in human studies and murine myoblasts after chronic 1 mM palmitate treatment [43,220]. In a study performed on rats infused with lipid emulsions, Barazzoni et al. pointed out that the activation of NF-κB is mediated by the enhancement of ROS production by fatty acids, therefore indicating ROS participation in IR induction [95]. Another study on rat hepatocytes treated chronically with palmitate shows that ROS has a part in desensitizing insulin signaling pathways by activating JNK, impairing the phosphorylation of IRS [221]. There are also reports from studies on human hepatocytes (chronic 250 μM palmitate exposure) and on high-fat fed mice that NOX2 and NOX3 may contribute to IR by mediating ROS generation and that inhibiting the activity of NOX oxidases may restore insulin sensitivity [80,222]. This suggests the connection between two FFA-induced processes: ROS and IR. Other than that,

ER stress may also have a role in the development of IR, as observed in murine mesentery arterioles after intraluminal palmitate infusion and in mice in vivo studies [138,223].

There is a study on murine and human myotubes treated chronically with various concentrations of palmitate (250 μM, 500 μM, and 750 μM) that showed that ER stress does not mediate IR, but a marked activation of UPR can induce IR at the level of IRS through the activation of the IRE-1/JNK pathway [224]. The matter of ER stress involvement is, therefore, debatable and needs further research to determine actual linkage; however, upon the aforementioned observations, there seems to be a connection of unknown nature between ER stress and IR, and we cannot state for sure that these processes are completely separate. There is evidence that the activation of TLR2 mediates proinflammatory responses that contribute to IR with the participation of MyD88, JNK, and NF-κB, as observed in mouse myocytes (acute and chronic 750 μM palmitate treatment), HAEC (acute 200 μM palmitate treatment), and murine preadipocytes (chronic 500 μM palmitate treatment) [177,225,226]. The fact worth mentioning is that the activation of TRL2 also mediates UPR, and that is another link potentially connecting ER stress with FA-induced IR.

FFAs are putative mediators of β-cell dysfunction, which may lead to the loss of functional β-cell mass and, therefore, contribute further to IR and the pathogenesis of T2DM. As demonstrated in human studies and in rat β-cells incubated chronically with 200 μM palmitate and glucose, FFAs inhibit insulin biosynthesis, leaving β-cells drained from their islet insulin content and with limited means to replenish it [227,228]. The disruption of insulin biosynthesis might occur due to the direct inhibitory effect of FFA on insulin gene expression. There are reports from studies on rat β-cells (chronic palmitate treatment), isolated rat islets (chronic palmitate treatment), and hamster β-cells (chronic palmitate treatment) that this effect takes place only in the presence of high glucose [229–231]. The mechanism behind this phenomenon is not yet elucidated, but there might be involvements of JNK activation (mouse hepatocytes after acute 500 μM palmitate treatment) [232], inhibition of insulin promoter activity, and de novo ceramide synthesis (rat islets after chronic 500 μM palmitate treatment) [233], or reduced binding of key regulatory factors (transcription factor MafA and pancreas-duodenum homeobox-1 (PDX-1)) to the insulin gene promoter (rat islets after chronic 500 μM palmitate treatment) [234]. As a consequence of decreased insulin gene expression, intensified lipogenesis is present, resulting in increased DAG and TAG content [231].

In addition to serving as an energy substrate, FFAs can also act as signaling molecules interacting with FFA receptors (FFARs), a family of G protein-coupled receptors (GPCRs). Currently, five types of GPCRs are known to be activated by FFA: FFAR1, FFAR2, FFAR3, FFAR4, and GPR84. These receptors are expressed in numerous sites, such as β-cells, immune cells, central nervous system, or adipose tissue, and they play a part in insulin secretion, inflammatory responses, and other significant signaling pathways [235]. Although the role of FFAR in cell metabolism is well described, their role in the molecular mechanisms of lipotoxicity requires further research. The majority of research concerning FFAR and lipotoxicity refers to FFAR1's role in β-cells. A study conducted by Marafie et al. demonstrated a possible regulatory role for FFAR1 in insulin signaling in rat β-cells under lipotoxic conditions (chronic palmitate treatment) [236]. Another study confirms that FFAR1 mediates, in part, the palmitate-induced (chronic palmitate treatment) potentiation of GSIS in mouse β-cells. Furthermore, inhibition and activation of FFAR1 seem to impose a different effect on the cell depending on FFA levels [237]. Graciano et al. showed that FFA-stimulated (acute palmitate treatment) superoxide production and insulin secretion are FFAR1-dependent in rat β-cells [238]. The level of FFAR1 expression is also negatively correlated with β-cell apoptosis. FFAR1 also plays a role in pioglitazone-mediated attenuation of FFA-induced oxidative stress and apoptosis, as observed in mouse β-cells after chronic palmitate treatment [239]. Similarly, exendin-4 prevents the proapoptotic effect of palmitate by reducing FFAR1 expression in human and murine pancreatic islets and rat β-cells [240]. Therefore, FFAR1 might serve as a direction of research on pharmacological agents with the ability to attenuate lipotoxicity in β-cells.

There also is a concept of glucolipotoxicity concerning β-cells, which includes the combined deleterious effect of both high FFA and glucose levels. The term was proposed to describe FFA lipotoxicity occurring in β-cells, which in this case is often dependent on high glucose levels, resulting in OS, loss of insulin secretory function, β-cell death, and inflammation, as observed in rat pancreatic islets and mouse and rat β-cells [241,242].

3.6. Autophagy

Autophagy is a process of cell self-digestion that regulates cellular component degradation through lysosomes by degrading malfunctioning organelles and proteins and plays an important role in maintaining cell homeostasis by regulating the synthesis and degradation of cellular components. Various data indicate that autophagy serves as an adaptive process that could overcome the deleterious effect of FFA on the cell, as observed in rat β-cells after chronic 400 μM palmitate treatment and in murine adipocytes after chronic treatment with 500 μM and 1 mM palmitate [243,244]. In physiological conditions, this process is upregulated by the deprivation of nutrients and growth factors.

There are many observations that excess FFA is able to increase the rate of autophagy concerning high-fat fed mice, mouse hypothalamic neuronal cell line, rat β-cells (after chronic palmitate exposure), and isolated rat pancreatic islets (after chronic palmitate exposure) [245–247]. A study on rat β-cells after chronic 400 μM palmitate treatment suggests that increased autophagy could overcome FFA-induced cell death and delay cell loss [243]. Evidence shows that the activation of autophagy under lipotoxic conditions occurs in a JNK-dependent manner, as observed in murine adipocytes after chronic treatment with palmitate, rat β-cells after acute treatment with 500 μM palmitate, and murine β-cells after chronic treatment with 500 μM palmitate [244,248,249]. It has been shown earlier that FFAs as TLR agonists are able to activate the JNK signaling pathway; therefore, it is possible that the activation of autophagy is a direct effect of saturated fatty acids. Another study on high-fat fed mice suggests that ceramides have their part in lipid-induced autophagy, pointing out that FFA could have imposed an indirect autophagy-inducing effect [250]. Additionally, Tan et al. indicated that FFA promoted DAG accumulation, and subsequent PKC activation is an upstream signaling mechanism for autophagy in mouse embryonic fibroblasts treated acutely with 250 μM palmitate [251].

The aspect of dependency of FFA-induced autophagy on other FFA-induced processes, such as ER stress or OS, is debatable. Quan et al. pointed out that autophagy is necessary for proper UPR action, leading to an assumption that both autophagy and ER stress are related processes, as observed in murine β-cells after chronic palmitate treatment [252]. Dependency of autophagy on ER stress was observed in another study on human osteoblast-like cells [139]. Yin et al. suggested that autophagy is a result of cellular stress induced by FFA, after the observation made on murine adipocytes after chronic treatment with palmitate that autophagy is induced subsequently to the activation of the ER stress-JNK pathway and plays a protective role against FFA-induced cell death, confirming the relationship between the processes [244]. Furthermore, Chen et al. suggested that FFA-induced autophagy activates Ca^{2+}/PKCα/NOX4 pathways to promoter ROS generation, as observed in HUVEC after chronic 300 μM palmitate treatment [253]. However, a different study conducted on rat β-cells after acute treatment with 500 μM palmitate demonstrated that FFA-stimulated autophagy occurs independently of oxidative or ER stress [248]. Further research is needed to determine the essence of relations between the processes.

There are many studies showing that FFAs are able to inhibit autophagy, worsening their detrimental impact. Nutrient abundance has been associated with the suppression of autophagic turnover, resulting in the accumulation of autophagosomes, autophagy substrates, ER stress, and lysosomal dysfunction, thus contributing to cell death, as observed in murine and rat β-cells and human pancreatic islets after chronic treatment with 400 μM palmitate and mouse astrocytes (inhibition of autophagic activity was observed after both acute and chronic palmitate treatment) [254,255]. The impairment of autophagy induced by chronic FFA treatment (500 μM palmitate) in mouse myoblasts may play a pivotal

role in inflammation regulation and OS might also serve as a mechanism of autophagy modulation by FFA [256]. A study conducted by Mei et al. on human hepatocytes suggests a concept that saturated FFAs induce apoptosis, decrease autophagy, and that autophagy and apoptosis are two antagonistic events that tend to inhibit each other [257]. Even though the views on the ability of FFA to induce or inhibit autophagy are divided, there is no doubt that autophagy is an important aspect of the lipotoxicity of the cell. Because of its protective actions, any autophagy inhibiting factor will, without a doubt, lead to the worsening of lipotoxicity, therefore naming those factors as beneficial for understanding the lipotoxic impact on the cell.

4. Conclusions

Although saturated fatty acids play a vital role in organelle structure, signaling pathways, and supplying the energy demand, the excess over the cell's ability to store fuel is proven to be detrimental for the proper functioning of the cell. Excess FFA might be caused by obesity, a high-fat diet, or dysfunction of adipose tissue manifested as reduced FFA storage capacity and increased releases of FFA from adipocytes. Lipotoxicity is referred to as deleterious processes caused by excess FFA in non-adipose tissues, such as OS, ER stress, inflammation, or insulin resistance. Despite many attempts to ascribe a specific metabolic pathway to the mechanism of toxic FFA actions, our review shows that lipids modulate cellular functions by multiple methods, resulting in many simultaneously occurring disturbances that tend to interfere with each other, creating a potential branched vicious cycle of self-driven lipotoxic processes. Therefore, being able to ameliorate lipotoxicity seems to be extremely difficult, because silencing one deleterious signaling cascade does not ensure that other signals will not disturb cell function. It seems that reducing excess FFA is the most eligible method to avoid toxic consequences.

Further understandings of the interrelation of molecular mechanisms of lipotoxicity may provide beneficial information about the development of pathological states. In addition to most obvious lipotoxicity-related diseases, such as T2DM or NAFLD, mechanisms of lipotoxicity may elucidate yet unknown etiologies of other diseases that are, at first glance, not related in any way to lipid metabolism, which constitutes further interesting research directions in this field.

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The Influence of Nucleoside Reverse Transcriptase Inhibitors on Mitochondrial Activity, Lipid Content, and Fatty-Acid-Binding Protein Levels in Microglial HMC3 Cells

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Abstract: Despite the availability of a wide range of preventive measures and comprehensive treatment options following infection, the development of acquired immunodeficiency syndrome (AIDS) remains a persistent challenge. Nucleoside reverse transcriptase inhibitors (NRTIs) represent the most commonly utilized therapeutic approach, despite being on the pharmaceutical market for nearly four decades. During this time, a spectrum of side effects ranging from mild discomfort and hypersensitivity reactions to the more prevalent nephrotoxicity and hepatotoxicity has been documented. In light of these considerations, our study aimed to investigate the impacts of two NRTIs, lamivudine and zidovudine, on lipid metabolism in HMC3 microglial cells. Our findings revealed statistically significant reductions in the ATP levels (nearly 8%) and increased mitochondrial superoxide levels (around 10%) after 24 h of treatment with the maximum therapeutic concentration of zidovudine compared to the untreated microglial cells. Furthermore, the concentrations of fatty-acid-binding proteins 4 and 5 were significantly lower (approximately 40%) in the microglial cells that were exposed to NRTIs than in the untreated cells. Notably, the total lipid concentration within the microglial cells markedly increased following NRTI administration with a 13% rise after treatment with 10 μM lamivudine and a remarkable 70% surge following the administration of 6 μM zidovudine. These results suggest that the prolonged administration of NRTIs may potentially lead to lipid accumulation, posing a significant risk to the delicate homeostasis of the neuronal system and potentially triggering a pro-inflammatory response in microglial cells.

Keywords: HIV; NRTIs; fatty acids; cell culture

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1. Introduction

Human immunodeficiency virus (HIV) infection poses a pervasive global health challenge, impacting millions of individuals worldwide (UNAIDS, 2021; World Health Organization, 2021). In the several decades since its inception, numerous therapeutic strategies have been developed to mitigate the effects of HIV infection. The primary goal of these therapeutic agents is to curtail the viral load, bolster immune functionality, and elevate the CD4+ cell counts, thereby diminishing the incidence of HIV-related ailments and lowering the risk of viral transmission [1].

Antiretroviral drugs, classified into six groups with distinct mechanisms of action, intervene at various stages of the virus's replication cycle, thereby hindering its proliferation within the host's organism. Within the phase of viral replication, reverse transcriptase inhibitors act, and they are further divided into two subgroups. The first subgroup comprises nucleoside reverse transcriptase inhibitors (NRTIs), which competitively inhibit the enzyme by integrating themselves into the DNA chain instead of the substrate for synthesis, leading to premature termination. Notable agents in this group include abacavir, lamivudine (3TC), zidovudine (AZT), and tenofovir. Presently, the conventional therapeutic approach is to amalgamate 2–3 pharmaceuticals of diverse classes, creating highly active antiretroviral therapy (HAART), also known as combined antiretroviral therapy (CART).

This regimen has proven to be more efficacious in impeding disease progression compared to monotherapy, extending patient survival by up to 7–10 years [2,3]. This therapeutic approach is suggested to be instituted as early as possible in all HIV-infected individuals, regardless of their CD4+ lymphocyte count. It is imperative to underscore that current antiretroviral therapy does not achieve the complete eradication of the virus from the host's body. Rather, it plays a pivotal role in retarding the progression of the disease. This is due to the existence of reservoirs housing latent viral forms, sustained viral replication notwithstanding therapeutic interventions, and the virus's localization within anatomical structures that are relatively inaccessible to pharmaceuticals, including the central nervous system (CNS) [4,5]. NRTIs also exhibit a spectrum of toxic effects, precipitating a multitude of side effects that constitute a significant clinical challenge for patients [3]. These side effects, stemming from nucleoside analogs, can be categorized into those arising from short-term toxicity and long-term toxicity. In the short term, the use of AZT may trigger symptoms such as nausea, vomiting, weakness, or headaches [6,7]. The concomitant administration of 3TC and AZT may induce hypersensitivity reactions (HSR), typified by symptoms including fever, rash, muscle pain, abdominal discomfort, drowsiness, and respiratory distress. Notably, repeated exposure to the drug following HSR may precipitate more severe anaphylactic reactions [8]. Anemia, a common side effect of AZT, results from impaired erythrocyte precursor synthesis in the bone marrow, giving rise to macrocytic anemia. [9]. This circulatory abnormality is primarily associated with thymidine analog drugs, particularly AZT, where macrocytosis is almost universally observed. An elevated mean erythrocyte volume in patients receiving AZT has emerged as an effective marker of treatment efficacy [10]. In the long term, NRTIs induce mitochondrial toxicity primarily via the inhibition of γ polymerase, which is responsible for mitochondrial DNA synthesis. Furthermore, NRTIs inflict oxidative damage, inhibit other mitochondrial enzymes, disrupt ATP synthesis, and trigger cell apoptosis, collectively culminating in the impairment of various systems and organs. The liver is particularly susceptible to mitochondrial toxicity, leading to the inhibition of fatty acid oxidation, ensuing lipid accumulation in the vesicles and transformation into triglycerides. This impedes gluconeogenesis, preventing the conversion of lactic acid into pyruvate, culminating in heightened blood lactate levels. The repercussions encompass symptoms such as malaise, weight loss, rapid breathing, nausea, and vomiting [3]. Patients frequently develop hepatic steatosis and hepatomegaly owing to triglyceride buildup. Severe cases, although relatively rare, may result in liver failure, cardiac arrhythmias, and elevated mortality rates (30–60%) [11]. Notably, among HIV-infected patients, a subtle decline in the bone mineral density is frequently observed, a phenomenon that is likely attributed to the impact of AIDS itself. However, the long-term toxicity of HAART, including NRTIs, significantly exacerbates this condition, ultimately resulting in osteopenia and osteoporosis [4]. AZT administration also contributes to the development of myopathy [12]. The use of NRTIs, coupled with resultant mitochondrial toxicity, induces various metabolic disorders, including disruptions in the lipid metabolism, ultimately giving rise to lipodystrophy—an accumulative fat tissue manifestation, predominantly in the abdominal region—along with, albeit less frequently, lipoatrophy, entailing the irreversible loss of subcutaneous fat tissue. These effects also extend to glucose metabolism due to the induction of insulin resistance [13]. This pertains to the excessive generation of reactive oxygen species, suppressing the expression of genes and proteins involved in pro-adipogenic transcription factors. This results in reduced adipocyte differentiation, accompanied by the impaired metabolism of both fatty acids and glucose [14].

Within the CNS, HIV infection can initiate neuroinflammation and cognitive impairment, collectively referred to as HIV-associated neurocognitive disorders (HANDs) [15,16]. During HIV infection, microglial cells become activated, subsequently releasing inflammatory mediators that contribute to neurotoxicity [17,18]. Notably, HIV proteins such as Tat and the viral envelope protein gp120 have been observed to activate microglial cells, eliciting the release of cytokines, chemokines, and reactive oxygen species (ROS) [19]. The activation of microglial cells during HIV infection has also been associated with the

stimulation of the NLRP3 (Nod-like receptor protein family, pyrin domain-containing 3) inflammasome, a multi-protein complex that activates caspase-1 and facilitates the secretion of pro-inflammatory cytokines, including IL-1β and IL-18 [20]. Metabolic perturbations, such as hyperglycemia and dyslipidemia, are commonplace in individuals living with HIV, contributing to the activation of microglial cells [21,22]. Additionally, palmitic acid, a saturated fatty acid, plays a role in promoting inflammation and oxidative stress across various cell types, including microglial cells [23]. The NRTI therapy efficacy in the CNS is constrained by the limited penetration of the blood–brain barrier (BBB) [24]. Furthermore, antiretroviral therapy has varying effects on microglial cells. 3TC mitigates microglial activation and reduces the production of pro-inflammatory cytokines, while AZT does not influence microglial activation but can reduce ROS production.

The research gap in this context lies in the critical need to comprehensively understand the molecular mechanisms underlying microglial activation and the various factors that can induce microglial dysfunction. While the activation of microglial cells during HIV infection is well established, there is a lack of clarity regarding the intricate processes and factors contributing to this activation. This gap in knowledge includes aspects such as the impacts of HIV proteins, metabolic disturbances, and antiretroviral therapy on microglial behavior. To bridge this gap and inform potential therapeutic strategies, our study was designed to investigate the effects of two nucleoside reverse transcriptase inhibitors (NRTIs), 3TC and AZT, on HMC3 microglial cells' homeostasis. By examining key intracellular parameters, including the ATP levels, ROS production, lipid content, and the expression of fatty-acid-binding proteins (FABP4 and FABP5), our research aims to provide fresh insights into how these molecules may function in the context of HIV infection within the CNS. This research is essential for the development of more effective treatment approaches for HIV and the prevention of HANDs. Furthermore, our findings have the potential to offer fresh insights into the mechanisms of action of these molecules in the treatment of HIV infection within the CNS.

2. Results

The HMC3 cell line, derived from transformed human microglial cells, faithfully maintains the essential characteristics of primary microglial cells. These cells form a homogeneous and nearly immortal population that is suitable for in-depth biochemical investigations into the functions of microglial cells. In addition to this, HMC3 cells demonstrate an amenability to transfection, providing a valuable platform for the study of gene regulation within microglial cells. Originating from primary human fetal brain-derived microglia, HMC3 cells display a resting state phenotype that includes a strong expression of the microglia/macrophage marker IBA1 and the endotoxin receptor CD14. Notably, they do not express the astrocyte marker GFAP. The transition to an activated microglial state, indicated by the upregulation of markers such as MHCII, CD68, and CD11b, can be achieved by exposure to IFN-gamma, highlighting their suitability for modelling activated microglia in studies. HMC3 cells are a valuable and adaptable resource for researchers investigating the intricacies of microglial cell function, particularly in the context of neuroinflammation and neurological disorders.

2.1. Cell Cytotoxicity Assay: XTT

The impact of the investigated NRTIs on microglial cell viability was evaluated using the XTT assay. The findings following a 24 h incubation period with DHA, PA, and NRTIs are depicted in Figure 1 and are presented as a percentage of viability relative to the control group (unexposed cells). As a positive control for measuring cytotoxic effects, incubation with 10% DMSO was employed.

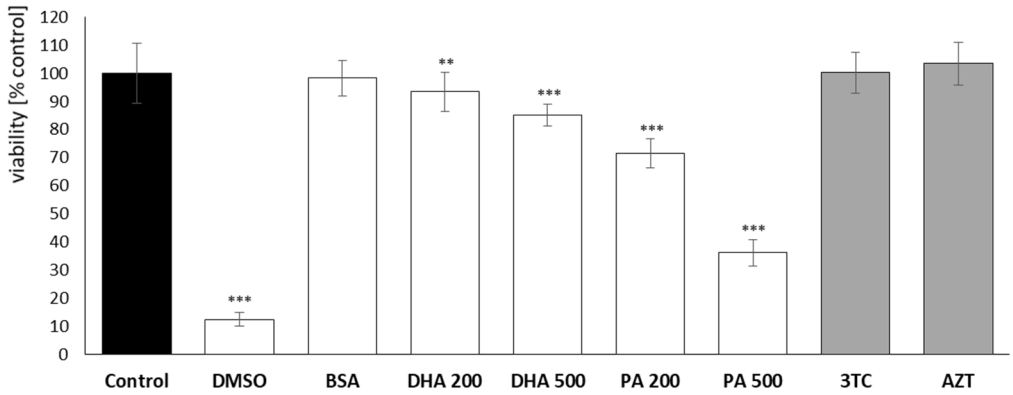

Figure 1. Cytotoxic effect of 24 h incubation with DHA, PA, 3TC, or AZT on microglial cells. Control—untreated HMC3 cells; DMSO—positive control, cells treated with 10% DMSO; BSA—incubation with 10% fatty acid-free BSA; DHA200 and DHA 500—incubation with 200 or 500 μM DHA; PA200 and PA 500—incubation with 200 or 500 μM PA; 3TC—incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine. Statistically significant differences compared to the control. ** $p < 0.01$; *** $p < 0.001$.

As anticipated, the most pronounced cytotoxicity was evident in the microglial cells following the introduction of 200 μM and 500 μM PA, resulting in cell mortalities of approximately 28% and 64%, respectively. An evaluation of the impact of docosahexaenoic acid (DHA), representing polyunsaturated omega-3 fatty acids, did not demonstrate a substantial adverse effect on the HMC3 cell viability, even at lipotoxic concentrations, with a mortality rate of approximately 15%. Notably, no indications of cytotoxicity were observed following a 24 h incubation with NRTIs.

2.2. ATP Measurement Assay, Bioluminescence Assay Kit HS II

The quantification of ATP levels following 1 h and 24 h of incubation with DHA, PA, or the evaluated NRTIs is depicted in Figure 2.

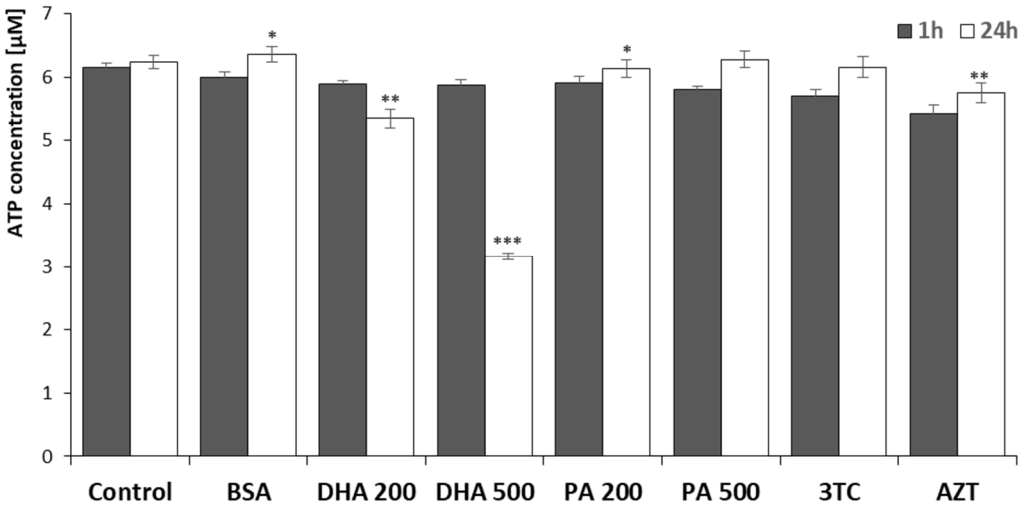

Figure 2. Mitochondrial ATP levels after 1 h and 24 h incubation with DHA, PA, 3TC, or AZT. Control—untreated HMC3 cells; BSA—incubation with 10% fatty acid-free BSA; DHA200 and DHA 500—incubation with 200 or 500 μM DHA; PA200 and PA500—incubation with 200 or 500 μM PA; 3TC—incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine. Statistically significant differences compared to the untreated HMC3 cells: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The initial analysis, involving a 1 h incubation period and focusing on the impact of NRTIs on the ATP levels, did not reveal significant differences. However, following a

24 h incubation, certain compounds exhibited a notable effect on ATP activation, resulting in statistically significant reductions in the ATP concentrations in specific instances. The most substantial influence was observed after incubation with DHA at concentrations of 200 μM and 500 μM, leading to decreases of 15% and 50% ($p < 0.01$ and $p < 0.001$, respectively). Additionally, a nearly 8% decrease was recorded after the administration of 6 μM of zidovudine. Furthermore, a statistically significant reduction in the ATP levels (in comparison to the untreated cells) was also observed after a 24 h incubation with 200 μM of PA.

2.3. Mitochondrial Superoxide Levels, MitoSOX™ Red Assay

Mitochondrial superoxide production can be visualized through fluorescence microscopy by employing the MitoSOX superoxide indicator. MitoSOX is particularly susceptible to rapid oxidation via superoxide, distinguishing it from other reactive oxygen species and reactive nitrogen species. The results of the fluorescence analysis are illustrated in Figure 3.

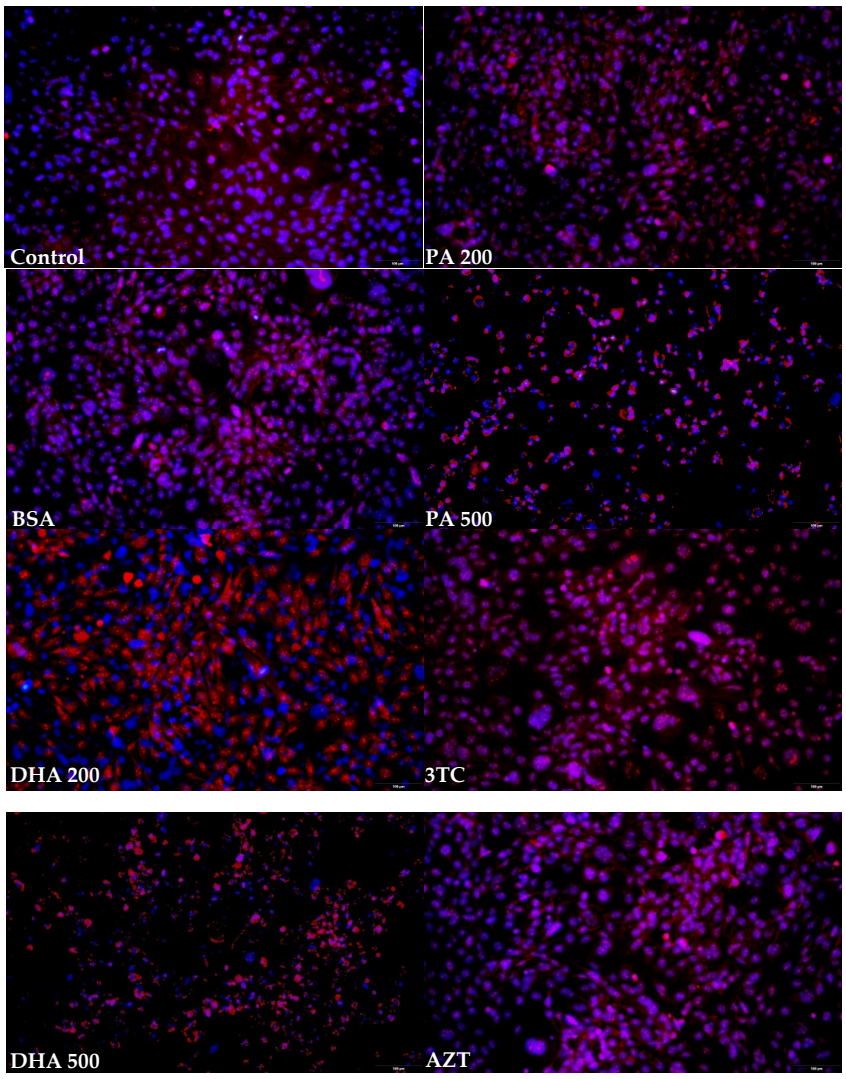

Figure 3. Examples of fluorescence microphotographs of fixed HMC3 cells stained with MitoSOX (red) and counterstained with DAPI (blue). Magnification: 20×. Control—untreated HMC3 cells; BSA—incubation with 10% fatty acid-free BSA; DHA200 and DHA500—incubation with 200 or 500 μM DHA; PA200 and PA500—incubation with 200 or 500 μM PA; 3TC—incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine.

An examination of the fluorescence micrographs revealed that the treatment with 500 μM DHA and 500 μM PA resulted in a distinct punctate fluorescence pattern, distinguishing it from the outcomes observed in the other experimental conditions. Conversely, the staining appeared more diffused and blurred in the case of 3TC and AZT when compared to the control cells. To quantitatively assess the differences between the various treatments, the MitoSOX fluorescence was spectrophotometrically measured at 580 nm.

The quantification of the mitochondrial superoxide levels was conducted in methanol-fixed HMC3 cells following a 24 h incubation with DHA, PA, or the tested NRTIs, as demonstrated in Figure 4.

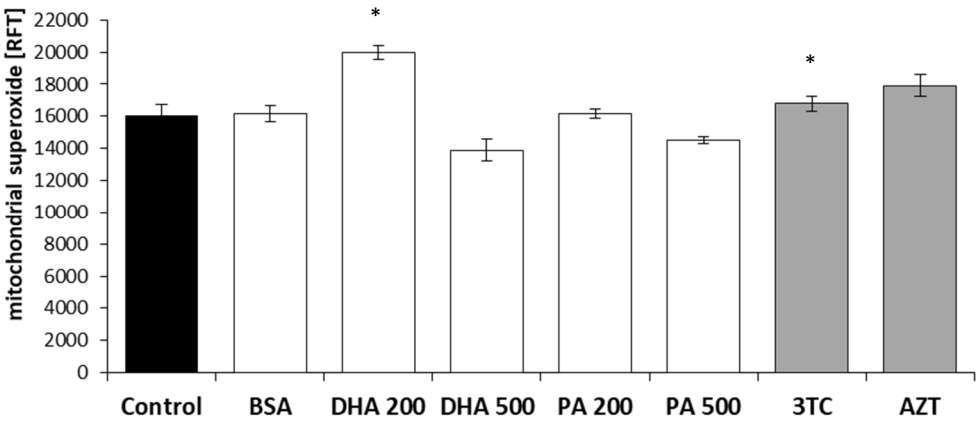

Figure 4. Mitochondrial superoxide levels after 24 h incubation with DHA, PA, 3TC, or AZT. Control—untreated HMC3 cells; BSA—incubation with 10% fatty acid-free BSA; DHA200 and DHA500—incubation with 200 or 500 μM DHA; PA200 and PA500—incubation with 200 or 500 μM PA; 3TC—incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine. Statistically significant differences compared to the untreated HMC3 cells: * $p < 0.05$.

The addition of 200 μM of DHA exhibited a statistically significant increase of approximately 25% in the detectable MitoSOX fluorescence. In the case of NRTI administration, we anticipated an elevation in the mitochondrial superoxide of around 10%, with statistical significance being achieved in the case of 3TC. Following incubation with the lipotoxic concentrations of DHA and PA, a reduction in the MitoSOX signal of nearly 10% and 13%, respectively, was observed.

2.4. The FABP 4 and 5 Concentrations, Human ELISA

The levels of FABP4 and FABP5 in HMC3 cell lysates following a 24 h incubation with DHA, PA, or the tested NRTIs are depicted in Figures 5 and 6. The results were normalized to a total protein concentration of 100 μg in the cell lysates, as determined using the BCA protein assay.

The intracellular levels of the FABP4 transporter exhibited a reduction in all experimental conditions, with the lowest values observed for the lipotoxic concentrations of 500 μM DHA and 500 μM PA, corresponding to 36% and 40% of the control, respectively. The physiological concentrations of 200 μM DHA or 200 μM PA showed slightly higher intracellular FABP4 levels. Incubation with 10 μM lamivudine or 6 μM zidovudine also led to a decrease of approximately 40%. Additionally, the results obtained after the administration of the carrier for polyunsaturated and unsaturated fatty acids (BSA) exhibited a decrease of 28% compared to the untreated cells, although this decrease was not statistically significant.

Similar to the observations with FABP4, the intracellular levels of the FABP5 transporter were reduced in all of the experimental conditions. The lowest levels of FABP5 were measured after the treatment with 200 μM DHA and 200 μM PA, resulting in a decrease of approximately 60%, while the lipotoxic concentrations of 500 μM DHA and 500 μM PA caused higher levels of FABP5, with concentration levels of 53% and 42% compared to the

untreated cells. Incubation for 24 h with 10 μM of lamivudine or 6 μM of zidovudine led to similar results as those obtained for 500 μM of PA.

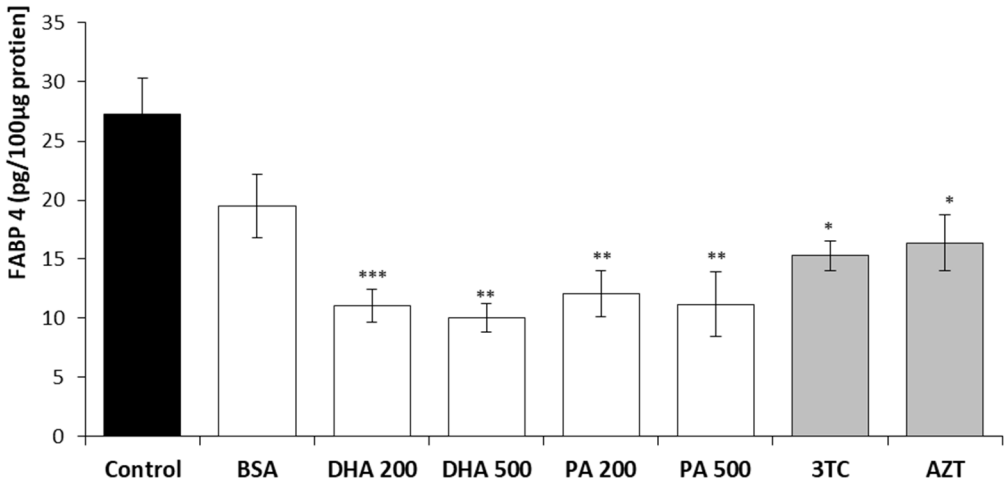

Figure 5. Intracellular FABP4 levels after 24 h incubation with DHA, PA, 3TC, or AZT. Control—untreated HMC3 cells; BSA—incubation with 10% fatty acid-free BSA; DHA200 and DHA 500—incubation with 200 or 500 μM DHA; PA200 and PA 500—incubation with 200 or 500 μM PA; 3TC10—incubation with 10 μM lamivudine; AZT6—incubation with 6 μM zidovudine. Statistically significant differences compared to the untreated HMC3 cells: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

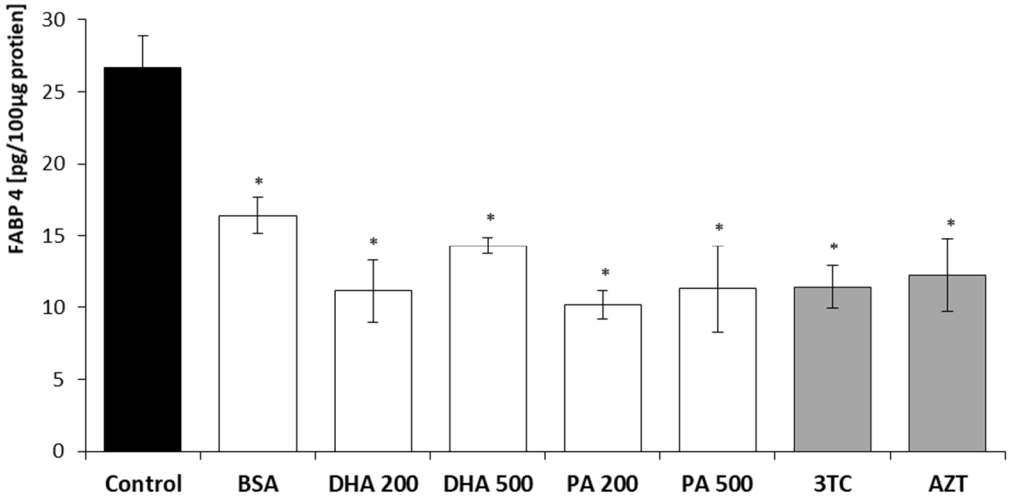

Figure 6. Intracellular FABP5 levels after 24 h incubation with DHA, PA, 3TC, or AZT. Control—untreated HMC3 cells; BSA—incubation with 10% fatty acid-free BSA; DHA200 and DHA 500—incubation with 200 or 500 μM DHA; PA200 and PA 500—incubation with 200 or 500 μM PA; 3TC 10—incubation with 10 μM lamivudine; AZT 6—incubation with 6 μM zidovudine. Statistically significant differences compared to the untreated HMC3 cells: * $p < 0.05$.

2.5. MAPK Family Activation Measurement, InstantOne ELISA™

The phosphorylation levels of the ERK, p38, and JNK1/2 proteins were analyzed in the HMC3 cell lysates following a 24 h incubation with DHA, PA, or the tested NRTIs. The results in Figure 7 are presented as multiples of the data obtained for the control, with the value for the untreated HMC3 cells (control) set as 1.

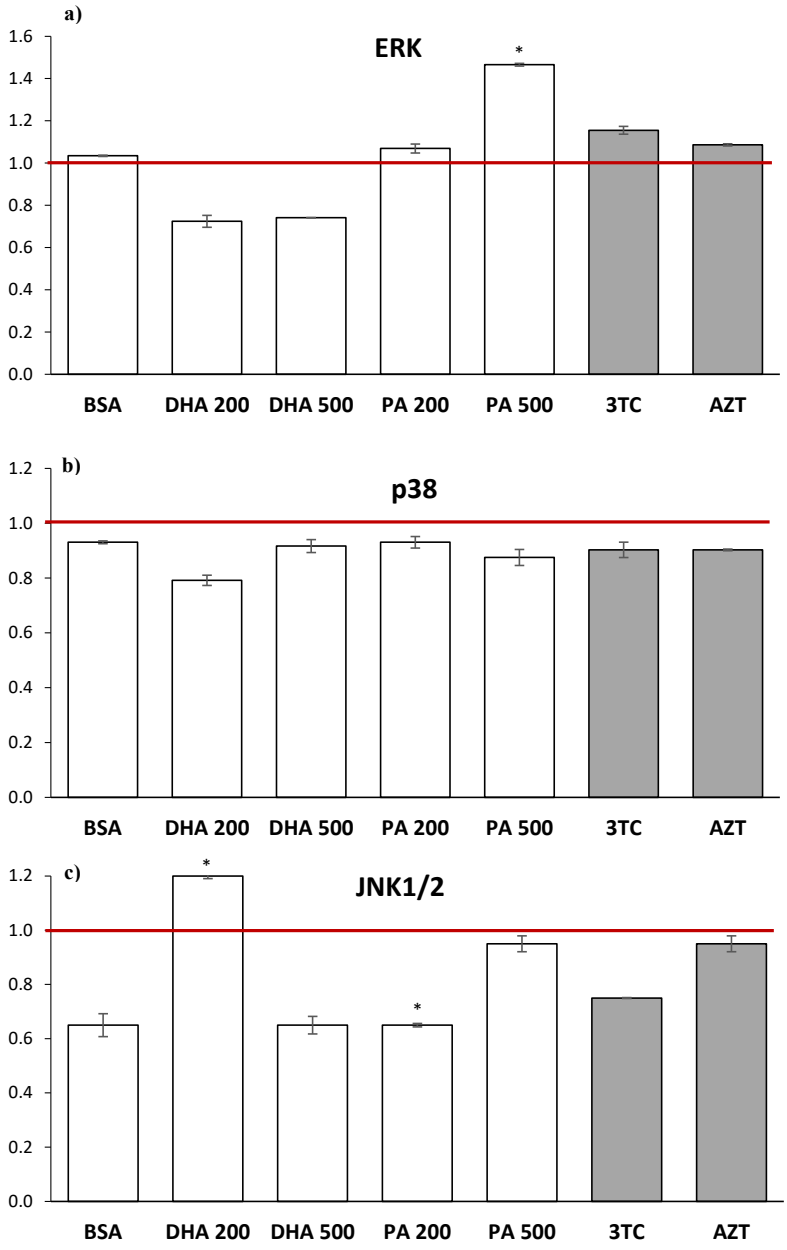

Figure 7. Intracellular ERK, p38, and JNK1/2 phosphorylation levels after 24 h incubation with DHA, PA, 3TC, or AZT. The outcomes are displayed as multiples of the data acquired from the control, where the untreated HMC3 cells are established as having a value of 1 (red line). Control—untreated HMC3 cells; BSA—incubation with 10% fatty acid-free BSA; DHA200 and DHA 500—incubation with 200 or 500 μM DHA; PA200 and PA 500—incubation with 200 or 500 μM PA; 3TC—incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine. Statistically significant differences compared to the untreated HMC3 cells: * $p < 0.05$,.

As depicted in Figure 7a, it appears that only high levels of PA (500 μM) cause a statistically significant activation of intracellular ERK phosphorylation, with an approximately 1.5-fold increase compared to the control. Additionally, a 24 h treatment with 10 μM of lamivudine resulted in the activation of intracellular ERK phosphorylation (approximately 1.15-fold compared to the control), although statistical significance was not achieved. When assessing the intracellular p38 protein phosphorylation levels, the administration of all substances led to a reduction in this process. The addition of NRTIs to HMC3 cells for 24 h did not significantly affect this process. In contrast, statistically significant increases in the intracellular JNK1/2 phosphorylation levels, compared to the untreated cells, were

observed after the treatment with physiological levels of DHA (approximately 1.2-fold), while the same concentrations of PA led to the inhibition of this process (approximately 0.65-fold). By analyzing the effect of NRTIs on intracellular JNK1/2 activation, it appears that 10 µM of lamivudine inhibits this process (approximately 0.75-fold), although statistical significance was not achieved.

2.6. Lipid Concentration Analysis, Oil Red O Staining

A microscopic evaluation of triglyceride staining and quantification was performed in PFA-fixed HMC3 cells after 24 h of incubation with DHA, PA, or the tested NRTIs. The results are presented in Figures 8 and 9.

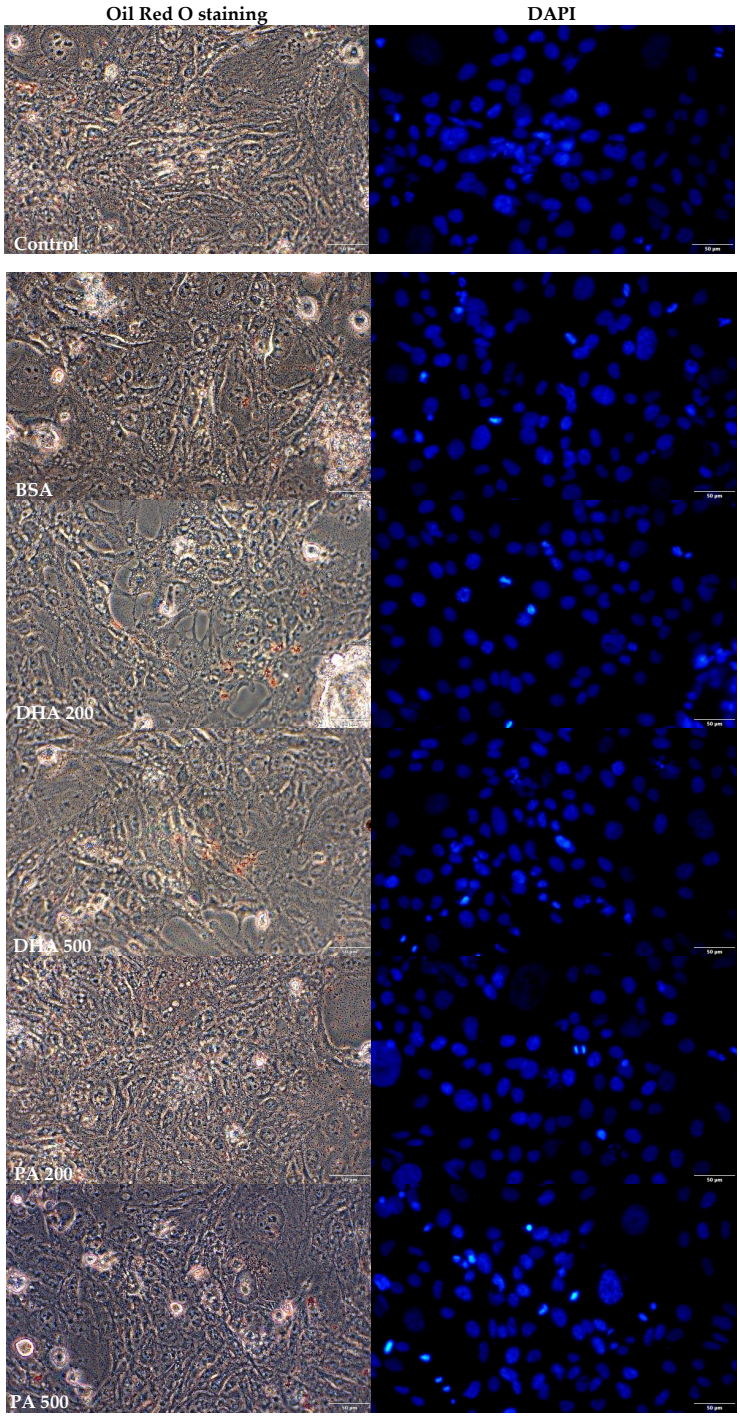

Figure 8. *Cont.*

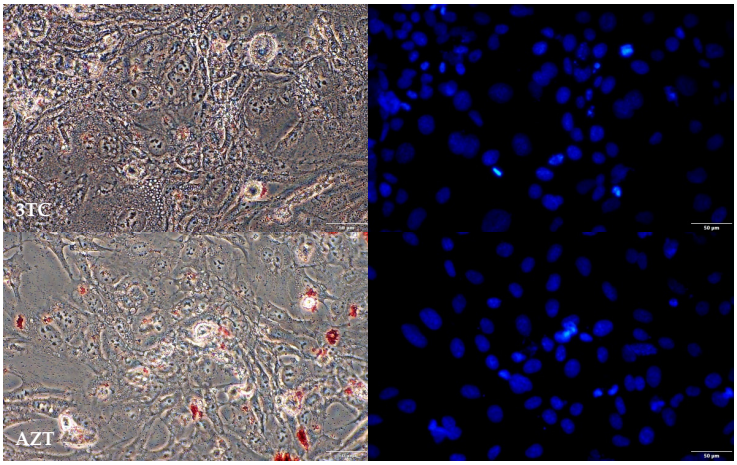

Figure 8. Examples of fluorescence microphotographs of fixed HMC3 cells stained with Oil Red O (red) and DAPI (blue). Magnification: 20×. Control—untreated HMC3 cells; BSA—incubation with 10% fatty acid-free BSA; DHA200 and DHA 500—incubation with 200 or 500 μM DHA; PA200 and PA 500—incubation with 200 or 500 μM PA; 3TC 10—incubation with 10 μM lamivudine; AZT 6—incubation with 6 μM zidovudine.

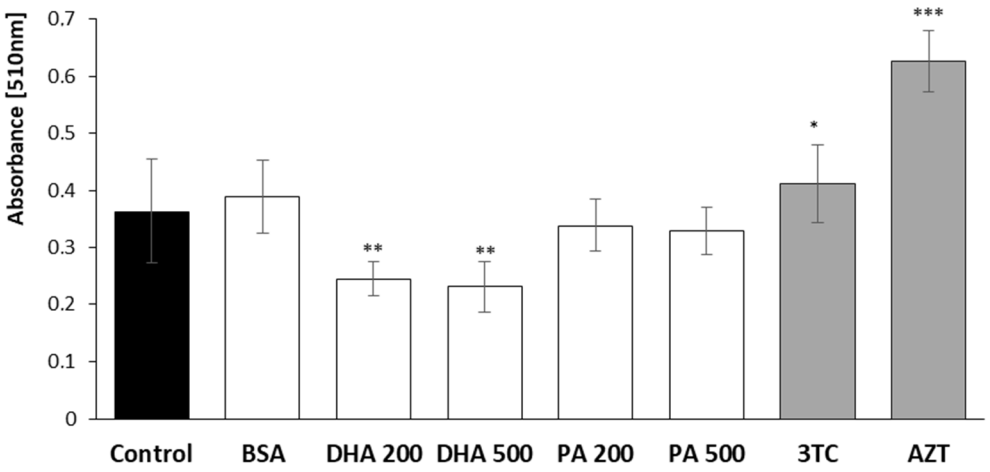

Figure 9. Quantification of the stained lipid performed using the eluted Oil Red O stain via measuring absorbance at 510 nm. Control—untreated HMC3 cells; BSA—incubation with 10% fatty acid-free BSA; DHA200 and DHA 500—incubation with 200 or 500 μM DHA; PA200 and PA 500—incubation with 200 or 500 μM PA; 3TC 10—incubation with 10 μM lamivudine; AZT 6—incubation with 6 μM zidovudine. Statistically significant differences compared to the untreated HMC3 cells: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The lipid content was determined through Oil Red O staining, and quantification of the stained lipid was achieved by measuring the absorbance at 510 nm. Interestingly, we observed a contrasting effect of the DHA on the lipid content in the HMC3 cells compared to the results obtained after 24 h of incubation with the NRTIs. Both concentrations of DHA led to a decrease in the lipid content, with a reduction of 32% observed at the physiological concentration and a reduction of 36% following the application of lipotoxic levels. In contrast, incubation with NRTIs for 24 h resulted in an increase in the lipid quantity, with a 13% rise after the treatment with 10 μM lamivudine and a remarkable 70% surge following the administration of 6 μM of zidovudine. Notably, there were no observable effects when the physiological nor lipotoxic concentrations of PA were added.

3. Discussion

In the context of the brain, lipids play multifaceted roles, including the involvement in brain development, neurogenesis, synaptogenesis, myelin sheath formation, and signaling processes. The dysregulation of lipid homeostasis is associated with brain damage, as well as various metabolic and neurological disorders. The term "lipotoxicity" refers to cellular damage resulting from an excess of free fatty acids (FFAs). Palmitic acid (PA), the most prevalent saturated fatty acid in the bloodstream, has been shown to induce apoptotic cell death in numerous cell types, including neonatal rat myocytes, pancreatic β cells, skeletal muscle cells, liver cells, podocytes, and hypothalamic neurons. Elevated levels of FFAs have been implicated as a potential risk factor for Alzheimer's disease, particularly in neurons and astrocytes, possibly contributing to cognitive impairment. The specific lipotoxic effects of PA on nerve cells remain an area of limited research. Furthermore, there is a knowledge gap concerning the mechanisms through which docosahexaenoic acid (DHA) safeguards cells against lipotoxicity [25] Many studies investigating the lipotoxic properties of NRTIs primarily focus on visceral tissues. Consequently, their effects on the human brain remain largely unexplored, giving rise to several unanswered questions and hypotheses [25–28].

Zidovudine, also known as 3-azido-3′-deoxythymidine, was the first NRTI to be approved for antiretroviral therapy. Initially approved for adults in 1987 and later for children in 1990, AZT exhibits activity against HIV-1 and HIV-2, as well as against other retroviruses like Spumavirinae, Lentivirinae, and Oncovirinae. AZT, a thymine analogue, competes with thymidine during DNA strand extension. Following oral administration, it is rapidly absorbed in the gastrointestinal tract, achieving peak serum concentrations within approximately 0.5 to 1.5 h, with an average bioavailability of 64%. The drug is widely distributed throughout the body, owing to its lipophilic nature, enabling excellent penetration into the CNS and genital secretions. Its plasma protein binding typically remains below 38%. Metabolized in the liver, the majority of AZT is excreted in the form of glucuronide in the urine, with only 14–18% of that excreted being unchanged. Lamivudine, often referred to as 2′-deoxy-3′-thiacytidine (3TC), is a hydrophilic analogue of cytidine, serving not only as an agent against HIV but also as a component of therapy for Hepatitis B Virus. Orally administered, 3TC is well absorbed in the gastrointestinal tract, reaching peak serum concentrations in a similar timeframe to AZT. Its bioavailability surpasses that of AZT, reaching 82–88% in adults and 66–68% in children, with a plasma protein binding rate of 38%. While 3TC penetrates the CNS to a lesser extent (about 5–10% of plasma concentration), it accumulates notably in semen and cervicovaginal secretions. Approximately 70% of the drug that is excreted in the urine is unchanged, with around 5% being excreted as a sulfoxide derivative. It is worth noting that 3TC is not recommended for monotherapy use due to the frequent development of drug resistance within the first three months of treatment, leading to reverse transcriptase mutations [6,29,30].

This investigation delved into the impact of the maximum therapeutic concentrations of two NRTIs, namely 3TC and AZT, on microglial cells. Utilizing the XTT assay, we did not find evidence of cytotoxic effects, as the cell viability closely paralleled that of the control sample. This finding aligns with the earlier research by Akay et al. [3], who did not observe a significant decrease in the survival of neuroglial cells that were exposed to AZT for 48 h. Additionally, Hung et al. [16] examined the effects of NRTIs (AZT, didanosine, tenofovir, and emtricitabine) on the survival of neuronal cells in the cerebral cortex. They reported that at the fifth day of evaluation, the cytotoxicity measurements resembled those of the control, while longer incubation periods (10 and 14 days) revealed substantial cytotoxic effects. This underscores the potential impact of the exposure duration of a given compound on its effects.

Mitochondrial toxicity is recognized as a prominent adverse effect of nucleoside analog treatment. To further explore these effects, we evaluated the ATP concentrations and superoxide production in HMC3 cells following the exposure to 3TC and AZT. A brief one-hour incubation did not yield any discernible differences in the ATP levels. However, after a 24 h exposure, we observed statistically significant reductions in the ATP levels

following incubation with 6 μM of AZT. Moreover, the 24 h treatment with NRTIs resulted in heightened levels of mitochondrial superoxide compared to the untreated cells. These observations might be linked to the activation of pathological mitochondrial pathways, initiating from mitochondrial DNA inhibition and extending to the production and accumulation of dysfunctional proteins, impaired fatty acid oxidation, and compromised oxidative phosphorylation, and culminating in an increased production of reactive oxygen species due to electron leakage from the electron transport chain. This deficit impacts ATP production and, consequently, leads to inadequate energy levels to maintain cellular homeostasis, potentially resulting in the death of microglial cells. The existing findings underscore that the duration of NRTI use and the use of NRTIs with substantial mitochondrial DNA inhibition constitute significant risk factors for lipoatrophy development [31].

Furthermore, we assessed the impacts of NRTIs on the levels of two fatty-acid-binding proteins, namely FABP4 and FABP5, in HMC3 cells. Following the exposure to both 3TC and AZT, we observed substantially lower concentrations of FABP4 compared to the control cells. FABP4 is known to play a role in inhibiting lipogenesis while promoting lipolysis, thereby influencing the composition of circulating free fatty acids [32]. Additionally, it significantly contributes to the promotion of lipotoxicity, mainly by triggering endoplasmic reticulum stress and oxidative stress through its actions on the mitochondria, leading to reactive oxygen species production and subsequent inflammation. Consequently, an increase in the FABP4 levels may indicate the presence of metabolic disorders [33]. Much of the research conducted thus far has focused on disorders of visceral and subcutaneous adipose tissue in association with NRTI use. For instance, Boothby et al. [6] measured FABP4 expression in patients undergoing various combinations of antiretroviral drugs over a six-month period and reported an approximately 2.5-fold increase in the FABP4 expression in patients receiving a combination of 3TC and AZT with a non-nucleoside reverse transcriptase inhibitor (NNRTI). Similarly, Escoté et al. [34] measured FABP4 in patients undergoing antiretroviral therapy, including prolonged stavudine treatment, and noted a significant correlation between increased FABP4 levels and the occurrence of lipodystrophy. Regarding FABP5, this protein is not exclusively associated with adipocytes and is expressed across various cell types. Researchers have suggested its involvement in the uptake of DHA by endothelial cells in brain blood vessels, playing a crucial role in preserving cognitive functions. Therefore, alterations in FABP5 expression may have an impact on the development of neurocognitive symptoms [35–37]. In our study, we observed a significant decrease in the FABP5 concentrations after exposing HMC3 cells to 3TC and AZT. Following a statistical analysis, both sets of results indicated these changes as statistically significant. At present, no studies have investigated the effects of NRTI drugs on FABP5 concentrations, making our findings a valuable reference point for future research. Furthermore, we found that 3TC and AZT had no discernible effects on the phosphorylation pathways of ERK1/2, p38, and JNK1/2, at least within the scope of our experimental settings. It might be worth exploring whether these compounds influence the genes that are responsible for activating the MAPK family. Importantly, we noted a considerable increase in the total lipid concentrations in the HMC3 cells following 3TC or AZT administration, supporting the notion that long-term NRTI use could potentially lead to lipid accumulation. This could pose a significant risk to the delicate homeostasis of the neuronal system and potentially trigger a pro-inflammatory response.

There is a limited body of literature addressing the impact of NRTIs on microglial functions within the brain. In a study conducted by Giunta et al. [38], the effects of various ART, including 3TC and AZT, on the microglial capacity to clear Aβ and potentially exacerbate amyloidosis were investigated. The results indicated a significant hindrance of the microglial phagocytosis of FITC-Aβ1-42 peptides in murine microglia in response to antiretroviral compounds. In contrast, Brown et al. [39] reported that AZT and 3TC alone did not reduce FITC-Aβ1-42 phagocytosis in primary mouse microglia. In an experimental study involving adult male Wistar rats treated with 3TC (6 mg/kg), a substantial increase in the microglial activity was observed, as evidenced by a significant upregulation

of reactivity for CD68. This heightened activation of microglia suggests an intensified phagocytic activity in response to pronounced neuroinflammation induced by 3TC [40]. Liuzzi et al. [41] conducted experiments on primary cultures of rat microglia treated with various doses of AZT for 20 h and simultaneously activated via an exposure to lipopolysaccharide. The assessment of the culture supernatants collected from the microglia did not reveal an increase in the MMP-2 mRNA and protein expression in response to LPS or combined AZT and indinavir treatment. While the LPS treatment induced the expression of MMP-9, it was dose-dependently inhibited by AZT and indinavir treatment in the LPS-stimulated microglia. Furthermore, there is supportive evidence suggesting that the pathogenesis of HIV-associated dementia is likely attributed to indirect effects of HIV infection on the brain, possibly mediated through the actions of macrophages and microglia [42]. Faria et al. [43] proposed the utilization of lipid nanocarriers for anti-HIV therapeutics to enhance penetration into HIV reservoir sites and surmount biological barriers such as BBB upon administration. Nanocarriers, when coated with specific surface stabilizers, offer potential utility in achieving elevated drug concentrations in the brain during CNS administration. The capacity of lipid nanocarriers to facilitate the brain delivery of anti-HIV drugs has been extensively documented, with liposomes, in particular, demonstrating the potential to enhance the brain accumulation of AZT [44,45].

4. Materials and Methods

4.1. Cell line and Conditions

The HMC3 adherent cell line, which possesses characteristics akin to primary microglial cells, was procured from the American Type Culture Collection (CRL-3304). These cells were cultivated in either 25 cm^2 or 75 cm^2 culture flasks under controlled conditions at 37 °C with 5% CO_2 in Eagle's Minimum Essential Medium (ATCC 30–2003). The culture medium was supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. To maintain cell viability and optimal growth, the culture medium was replenished every 2–3 days. When the cells reached 80–90% confluence, they were subcultured using TrypLE^{TM} Express Enzymesolution (12604013) from Gibco, Thermo Fisher Scientific, Waltham, MA, USA.

4.2. Tested Compounds

The concentrations of lamivudine (Sigma-Aldrich Saint Louis, MO, USA, Y0000425) and zidovudine (Merck, cat. nr. Z1900000) selected for this study were chosen to align with the therapeutic concentrations achieved in blood serum during drug treatment—lamivudine at 10 μM and zidovudine at 6 μM, as previously established [46–48]. In accordance with the FDA's National Drug Code, the following abbreviations will be used: 3TC for lamivudine and AZT for zidovudine. Stock solutions of 3TC and AZT were prepared by dissolving the substances in phosphate-buffered saline (PBS). To exemplify the effects of saturated fatty acids, palmitic acid (PA) was selected for the study. The following concentrations of PA were chosen to represent both physiological and elevated levels of free fatty acids (FFAs) in blood serum: palmitic acid at a physiological concentration of 200 μM and at a lipotoxic concentration of 500 μM [49]. For the conjugation of PA with fatty acid-free bovine serum albumin (BSA, Sigma-Aldrich Saint Louis, MO, USA, A7030), a three-step process was employed. Initially, 1 g of PA powder was dissolved in 7.8 mL of 99% ethanol to create a 500 mM solution at 37 °C. Subsequently, this solution was filtered using a 0.2 μm sterile filter. In the next step, 1.5 g of fatty acid-free BSA was diluted in 15 mL of serum-free media at 37 °C and also filtered using a 0.45 μm sterile filter. Finally, a 5 mM PA-BSA solution was obtained by combining these two solutions at a 100:1 ratio (BSA:PA) [50]. A solution of the positive control, cis-4,7,10,13,16,19-docosahexaenoic acid (DHA, Sigma-Aldrich, catalog number D2534), was prepared using the same protocol and used in concentrations equivalent to those of PA.

4.3. Cell Cytotoxicity Assay

Cell viability following treatment with DHA, PA, 3TC, or AZT was assessed using a colorimetric XTT assay (Roche Basel, Switzerland, 11465015001). To initiate the assay, 4×10^4 cells were seeded into wells in a 96-well plate. After a 24 h incubation period, allowing for cellular attachment, the culture medium was replaced, and the specified concentrations of PA, DHA, 3TC, or AZT were introduced to the cells. The incubation was carried out for 24 h at 37 °C, under conditions of 5% CO_2 and 95% humidity. A freshly prepared XTT mixture was employed for the assay, created by blending the XTT labeling reagent with the electron coupling reagent at a ratio of 50:1. Subsequently, 50 µL of the XTT mixture was added to the cells, and the cells were further incubated for 18 h at 37 °C, maintaining an environment of 5% CO_2 and 95% humidity, adhering to the manufacturer's protocol. Following this incubation period, the absorbance of the samples was determined at 450 nm, with reference measurements taken at wavelengths exceeding 650 nm. This analysis was conducted utilizing a Synergie multi-well scanning spectrophotometer (STAT FAX 2100, Awareness Technology, Inc., Palm City, FL, USA).

4.4. Intracellular ATP Test

Intracellular ATP levels were quantified by employing the ATP Bioluminescence Assay Kit HS II (Roche, 11699709001) following the manufacturer's protocol. Concisely, HMC3 cells were seeded at a density of 4×10^4 cells per well in 96-well plates and allowed to adhere for 24 h. Subsequently, they were exposed to the designated concentrations of PA, DHA, 3TC, or AZT for a 24 h incubation period. Upon completion of the treatment, the cells were lysed, and luciferase reagent was introduced into both the experimental samples and standards. The resulting green luminescence was quantified utilizing a luminometer (Spark® multimode microplate reader, Tecan, Männedorf, Switzerland) with measurements taken at 562 nm after a brief 1 s delay and integrated over the span of 1 to 10 s. To derive the actual ATP concentrations, blank readings were subtracted from the raw data, and the values were calculated using a log–log plot based on the standard curve data.

4.5. Levels of Mitochondrial Superoxide

The assessment of mitochondrial superoxide levels was conducted using the MitoSOX™ Red assay (Thermo Fisher Scientific Waltham, MA, USA, M36008) in accordance with the manufacturer's instructions. In brief, a 5 mM stock solution of MitoSOX™ reagent was freshly prepared by dissolving the contents of the vial in 13 µL of anhydrous dimethyl sulfoxide (DMSO). A working solution of 500 nM was created by adding 5 µL of the stock solution to 50 mL of phosphate-buffered saline (PBS). HMC3 cells were seeded at a density of 4×10^4 cells per well in 96-well plates and allowed to adhere for 24 h. Subsequently, they were subjected to specified concentrations of PA, DHA, 3TC, or AZT for a 24 h incubation period. Following the incubation, supernatants were collected, and the HMC3 cells were gently washed with room-temperature PBS. The cells were then fixed with 100% cold methanol for 5 min at 4 °C. After fixation, the cells were rinsed three times with PBS, and 100 µL of the MitoSOX™ reagent working solution was added to each well. The cells were incubated in the dark at 37 °C for 10 min. Following incubation, the HMC3 cells were washed once with PBS, and the fluorescence was measured for the entire well using a Spark® multimode microplate reader from Tecan, Männedorf, Switzerland (excitation at 510 nm and emission at 560 nm).

For fluorescence imaging, 4×10^4 HMC3 cells were seeded in 48-well plates and treated as described above. Following methanol fixation, the cells were counterstained with diamidino-2-phenylindole (DAPI) for 15 min, and the mitochondrial superoxide production was visualized using a fluorescence microscope (CKX53, Olympus, Hamburg, Germany.

4.6. Protein Concentration Assessment

The Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was employed to quantify the total protein concentration in the samples. The working range for total protein measurement spanned from 5 to 250 ng/mL. Cell pellets from a

96-well plate, each containing 4×10^4 cells, were spun down, and 10 µL from each cell pellet was pipetted into a well of a microplate. Subsequently, 200 µL of the working reagent was added to both the samples and standards. The plate was then placed on a plate thermostatic shaker (DTS-2, Elmi SIA, Riga, Latvia) and mixed for 30 s. Following the mixing step, the plate was covered and incubated at 37 °C for 30 min, after which it was allowed to cool to room temperature. The absorbance was measured at 562 nm using a Synergie multi-well scanning spectrophotometer (STAT FAX 2100, Awareness Technology, Inc., USA), and the total protein concentration was determined based on a standard curve.

4.7. FABP4 and FABP5 Concentrations

The intracellular concentrations of fatty-acid-binding protein 4 (FABP4) and 5 (FABP5) were quantified through the utilization of enzyme-linked immunosorbent assays. The Human FABP4 ELISA Kit (ab234565, Abcam, Cambridge, UK) and the Human FABP5 ELISA Kit (E1399Hu, Bioassay Technology Laboratory, Birmingham, UK) were employed for these measurements. HMC3 cells were plated in 96-well plates at a density of 4×10^4 cells per well and allowed to adhere for 24 h. Subsequently, they were exposed to the respective concentrations of DHA, PA, 3TC, or AZT for a 24 h duration. Following the treatment, the cells were lysed using radioimmunoprecipitation assay buffer. The subsequent FABP4 and FABP5 ELISA assays were conducted following the guidelines provided by the manufacturers. The cellular pellet was standardized for cellular protein concentration using the BCA assay, and the protein concentration was measured. Adjustments were made to ensure that the protein concentration in the cell lysates was maintained at 100 µg/mL total protein concentration.

4.8. MAPK Family Activation Measurement

The intracellular activation of ERK1/2, p38, and JNK1/2 was assessed in HMC3 cell lysates using the InstantOne ELISA™ kit (IOAP96, Thermo Fisher Scientific, Waltham, MA, USA). The ELISA test was executed in accordance with the provided manufacturer's protocol. HMC3 cells were seeded in 96-well plates at a density of 4×10^4 cells per well and allowed to adhere for 24 h. Subsequently, they were exposed to the respective concentrations of DHA, PA, 3TC, or AZT for a 24 h period. Following treatment, the cells were lysed using RIPA buffer. The extent of activation/phosphorylation of ERK1/2, p38, and JNK1/2 proteins in HMC3 cell lysates was quantified, with reference to both negative and positive control samples.

4.9. Lipid Concentration and Staining Protocol

The HMC3 cells, incubated for 24 h with either NRTIs or fatty acids, underwent Oil Red O staining (00625-25G, Sigma Aldrich, Saint Louis, MO, USA) to assess the accumulation of lipid droplets. The Oil Red O working solution was meticulously prepared by adhering to the manufacturer's instructions.

For lipid quantification, HMC3 cells were initially seeded at a density of 4×10^4 cells per well in a 96-well plate. Subsequently, they were left undisturbed for 24 h to ensure proper adherence before being exposed to varying concentrations of DHA, PA, 3TC, or AZT for a 24 h duration. Following the treatment period, the cells were fixed with 4% paraformaldehyde for 1 h at room temperature. Subsequent to fixation, the cells were incubated with 0.5% Triton X100 in PBS for 30 min at room temperature, washed thrice with PBS, and incubated with the Oil Red O working solution for 2 h. After Oil Red O staining, the cells underwent three PBS washes and were air-dried at 32 °C. To extract and dissolve the Oil Red O that had adhered to the HMC3 cells, 100 µL of isopropanol was added to each well. Then, 100 µL of this extract was transferred to fresh wells, and the absorbance at 510 nm was measured using a Synergie multi-well scanning spectrophotometer (STAT FAX 2100, Awareness Technology, Inc., Palm City, FL, USA).

For microscopic evaluation, HMC3 cells were seeded in 6-well plates at a density of 5×10^5 cells per well and allowed to adhere for 24 h. Subsequently, they were treated with various concentrations of DHA, PA, 3TC, or AZT for 24 h. Following the treatment period,

the cells were fixed with 4% paraformaldehyde for 1 h at room temperature. After fixation, the cells were incubated with 0.5% Triton X100 in PBS for 30 min at room temperature, followed by three PBS washes. The cells were then incubated with the Oil Red O working solution for 2 h. Following Oil Red O staining, the cells underwent three PBS washes and were counter-stained with DAPI for 15 min. The lipid droplets were visualized using an inverted microscope (CKX53 Olympus, Hamburg, Germany).

4.10. Statistics

Statistical analysis was conducted by employing parametric tests, specifically ANOVA, along with the application of appropriate post hoc tests. These statistical methods were chosen due to the normal distribution of the data and the equality of variance within the dataset. The results are expressed in the format of mean ± standard deviation (SD). The statistical analysis was executed using Statistica 13.1 software by Dell Software Inc., Port St. Lucie, FL, USA. For each experimental condition, a minimum of three independent experiments were performed to ensure the reliability of the results. The descriptive statistics are presented as mean ± SD. Significance levels are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

5. Conclusions

In the context of the CNS, it is important to note that HIV primarily resides within the cerebral microglia or macrophages, which are the innate immune cells in the brain parenchyma, as opposed to astrocytes, as observed in the brains of HIV-1-infected aviremic individuals undergoing suppressive antiretroviral therapy [42]. The various cellular reservoirs for HIV-1 within the CNS may play crucial roles in the molecular mechanisms associated with HIV-1 neuropathogenesis. In summary, this study sheds light on the potential impact of nucleoside reverse transcriptase inhibitors (NRTIs) on the development of lipotoxicity in neuronal cells. However, it is imperative to recognize that the observed effects may vary according to the duration of exposure to these compounds. Lipid toxicity appears to have been induced in microglial cells by the tested drugs, although the extent of this effect was insufficient to trigger cell death. Nevertheless, it is essential to acknowledge the various limitations of this research. One key limitation is that the study was conducted on an in vitro cell line, which means that the results obtained here may not perfectly mirror the responses observed in patient-derived materials. The microenvironmental conditions in vivo and the influence of numerous other factors may yield different outcomes. Additionally, the relatively short-term exposure of cultured cells to these drugs contrasts with the prolonged drug regimens taken by patients, thereby potentially leading to varying consequences. To gain a more comprehensive understanding of the cellular changes associated with lipid metabolism and to confirm the link between these changes and the mitochondrial dysfunction and reactive oxygen species (ROS) production induced by the drugs, as postulated by several researchers, future investigations should include assessments of ROS concentrations, lipid peroxidation, and the presence of other oxidative stress markers. The subject of lipid disorders within the CNS under the influence of NRTIs remains an area of inquiry with many unanswered questions. Therefore, it is imperative to continue researching in order to unravel the intricate mechanisms behind this phenomenon. Such knowledge is of paramount importance as it holds the potential to mitigate the risks of complications related to lipotoxicity in patients undergoing NRTI therapy. Further research endeavors are warranted to provide a more comprehensive understanding of the intricate interplay between NRTIs and lipid metabolism within the CNS.

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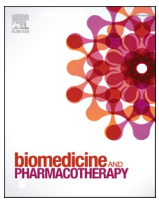

Investigating the role of nucleoside reverse transcriptase inhibitors in modulating lipotoxicity: Effects on lipid dynamics stress pathways, and insulin resistance on the function of dopaminergic neurons

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ABSTRACT

Despite decades of advancements in HIV treatment, the persistence of viral reservoirs necessitates lifelong therapy, complicating efforts to fully control the infection. Nucleoside reverse transcriptase inhibitors (NRTIs) remain a cornerstone of HIV treatment, but long-term use is associated with side effects, including lipid metabolism disruption and neurocognitive disorders. There is a gap in understanding the safety of antiretrovirals and their impact on lipid toxicity in the central nervous system. To address this issue, our study investigated the impact of NRTIs, specifically lamivudine and zidovudine, on lipid metabolism and insulin resistance in the SH-SY5Y dopaminergic neuronal cell line. We also compared these effects to those induced by two free fatty acids, palmitic and docosahexaenoic acids. We measured mitochondrial superoxide levels, fatty acid binding proteins 4 and 5, and overall lipid content. Additionally, we assessed insulin resistance by analyzing the phosphorylation of mitogen-activated protein kinases and phosphoinositide 3-kinase, as well as the concentrations of insulin receptor substrate 1 and insulin receptor. The results demonstrated that NRTIs led to reduced fatty acid binding protein 4 levels and lipid content, similar to the effects observed with fatty acids. Moreover, lamivudine and zidovudine increased mitochondrial superoxide levels. Lamivudine also amplified the phosphorylation level of ERK. These findings suggest that NRTIs may contribute to lipotoxicity in dopaminergic neurons, warranting further investigation into their long-term effects on the central nervous system.

1. Introduction

The current conventional approach in HIV infection treatment is the administration of highly active antiretroviral therapy (HAART), also referred to as combined antiretroviral therapy (cART) which comprises 2–3 pharmaceutical agents of diverse classes of drugs. A notable class of antiretroviral pharmaceuticals is a group of nucleoside reverse transcriptase inhibitors (NRTIs), represented by such agents as abacavir, lamivudine (3TC), zidovudine (AZT), tenofovir. Regardless of the imposed therapy, certain anatomic sites, such as the central nervous system (CNS), are at risk of persistent viral replication which can lead to systemic viral rebound [1]. CNS poses as a potential HIV reservoir, housing latent virus forms either not exposed to the therapeutic agents due to their inaccessibility or resistance to said agents [2]. HIV-associated neurocognitive disorders (HAND) are commonly observed in HIV-infected individuals [3]. HAND encompasses a wide range of conditions, from mild cognitive impairment to severe dementia described as HIV-associated dementia (HAD). The extended life expectancy of HIV-positive individuals is related to a growing number of people living with HAND. Approximately 30–50 % of HIV-infected individuals experience mild to severe neurocognitive impairments [4,5]. Despite the likely multidimensional pathophysiology of HAND and affective changes in the post-cART era, dysfunction in the dopaminergic system remains prevalent [6–8]. The distinct abnormalities in the white matter and subcortical structures, including the basal ganglia, were observed in the brains of individuals with progressive dementia associated with acquired immunodeficiency syndrome (AIDS) as far back as in the 1980s [9]. These observations led researchers to hypothesize dopaminergic system dysfunction as a potential mechanism underlying the disease [10]. HIV-associated lipodystrophy is an undesirable effect of antiretroviral therapy (ART) that occurs due to the redistribution of adipose tissue. HIV-associated lipodystrophy can manifest as two

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distinct phenotypes: fat accumulation (lipohypertrophy) or fat loss (lipoatrophy). In some patients, the two manifestations may coexist as well. Lipodystrophy also contributes to morbidity via the development of insulin resistance (IR), hyperlipidemia, and endothelial dysfunction, which can increase the risk of cardiovascular disease [11]. Therefore identification and prompt management of HIV-associated lipodystrophy are of utmost importance. The exact cause of lipodystrophy is unknown. However, the use of specific thymidine analog NRTIs, such as zidovudine or stavudine, is associated with the development of lipoatrophy [12]. These effects also extend to glucose metabolism due to the induction of IR [13]. In addition, host lifestyle factors, such as high caloric intake with resultant elevated baseline triglyceride levels, have also been implicated as a risk factor for lipohypertrophy in patients with HIV [14].

The impact of certain fatty acids varies due to chemical classification as saturated or unsaturated. The research confirms that most deleterious effects are provided by saturated fatty acids (such as palmitic acid, also referred to as palmitate), whereas unsaturated fatty acids relieve cells from lipotoxic effects. Oleic acid, containing one unsaturated bond in its structure, was shown to be more steatogenic but less damaging than palmitic acid. Furthermore, oleic acid prevents oxidative stress and apoptosis caused by palmitic acid in human hepatocytes and rat and mouse myocytes [15–17]. Moreover, docosahexaenoic acid (DHA) is known for ameliorating or protecting against the lipotoxic effects [18–20]. On the other hand, palmitic acid (PA) plays a role in promoting inflammation and oxidative stress across various cell types, including microglial cells [21]. By taking into account the subsequent character of the possible effects of both HIV infection and antiretroviral therapy on lipid homeostasis, the phenomenon described as lipotoxicity might occur, leading to cellular metabolic perturbations. Furthermore, palmitic acid has been documented to induce neuroinflammation and oxidative stress, negatively impacting dopaminergic neurons. Studies have shown that PA can impair mitochondrial function and promote apoptosis in neuronal cells, contributing to neurodegenerative conditions [22,23]. Over long-term administration, NRTIs cause mitochondrial toxicity primarily by inhibiting polymerase, the enzyme responsible for mitochondrial DNA synthesis. Additionally, NRTIs induce oxidative damage, inhibit other mitochondrial enzymes, disrupt ATP synthesis, and trigger cell apoptosis. Furthermore, the effectiveness of NRTI therapy in the CNS is limited by the restricted penetration of the blood-brain barrier (BBB) [24]. Given the adverse phenomenons surrounding both HIV infection and antiretroviral therapy, it can be assumed that the BBB's condition might deteriorate under such circumstances, further contributing to NRTIs adverse reactions.

While antiretroviral therapy, including lamivudine and zidovudine, has significantly improved the prognosis of individuals with HIV, emerging evidence suggests potential neurotoxic effects associated with these medications. Moreover, elevated levels of palmitic acid resulting from the breakdown of adipose tissue due to lipoatrophy, may exacerbate neuronal damage. While the various components of the intricate aforementioned processes are well examined, there is a research gap concerning the correlation between them and the potential escalation of lipotoxicity and neurotoxicity. This study was formulated to explore the impact of two nucleoside reverse transcriptase inhibitors (3TC and AZT), as well as FFA (PA, DHA) on the homeostasis of the SH-SY5Y dopaminergic neuronal cell line. Measurements with PA would serve as a model of lipotoxicity, whereas the effects of DHA are investigated for use as a positive reference.

This research's objective is the examination of NRTI and FFA-induced influence on the critical intracellular parameters, including insulin receptor (InsR) and insulin receptor substrate 1 (InsRS1) levels, reactive oxygen species (ROS) production, lipid content, and the expression of fatty acid binding proteins 4 and 5 (FABP4 and FABP5). Our findings have the potential to shed light on the mechanisms underlying the actions of these molecules on lipid homeostasis within the CNS.

2. Materials and methods

2.1. Cell culture and conditions

The adherent SH-SY5Y cell line obtained from ECACC (European Collection of Authenticated Cell Cultures, number 94030304, Salisbury, UK) was used as a model of dopaminergic neurons to conduct assays. The cells were cultivated in culture flasks (Nunc™ EasYFlask™, 156367, 148576, Thermo Fisher Scientific, Denmark). The conditions for the cell culture included incubation at 37°C with 5 % CO_2. The cells were grown in Ham's F12 (234700712, Sartorius, Israel): EMEM (820100a, CLS, Eppelheim, Germany) medium in a 1:1 ratio with the addition of 2 mM glutamine (Glutamax, 35050061, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 1 % Non-Essential Amino Acids (M7145–ml, Sigma-Aldrich, UK), 15 % v/v fetal bovine serum (FBS), and with the antibiotics - streptomycin (50 U/ml) and penicillin (10 mg/ml). The cells were subcultured after reaching 80–90 % confluence using TrypLE Enzyme solution (12604013, Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The cell line was differentiated. In the process, the cells were seeded in the 25-cm^2 culture cell flasks in the amount of 650 000 cells per flask and treated with 10 μM retinoic acid in the course of five days. In order to subject the cells to the differentiation agent, the culture medium was changed to Neurobasal™ Medium (21103049, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with an addition of B-27 supplement (A35828–01, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 3 % v/v FBS and 2 mM glutamine.

2.2. Preparation of tested compounds

Drugs representing the therapeutic group of nucleoside reverse transcriptase inhibitors selected for this study are lamivudine and zidovudine. The concentrations utilized in the assays correspond to the therapeutic levels observed in blood serum during drug treatment, specifically lamivudine (PHR1365–1G, Sigma-Aldrich, St. Louis, MO, USA) at 10 μM and zidovudine (PHR1292–1G, Sigma-Aldrich, St. Louis, MO, USA) at 6 μM, as previously documented [25–27]. Stock solutions of 3TC and AZT were prepared by dissolving the weighed amount of the compounds in phosphate-buffered saline (PBS). Palmitic acid (PA, P0500–25G, Sigma, Malaysia) was selected as a representative of the saturated free fatty acid (FFA) group as the most abundant and toxic fatty acid present in blood serum. Both physiological and elevated levels of FFA in blood serum were represented by palmitic acid concentrations of 200 μM and 500 μM, respectively [28]. A three-step process was employed for the conjugation of palmitic acid with serum. First, 1 g of PA powder was dissolved in 7.8 ml 99 % ethanol (32294–1 L, Honey-Well, France) at 35–40°C, creating a 500 mM stock solution. The solution was filtered using a 0.2 mm filter. Subsequently, 1.5 g of fatty acid-free bovine serum albumin (BSA, A7030, Sigma-Aldrich Saint Louis, MO, USA) was dissolved in 15 ml of serum-free media (Ham's F12: EMEM in a 1:1 ratio) at 37°C. Finally, the 5 mM PA-BSA solution was created by mixing the two solutions at a 100:1 (BSA:PA) ratio [29]. Cis-4,7,10,13,16,19-docosahexaenoic acid (DHA, D2534, Sigma-Aldrich, St. Louis, MO, USA) was selected as the positive control for the assays and was prepared utilizing the same protocol of preparation as PA. The concentration of DHA used in the study was equivalent to that of PA.

2.3. Cell viability assay

The cell viability was determined using a colorimetric XTT assay (Roche Basel, Switzerland, 11465015001) following the treatment of cell culture with DHA, PA, 3TC, or AZT. In order to perform the assay, the cells were seeded in a 96-well plate with a density of 1×10^4 cells per well. The cells were allowed to adhere and, subsequently, the culture medium was changed. The selected concentrations of DHA, PA, 3TC, or AZT were introduced to the cells and the 24-hour incubation was

performed at 37 °C with 5 % CO_2 and 95 % humidity. The XTT reagent mixture was prepared prior to use by blending the XTT labeling reagent with the electron coupling reagent at a 50:1 ratio. In the next step, 50 μL of XTT mixture was added to each well with the cells and then the plates were incubated for 18 hours in the conditions of 5 % CO_2, 95 % humidity, and 37 °C. Following the incubation, the absorbance of the samples was measured at the wavelength 450 nm with the reference measurements at 650 nm. The measurements were conducted on a Synergie multi-well scanning spectrophotometer (STAT FAX 2100, Awareness Technology, Inc, Palm City, FL, USA). The assay was performed in accordance with the manufacturer's protocol.

2.4. Mitochondrial superoxide content measurement

The differentiated SH-SY5Y cells were seeded at a density of 1×10^4 cells per well in 96-well plates. After allowing the cells to adhere, they were treated with the selected concentrations of DHA, PA, 3TC, or AZT and incubated for 24 h at 37 °C, 5 % CO_2, and 95 % humidity. The measurement of mitochondrial superoxide content was assessed using Mitosox™ Red Assay (Thermo Fisher Scientific Waltham, MA, USA, M36008) according to the manufacturer's protocol. A fresh 5 mM stock solution of Mitosox™ reagent was prepared: 50 μg of Mitosox™ mitochondrial superoxide indicator was dissolved in 13 μl of dimethylsulfoxide (DMSO). Then, the stock solution was diluted in PBS to make a 5 μM working solution by adding 5 μl of stock solution to 4555 μl of PBS. Following the incubation with tested compounds, the cells were fixed with 100 % cold methanol for 5 min at 4 °C. Subsequently, 100 μl of Mitosox™ reagent working solution was added to each well. The plate with cells was incubated in the dark, at 37 °C for 10 min. Finally, the fluorescence was measured with excitation/emission maxima at 510 nm and 560 nm respectively. For the fluorescence measurement, the SparkR multimode microplate reader (Tecan, Mannedorf, Switzerland) was used.

2.5. Protein concentration assessment

To quantify the total protein in the samples the Pierce™ BCA Protein Assay Kit (nr cat. 23225, Thermo Fisher Scientific, Waltham, MA, USA) was used. A working solution was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B. The differentiated SH-SY5Y cells (seeded in the density of 4×10^4 cells per well in a 96-well plate) were lysed after the 24 h incubation with the tested compounds. The 25 μl of tested samples were pipetted into microplate wells and then a 200 μl of the working solution was added to each well. Subsequently, the microplate was then covered and incubated for 30 min at 37 °C. The absorbance was measured at 562 nm using a Synergy multi-well scanning spectrophotometer (STAT FAX 2100, Awareness Technology Inc., USA). The total protein concentration was calculated based on a standard curve.

2.6. FABP4 and FABP5 concentration assessment

The expression of fatty acid binding protein 4 (FABP4) and 5 (FABP5) was measured using two enzyme-linked immunosorbent assays: The Human FABP4 ELISA Kit (ab234565, Abcam, Cambridge, UK) and the Human FABP5 ELISA Kit (E1399Hu, Bioassay Technology Laboratory, Birmingham, UK). To perform the assays, the differentiated SH-SY5Y cells were seeded at the density of 4×10^4 cells per well on a 96-well microplate. Following the 24 h period in which the cells were allowed to adhere, they were treated with the designed concentrations of DHA, PA, 3TC, or AZT and incubated for 24 h at 37 °C with 5 % CO_2 and 95 % humidity. Following the treatment, the cells were lysed. The FAPB4 and FAPB5 ELISA assays were performed in accordance with the manufacturer's guidelines. The total protein concentration was measured in samples using the BSA assay, and the adjustment was performed in order to ensure the protein concentration in the cell lysates was maintained at 100 μg/ml total protein concentration. The absorbance was measured at 450 nm using a Synergy multi-well scanning spectrophotometer (STAT FAX 2100, Awareness Technology Inc., USA). The final concentrations of FABP4 and FABP5 were calculated using a standard curve.

2.7. MAPK family kinases activation measurement

The InstantOne ELISA™ Kit (IOAP96, ThermoFisher Scientific, Waltham, MA, USA) was utilized to determine the intracellular content of the activated kinases representative of mitogen-activated protein kinase (MAPK) family: ERK, p38 and JNK. The ELISA assay was performed following the guidelines of the manufacturer. The differentiated SH-SY5Y cells were seeded on a 96-well plate with a density of 4×10^4 cells per well. The cells were allowed to adhere for 24 h at 37 °C with 5 % CO_2 and 95 % humidity and then treated with the selected concentrations of DHA, PA, 3TC, or AZT for 24 h at 37 °C, 5 % CO_2, and 95 % humidity. The cells were lysed according to the manufacturer's protocol. The absorbance of the samples was measured at 450 nm using a Synergy multi-well scanning spectrophotometer. The activation/phosphorylation extent of ERK, p38, and JNK kinases was determined in reference to both negative and positive control samples provided with the InstantOne ELISA™ Kit.

2.8. Phosphoinositide 3 kinase (PI3K) activity assessment

To quantify the phosphorylation of PI3K, a Phospho-PI 3 kinase p85 + Total In-cell ELISA Kit (ab207484, Abcam, Cambridge, UK) was used. In order to perform the assay, the differentiated SH-SY5Y cells were seeded on a 96-well microplate with a density of 4×10^4 cells per well. After the 24 h incubation at 37 °C with 5 % CO_2 and 95 % humidity for cell adherence, the treatment with selected concentrations of DHA, PA, 3TC, or AZT was introduced and the cells were again incubated for 24 h in the same conditions as aforementioned. The supernatants were removed from the wells, and the microplate with adhered cells was used for the phosphorylated PI3K determination in accordance with the manufacturer's protocol. As the final step, the absorbance of the samples was measured at 450 nm using a Synergy multi-well scanning spectrophotometer. In order to normalize each reading, the Crystal Violet staining was utilized. The Crystal Violet solution was added to each well in the quantity of 100 μl. In the next step, the microplate was incubated at room temperature for 30 min. Then, the 100 μl of 1 % SDS solution was added to each well and incubated at room temperature for 1 hour. The absorbance was measured on a spectrophotometer at 595 nm. To determine the extent of phosphorylation of PI3K, the ratio between total PI3K and phospho-PI3K was calculated. In order to normalize the readings at 450 nm, they were corrected by dividing by the reading at 595 nm, resulting in the relative cell number, as Crystal Violet binds to the cell nuclei.

2.9. Insulin Receptor Substrate 1 concentration assessment

To quantify the content of the InsRS1 the Human IRS1 SimpleStep ELISAR Kit (ab289646, Abcam, Cambridge, UK) was utilized. The differentiated SH-SY5Y cells were seeded on a 96-well plate with a density of 4×10^4 cells per well. The cells were allowed to adhere for 24 h at 37 °C with 5 % CO_2 and 95 % humidity and then treated with the selected concentrations of DHA, PA, 3TC, or AZT for 24 h at 37 °C, 5 % CO_2, and 95 % humidity. The cells were lysed and the solutions for the standard curve were prepared. The ELISA assay was performed following the guidelines of the manufacturer. The absorbance of the samples and standards was measured at 450 nm using a Synergy multi-well scanning spectrophotometer. The final quantity of the InsRS1 was calculated using a standard curve.

2.10. Insulin receptor expression assay

The InsR expression was determined using a Human InsR (Insulin Receptor) ELISA kit (HUFI00828, AssayGenie, Dublin, Ireland). To perform the assay, the differentiated SH-SY5Y cells were seeded at the density of 4×10^4 cells per well on a 96-well microplate. Following the 24 h period in which the cells were allowed to adhere, they were treated with the designated concentrations of DHA, PA, 3TC, or AZT and incubated for 24 h at 37 °C with 5 % CO_2 and 95 % humidity. The cells were then lysed. The assay was carried out according to the manufacturer's protocol. The series of dilutions of the standard solution was made for the standard curve. The absorbance of the samples and standards was measured at 450 nm using a Synergy multi-well scanning spectrophotometer. The protein concentrations in sample lysates were also determined using a BCA assay. The final expression of InsR was calculated using a standard curve.

2.11. Lipid staining

To assess the accumulation of lipids, the staining of the cytoplasmic drops of neutral lipids with Red Oil O (00625–25 G, Sigma Aldrich, Saint Louis, MO, USA) was carried out. To perform the staining, the differentiated SH-SY5Y cells were seeded at the density of 1×10^4 cells per well on a 96-well microplate. The cells were allowed to adhere, and then they were treated with the studied concentrations of DHA, PA, 3TC, or AZT and incubated for 24 h at 37 °C with 5 % CO_2 and 95 % humidity. The Red Oil O working solution was prepared following the manufacturer's protocol. Firstly, the 60 mg of Red Oil O was reconstituted with 20 ml of 100 % isopropanol, thus creating a Red Oil O stock solution. The stock solution was mixed well and left undisturbed for 20 min. To achieve the working solution, 3 parts of the stock solution were added to 2 parts deionized H_2O. The working solution was mixed well, left undisturbed for 10 min and then filtered through a Whatman No. 1 filter paper. The cells were fixed with 4 % paraformaldehyde (22023–20 ML, Biotium, Fremont, CA, USA) and incubated for 1 hour at room temperature. Subsequently, the cells were incubated with 0,5 % Triton X100 in PBS at room temperature for 30 min, then washed three times with PBS. The Red Oil O working solution was added to each well in the amount enabling the complete immersion of the cells in the solution for 2 h at room temperature. Then, the cells were washed thrice with PBS and the excess of PBS was evaporated by placing the microplate at 32 °C for about 30 min. In the next step, the Red Oil O which remained in the cells was extracted by adding 100 µl of isopropanol to each well. Each extract was transferred to a fresh well and the absorbance of the extracts was measured at 510 nm using a Synergy multi-well scanning spectrophotometer.

2.12. Statistics

The data were analyzed using GraphPad Prism 9 (GraphPad Software, Boston, MA, USA). Statistical significance was determined through one-way ANOVA followed by post hoc multiple comparison tests, specifically Tukey's test, or Dunnett's test for cell viability assays involving individual compounds. Results are presented as the mean and standard deviation (SD). Each experiment was conducted in triplicate to ensure reproducibility. Differences among means were considered significant at a threshold of $p < 0.05$.

3. Results

3.1. Cell viability assay

The results of the cell viability assay are presented as a percentage of cell viability in relationship with the control (untreated cells) in Fig. 1.

We observed that 200 µM DHA slightly improved the cell viability (p_{DHA200}= 0,0351) while the other studied compounds imposed no significant effect. Further statistical analysis revealed that there is also a relation between the viability levels after subjecting dopaminergic neuronal cells to the treatment with 200 µM DHA and NRTIs (p_{3TC}= 0,0123, p_{AZT}= 0,0132), as presented in Fig. 1. These findings suggest that NRTIs do not improve cell viability in a similar manner as 200 µM DHA. However, studied NRTIs as well as PA do not show any significant cytotoxic effect in given conditions.

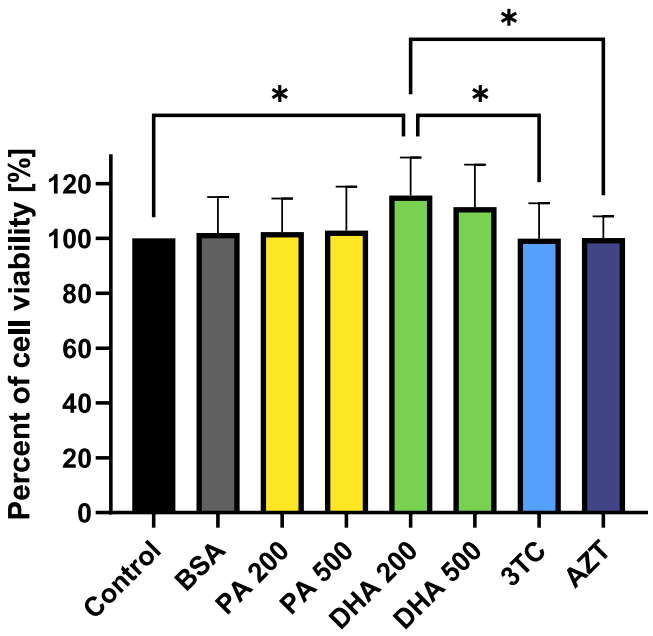

Fig. 1. The cytotoxic effect after 24 h incubation with DHA, PA, 3TC, or AZT. Control— untreated SH-SY5Y cells; BSA—incubation with 10 % fatty acid-free BSA; DHA 200 and DHA 500— incubation with 200 µM or 500 µM DHA; PA 200 and PA 500—incubation with 200 µM or 500 µM PA; 3TC—incubation with 10 µM lamivudine; AZT—incubation with 6 µM zidovudine. Statistically significant differences compared to the untreated SH-SY5Y cells, and NRTIs and FFAs: * $p < 0.05$.

3.2. Mitochondrial superoxide content measurement

The results of the quantification of the mitochondrial superoxide levels conducted in SH-SY5Y cells are presented in Fig. 2.

As demonstrated in Fig. 2, all studied FFAs significantly and comparably decreased the superoxide levels in SH-SY5Y cells (p_{PA200}= 0,0011, p_{PA500}= 0,0003, p_{DHA200}= 0,0092, p_{DHA500}= 0,0068). The decrease was at the levels of approximately 15 % for PA and 13 % for DHA in comparison with the control.

The NRTIs have contributed to the growth of the superoxide levels of approx. 5 % and 18 % for 3TC and AZT respectively (p_{AZT}= 0,0003).

The analysis of the differences between the effects of NRTIs and FFAs is presented in Fig. 3.

In relation to all given FFAs, 3TC, and AZT demonstrated a significant increase in superoxide levels, revealing the opposite effect between those two groups.

3.3. Intracellular FABP4 and FABP5 levels

The quantification of the FABP4 concentrations is presented in Fig. 4. The results were adjusted to a total protein concentration of 100 µg in the cell lysates, as measured using the BCA protein assay.

A decrease in the FABP4 levels was observed in all experimental conditions (Fig. 4a). The change in comparison to control corresponds to 30 % and 55 % for 200 µM PA (p_{PA200}=0,0092) and 500 µM PA (p_{PA500} <0,0001). 200 µM DHA and 500 µM DHA led to a decrease of approx. 40 % and 63 % respectively (p_{DHA200}= 0,0009, p_{DHA500} <0,0001). 3TC

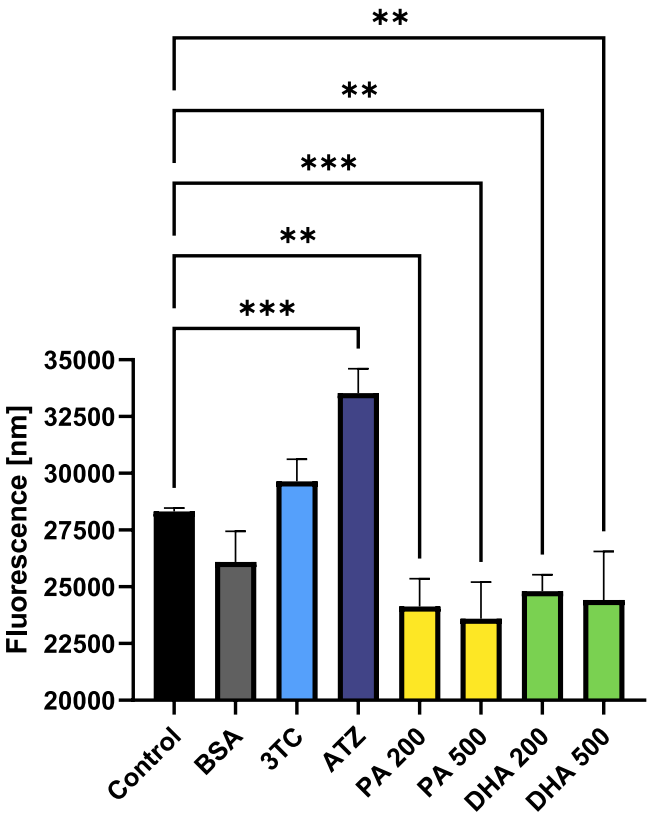

Fig. 2. Mitochondrial superoxide levels after 24 h incubation with DHA, PA, 3TC, or AZT. Control— untreated differentiated SH-SY5Y cells; BSA—incubation with 10 % fatty acid-free BSA; DHA 200 and DHA 500— incubation with 200 μM or 500 μM DHA; PA 200 and PA 500—incubation with 200 μM or 500 μM PA; 3TC— incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine. Statistically significant differences compared to the untreated SH-SY5Y cells: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

decreased FABP4 value by approx. 24 %, and AZT by approx. 30 % (p_{AZT}=0,0099).

The BSA, the environment control, decreased FABP4 concentration to the same level as 200 μM PA and 200 μM DHA (a similar level was achieved in the case of NRTIs, however, they have not been conjugated with BSA). Therefore, it must be considered that the changes observed after treatment with those conditions are related not to the given FFA amount, but to the carrier they have been conjugated to. Statistical analysis confirms that among the treatment conditions with FFA, only 500 μM PA (p_{PA500}= 0,0448) and 500 μM DHA (p_{DHA500}=0,0121) presented a significant change in FABP4 concentration (Fig. 4b). The statistical analysis of the changes in FABP4 concentration after treatment with FFAs and NRTIs is presented in Fig. 5.

In both cases, only the treatment with a higher concentration of FFA demonstrated a significant change in FABP4 concentration in comparison with NRTIs.

The results of FABP5 quantification were adjusted to a total protein concentration of 100 μg in the cell lysates, as determined using the BCA protein assay, and are presented in Fig. 6.

FFAs notably decreased FABP5 levels, an approx. 30 % and 25 % decrease compared to the control was observed after the administration of 200 μM PA (p_{PA200}=0,0102) and 500 μM PA (p_{PA500}=0,0351) respectively.

The DHA 200 μM and 500 μM imposed a 25 % and 33 % decreasing effect on FAPB5 concentration respectively (pa_{DHA200}= 0,0309, p_{DHA500}= 0,0051). In the case of NRTI administration, the decrease was slight (approx. 3–7 %) and the statistical significance was not achieved.

Fig. 7 demonstrates the statistically significant changes between the FABP5 concentrations after treatment with FFAs and NRTIs.

The only statistically significant difference in this case was found between 500 μM DHA and AZT, suggesting that these two compounds impose an alternative effect on dopaminergic neuronal cells.

3.4. MAPK family protein kinase phosphorylation

The phosphorylation levels of ERK, p38, and JNK were determined as a ratio in relationship with positive control for each protein kinase and are presented in Fig. 8a, b, and c. The relationship between FFAs and NRTIs was captured in Fig. 8d and e.

The most notable change was the amplification of ERK phosphorylation by 3TC (p_{3TC}=0,0280) with a 2.5-fold increase in comparison with the untreated control. A slight decrease of approx. 25 % was imposed by 500 μM PA and 500 μM DHA. NRTIs and DHA also increased p38 activity by approx. 30 % for NRTIs, and 200 μM DHA and 95 % for 500 μM DHA. JNK activity was amplified by approx. 40 % for 3TC, 140 % for AZT, 58 % for 200 μM DHA, and 140 % for 500 μM DHA.

The phosphorylation levels of ERK after the treatment with 3TC proved to be statistically significantly greater than the levels determined by 200 μM PA and 200 μM DHA (Figs. 4d, 4e).

3.5. PI3K activity assessment

The measurements for PI3K were standardized using a Crystal Violet staining. The measurements for both DHA concentrations exhibited no signal in the assay and, therefore were not included in the figures. Fig. 9 demonstrates the ratios between the phosphorylated PI3K and total PI3K after given treatments.

500 μM PA led to a 44 % decrease in PI3K activity (p_{PA500}= 0,0289). In opposition, both studied NRTIs increased PI3K phosphorylation by approx. 46 % for 3TC and 17 % for AZT (p_{3TC}= 0,0210).

The differences between the effect of 3TC and PA on PI3K activity proved to be statistically significant for both studied concentrations of PA underlining the alternate effect. As for AZT, only 500 μM PA imposed an effect statistically significantly different on the phosphorylation levels of PI3K (Fig. 9b).

3.6. InsRS1 concentration

The concentrations of InsRS1 in SH-SY5Y cell lysates are presented in Fig. 10.

No statistically significant change was captured after conducting the assay. The concentrations of InsRS1 were slightly decreased after the treatment with 200 μM PA, 500 μM DHA, and 3TC and slightly increased after the exposure to 500 μM PA.

3.7. InsR expression determination

The captured insulin receptor concentrations are depicted in Fig. 11. The BCA protein assay was used to normalize the results of insulin receptor quantification to a total protein concentration of 100 μg in the cell lysates.

All experimental conditions led to a reduction in insulin receptor concentrations. The most notable decrease in InsR concentration was observed after the incubation with 500 μM DHA, corresponding to approx. 35 % in relation to untreated control (p_{DHA500}= 0,0159). This data point was also the only one with which statistical significance was achieved.

3.8. Lipid content measurement

Fig. 12 demonstrates the obtained result from the lipid staining in the form of absorbance values.

Under all experimental conditions, there was a noticeable decline in

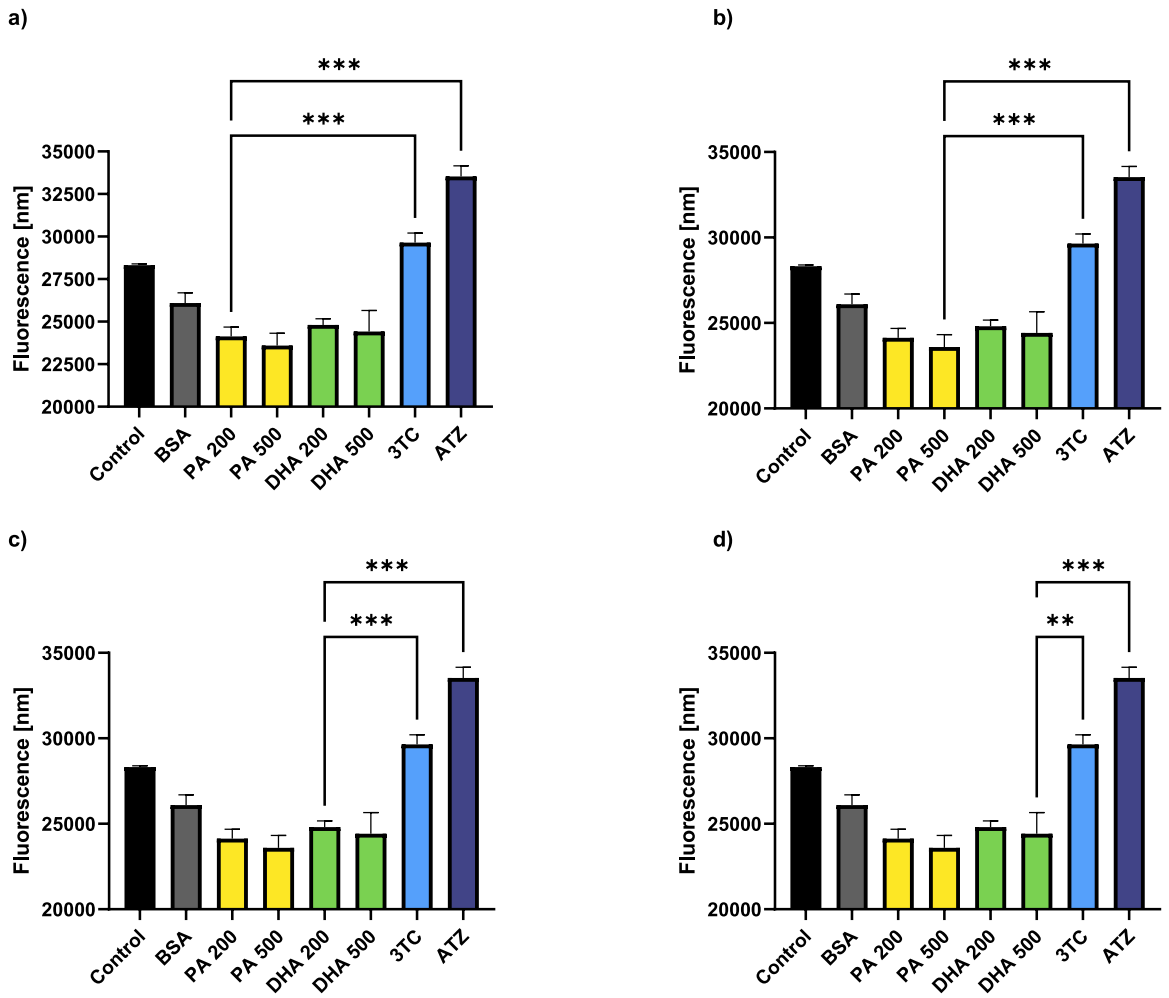

Fig. 3. Mitochondrial superoxide levels after 24 h incubation with DHA, PA, 3TC, or AZT. Control— untreated differentiated SH-SY5Y cells; BSA—incubation with 10 % fatty acid-free BSA; DHA 200 and DHA 500— incubation with 200 μM or 500 μM DHA; PA 200 and PA 500—incubation with 200 μM or 500 μM PA; 3TC—incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine. Statistically significant differences: a) between 200 μM PA and NRTs, b) between 500 μM PA and NRTIs, c) between 200 μM DHA and NRTIs, d) between 500 μM DHA and NRTIs: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

the absorbance values after lipid staining. 500 μM PA led to a most notable decrease of approx. 29 % (p_{PA500} <0,0001). 200 μM PA, 200 μM DHA, and 500 μM DHA caused a similar change in absorption as BSA, a carrier for FFAs (p_{PA200}= 0,0020, p_{DHA200}= 0,0002, p_{DHA500}= 0,0002). Therefore, further statistical analysis was performed to determine which conditions led to a change statistically significantly different than the treatment with BSA (Fig. 12b). The tested compounds did not demonstrate a statistically significant difference in comparison with the BSA. This fact suggests that the changes in absorbances after the treatment with FFAs might be a result of the applicated carrier only.

The treatment with selected NRTIs led to a slight decrease in lipid accumulation (p_{3TC}= 0,0216). The relationships between the effect imposed by FFAs and NRTIs are shown in Fig. 13.

The results demonstrated in Fig. 13 suggest that treatment of the dopaminergic neuronal cells with 500 μM PA, 200 μM DHA, and 500 μM DHA led to a statistically significantly different lipid accumulation than the AZT.

4. Discussion

This study explores the potential impact of NRTIs, PA, and DHA on dopaminergic neurons and compares the results between the groups to determine whether they might act in the same manner. The experiments were designed to concentrate on the three critical aspects of lipotoxicity: IR, lipid transport and accumulation, and general cellular homeostasis, including ROS generation and MAPK signaling pathways. Moreover, despite the well-documented impacts of PA on visceral tissues, its effects on brain tissues have been comparatively less explored. Emerging research underscores the importance of lipidomics in elucidating the molecular underpinnings of neurodegenerative diseases, offering potential biomarkers for early diagnosis and novel therapeutic targets. Therefore, a comprehensive understanding of lipid homeostasis and its disruption in the CNS is crucial for advancing the development of effective interventions for neurodegenerative diseases.

The investigation of dopaminergic neuron cellular metabolism included measurement of cytotoxicity, mitochondrial superoxide levels, fatty acid binding protein 4 and 5 concentration, lipid accumulation, and phosphorylation of selected mitogen-activated protein kinases after the treatment with NRTIs and FFAs. The induction of the IR was assessed via the levels of PI3K, InsRS1, and InsR. Also, the effects of NRTIs and FFAs were compared to investigate whether there is a similarity in results between the two groups.

Our analysis of the impact of studied compounds on cell viability revealed no significant cytotoxic effects, as the cell viability remained comparable to that of the control group. The results indicate that the treatment did not adversely affect cell survival, with viability percentages aligning closely with those observed in untreated cells. However, it is noteworthy that PA under the set experimental conditions also failed

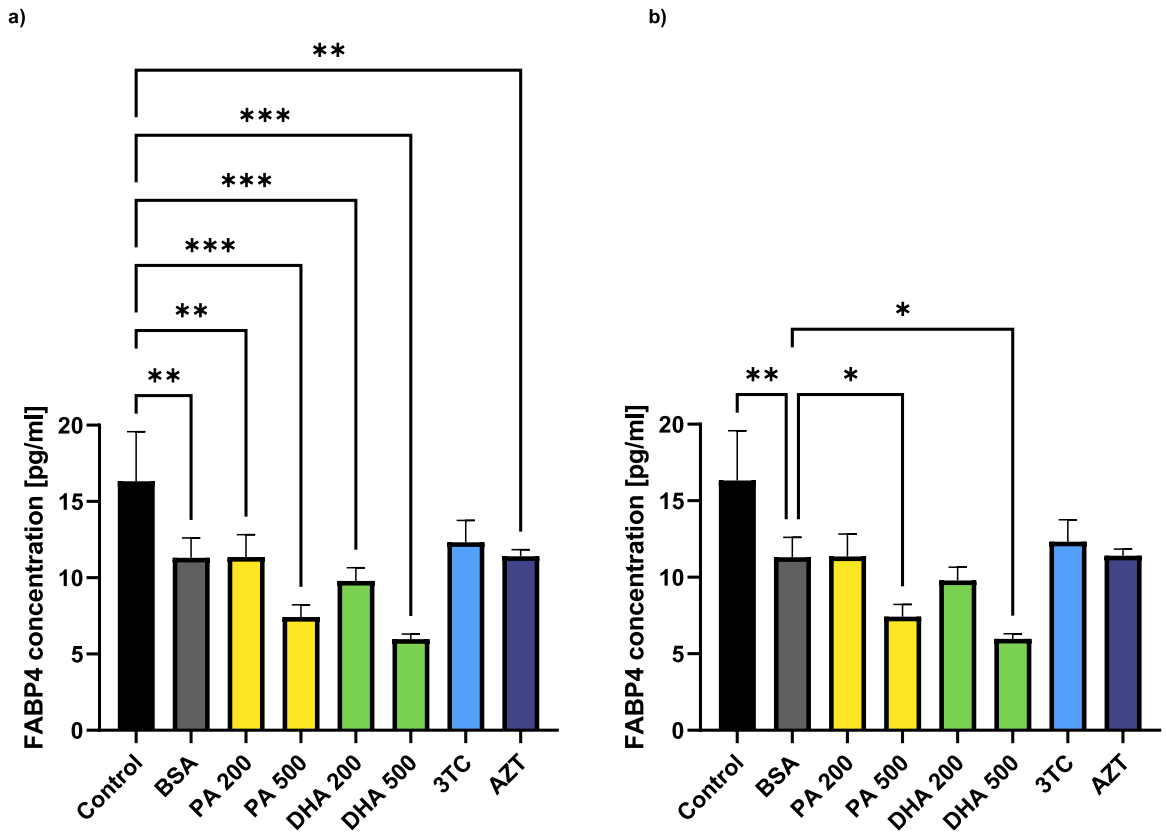

Fig. 4. Intracellular FABP4 levels after 24 h incubation with DHA, PA, 3TC, or AZT. Control— untreated differentiated SH-SY5Y cells; BSA—incubation with 10 % fatty acid-free BSA; DHA 200 and DHA 500— incubation with 200 μM or 500 μM DHA; PA 200 and PA 500—incubation with 200 μM or 500 μM PA; 3TC— incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine. Statistically significant differences: a) compared to the untreated SH-SY5Y cells, b) compared to the BSA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

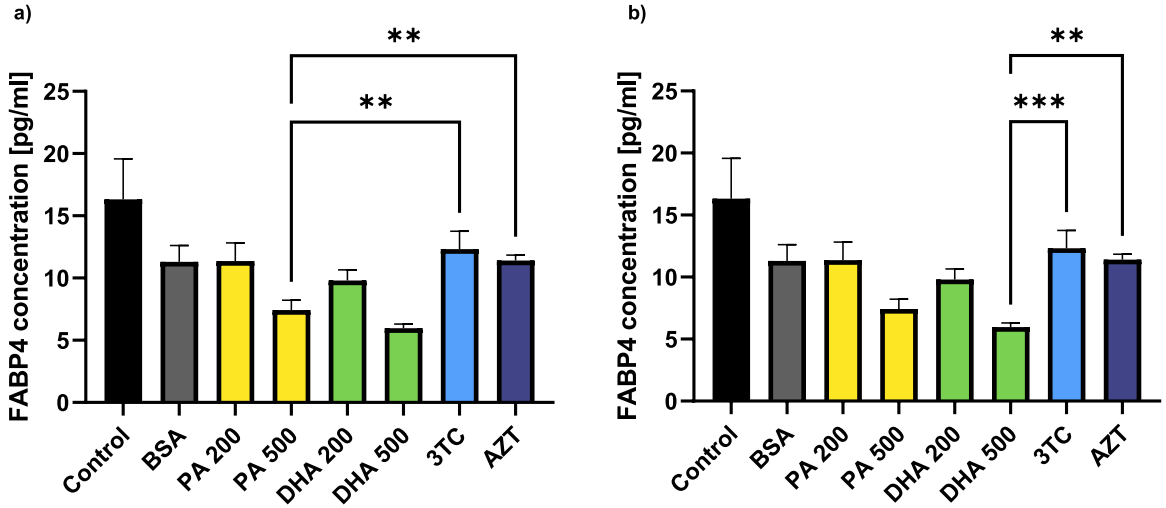

Fig. 5. Intracellular FABP4 levels after 24 h incubation with DHA, PA, 3TC, or AZT. Control— untreated differentiated SH-SY5Y cells; BSA—incubation with 10 % fatty acid-free BSA; DHA 200 and DHA 500— incubation with 200 μM or 500 μM DHA; PA 200 and PA 500—incubation with 200 μM or 500 μM PA; 3TC— incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine. Statistically significant differences: a) between PA and NRTs, b) between DHA and NRTIs: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

to show such properties, despite numerous literature reports supporting them [30–32]. This would suggest that the experimental conditions may not have provided a reliable determination of cytotoxic properties, so this result will not be analyzed further. On the other hand, DHA led to a slight increase in cell viability, in this case confirming the positive influence exerted on the cells.

A study of the effects of NRTIs and FFAs on the content of the mitochondrial fraction of superoxide showed that both lamivudine and zidovudine cause an increase in its amount, in opposition to both FFAs, which lead to a decrease. In this case, the result for NRTIs is consistent with literature data, for example, with the study by Kohler et al., which implicates AZT in the development of mitochondrial dysfunction

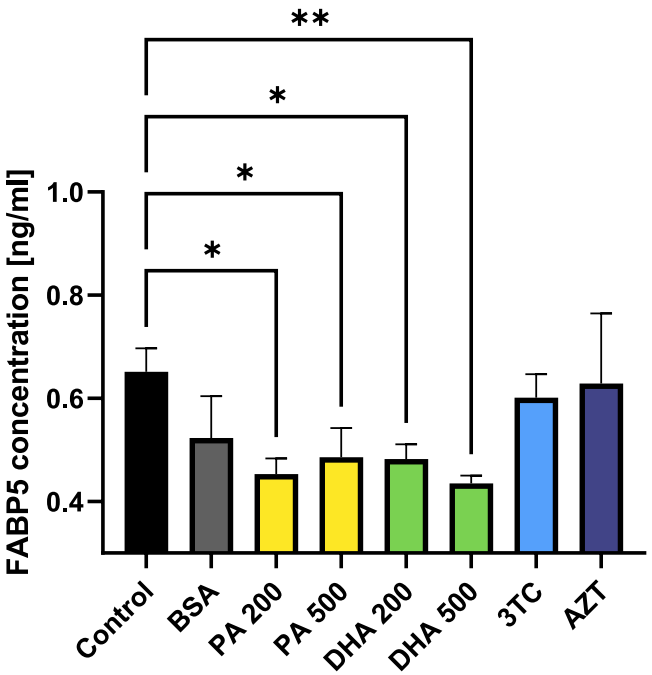

Fig. 6. Intracellular FABP5 levels after 24 h incubation with DHA, PA, 3TC, or AZT. Control— untreated differentiated SH-SY5Y cells; BSA—incubation with 10 % fatty acid-free BSA; DHA 200 and DHA 500— incubation with 200 μM or 500 μM DHA; PA 200 and PA 500—incubation with 200 μM or 500 μM PA; 3TC— incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine. Statistically significant differences compared to the untreated SH-SY5Y cells: * $p < 0.05$, ** $p < 0.01$.

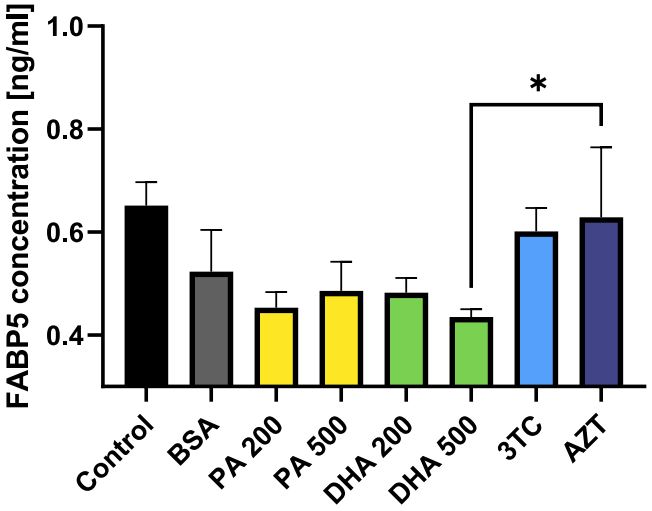

Fig. 7. Intracellular FABP5 levels after 24 h incubation with DHA, PA, 3TC, or AZT. Control— untreated differentiated SH-SY5Y cells; BSA—incubation with 10 % fatty acid-free BSA; DHA 200 and DHA 500— incubation with 200 μM or 500 μM DHA; PA 200 and PA 500—incubation with 200 μM or 500 μM PA; 3TC— incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine. Statistically significant differences between the FFAs and NRTIs: * $p < 0.05$.

through induction of ROS generation [33]. Mitochondrial superoxide levels may be increased due to a variety of factors associated with mitochondrial dysfunction and cellular stress. Since the mechanism of action of NRTIs involves the inhibition of mitochondrial DNA, an important cause of increased ROS levels may be the accumulation of dysfunctional proteins, impaired fatty acid oxidation, and impaired oxidative phosphorylation. The incubation of dopaminergic neurons with 3TC led to an increase in mitochondrial superoxide levels compared to the untreated cells, however to a noticeably lesser extent than AZT. Lamivudine's potential to cause mitochondrial toxicity is recognized as lower in relationship to other NRTIs, including AZT, therefore it is possible, that 3TC's concentration used in this study does not conclusively lead to mitochondrial toxicity [34]. The result for DHA was expected, as this compound is generally recognized as a positive factor for the organism. An unexpected observation is the counteracting effect of FFA on superoxide levels. Despite many reports on the involvement of PA in the induction of cellular stress, there are few studies conducted on dopaminergic neurons. Ng et al. showed that PA leads to the development of OS in cells of the SH-SY5Y line, while an increase in H_2O_2 levels was confirmed [35]. However, a study conducted on chondrocytes in which PA was observed to increase superoxide content [36]. Therefore, it might be possible that PA does not influence the ROS content in the dopaminergic cells, however, it is necessary to perform additional assays to unambiguously analyze the effect of PA. On the other hand, reducing superoxide generation may contribute to mitigating one of the mechanisms of NRTI toxicity, but a new research model would also need to be constructed to make this conclusion.

FABP4, or adipocyte fatty acid binding protein, exerts a significant influence on lipid metabolism by suppressing lipogenesis and enhancing lipolysis. Furthermore, changing levels of this protein have been correlated with an increased risk of cardiovascular diseases, type 2 diabetes, and non-alcoholic fatty liver disease among others. Both AZT and 3TC demonstrated a noticeable decreasing effect on FABP4 concentrations in dopaminergic neuronal cells compared to the untreated control and statistical significance has been achieved for the AZT. This might indicate the lesser extent of lipolysis and therefore reduced release of FFAs into the bloodstream from the cells. Such an effect can have a twofold impact on the lipid homeostasis. Firstly, it might lead to decreased availability of FFAs for energy production, thereby impairing cellular energy metabolism, especially in tissues reliant on fatty acids as a primary energy source. On the other hand, reduced breakdown of triglycerides can potentially lower the risk of lipotoxicity and related conditions such as insulin resistance and inflammation. To our knowledge, there is very little research concerning the role of FABP4 in NRTIs' influence on lipid homeostasis. A study conducted by Gibellini et al. on adipocyte stem cell lines showed an increasing effect of NRTIs on FABP4 gene expression in human Dental Pulp Stem Cells (hDPSCs) and a decreasing effect in human Bone Marrow-Derived Mesenchymal Stem Cells (hBM-MSCs) [37]. Boothby et al. demonstrated that the gene expression of FABP4 in patients after a 6-month treatment with AZT showed a 2.5-fold increase compared with pre-treatment [38]. A significant positive correlation between FABP4 plasma concentration in patients and lipodystrophy was demonstrated by Escote et al. [39]. Selected NRTIs, as well as both FFAs, imposed a decreasing effect on FABP4 concentrations. However, the differences between the decrease caused by NRTIs and FFAs proved to be statistically significantly greater for FFAs. Such results suggest that NRTIs, as well as DHA, do not contribute to the potential toxic action. Regarding DHA, such data was expected. However, PA has a confirmed increasing effect on the expression of FABP4 in studies on other cell lines at the same concentration and incubation time [40,41]. As PA also did not impose an increasing effect on FABP4 in our study, it might be speculated that this transporter does not mediate lipotoxicity in dopaminergic neurons at all. There is a possibility that dopaminergic neurons are less susceptible to lipotoxicity than other cell types. This might be due to the greater emphasis on glucose as the primary energy source in the CNS.

FAPB5 is expressed highly in the developing brain and the adult brain, its expression is observed in both neurons and glia [42]. Our study demonstrated that 3TC and AZT had no discernible effects on the concentration of FABP5, suggesting that either there is no relationship between NRTIs and studied protein or the scope of designed experimental settings does not capture the relationship. The decrease in FABP5

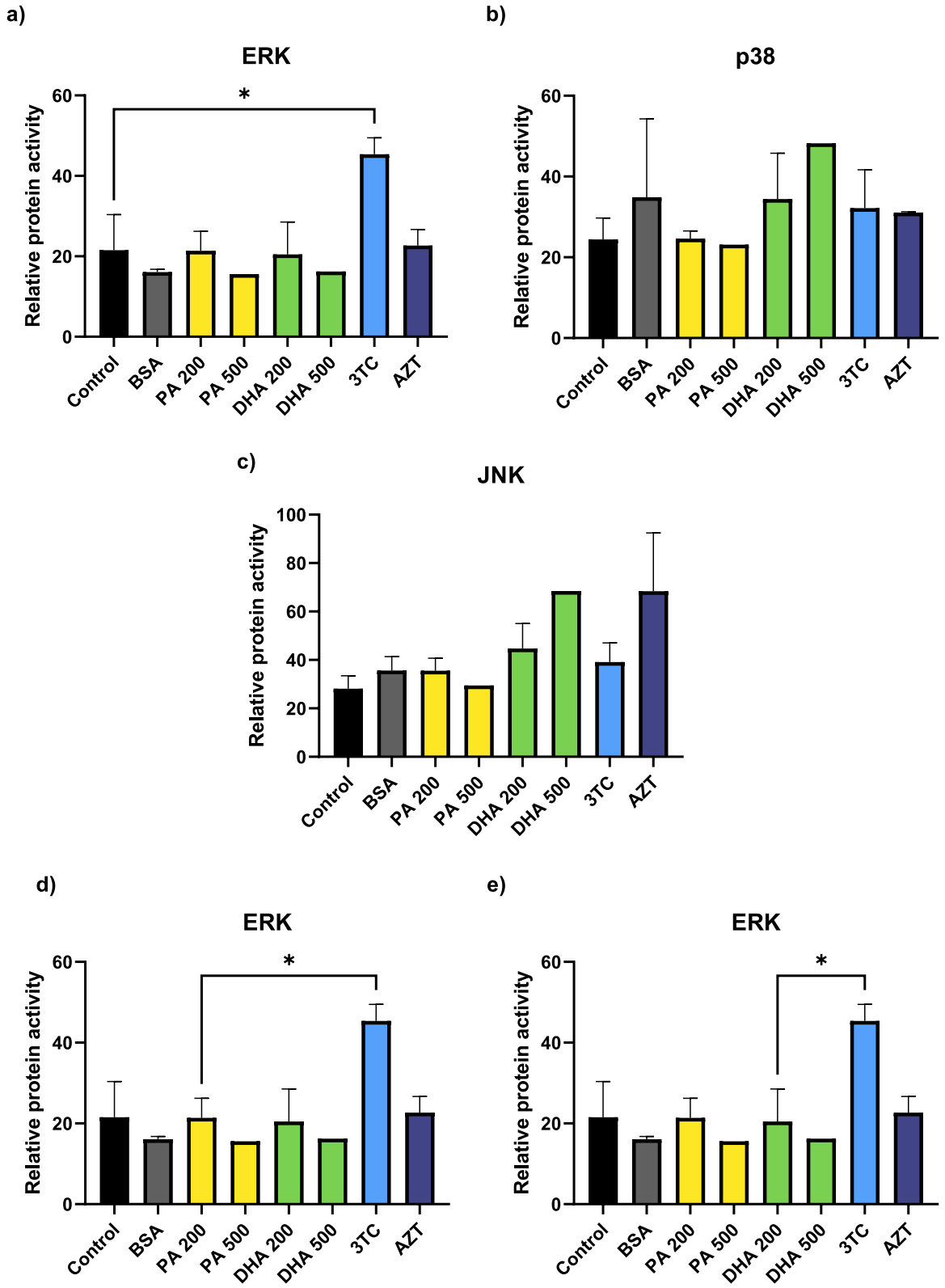

Fig. 8. The ratio between ERK, p38, and JNK and positive control after 24 h incubation with DHA, PA, 3TC, or AZT. Control— untreated differentiated SH-SY5Y cells; BSA—incubation with 10 % fatty acid-free BSA; DHA 200 and DHA 500— incubation with 200 μM or 500 μM DHA; PA 200 and PA 500—incubation with 200 μM or 500 μM PA; 3TC— incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine. Statistically significant differences: a) for ERK compared to the untreated SH-SY5Y cells, b) for p38 compared to the untreated SH-SY5Y cells, c) for JNK compared to the untreated SH-SY5Y cells, d) between PA and NRTIs, e) between DHA and NRTIs: * $p < 0.05$.

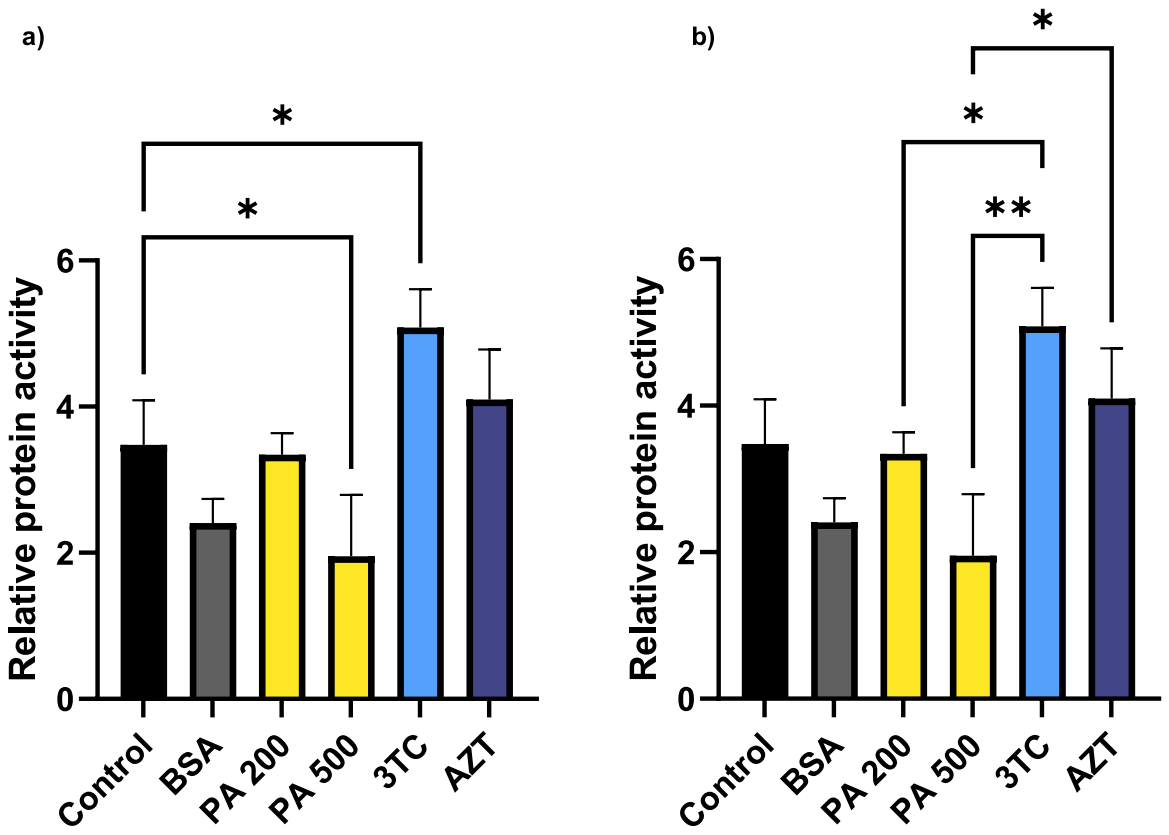

Fig. 9. The ratio between phosphorylated PI3K and total PI3K after 24 h incubation with DHA, PA, 3TC, or AZT. Control— untreated differentiated SH-SY5Y cells; BSA—incubation with 10 % fatty acid-free BSA; DHA 200 and DHA 500— incubation with 200 μM or 500 μM DHA; PA 200 and PA 500—incubation with 200 μM or 500 μM PA; 3TC— incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine. Statistically significant differences: a) compared to the untreated SH-SY5Y cells, b) between PA and NRTIs: * $p < 0.05$, ** $p < 0.01$.

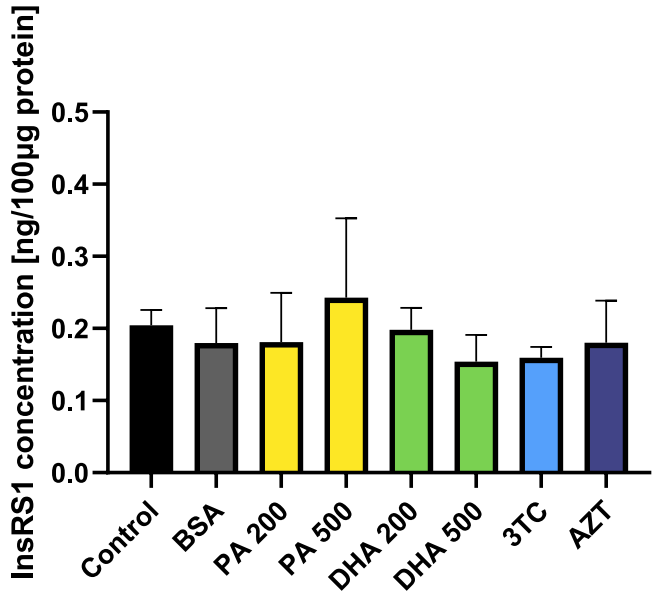

Fig. 10. The concentration of insulin receptor substrate 1 (InsRS1) after 24 h incubation with DHA, PA, 3TC, or AZT. Control— untreated differentiated SH-SY5Y cells; BSA—incubation with 10 % fatty acid-free BSA; DHA 200 and DHA 500— incubation with 200 μM or 500 μM DHA; PA 200 and PA 500—incubation with 200 μM or 500 μM PA; 3TC— incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine.

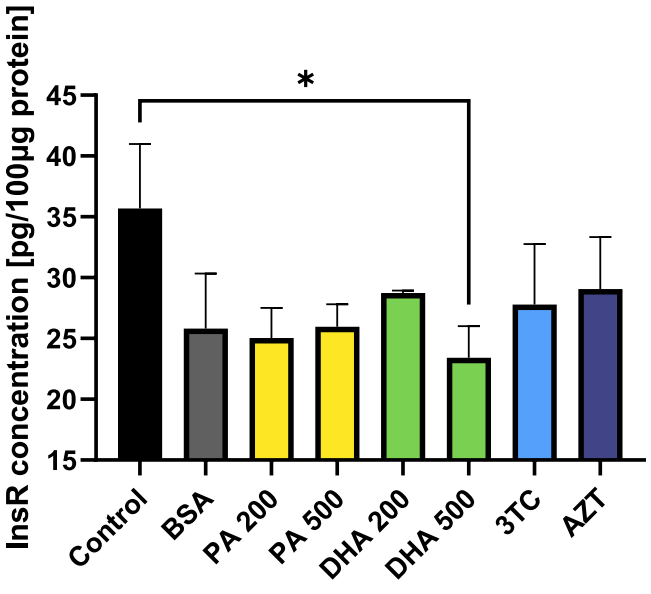

Fig. 11. The concentration of insulin receptor after 24 h incubation with DHA, PA, 3TC, or AZT. Control— untreated differentiated SH-SY5Y cells; BSA—incubation with 10 % fatty acid-free BSA; DHA 200 and DHA 500— incubation with 200 μM or 500 μM DHA; PA 200 and PA 500—incubation with 200 μM or 500 μM PA; 3TC— incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine. Statistically significant differences compared to the untreated SH-SY5Y cells: * $p < 0.05$.

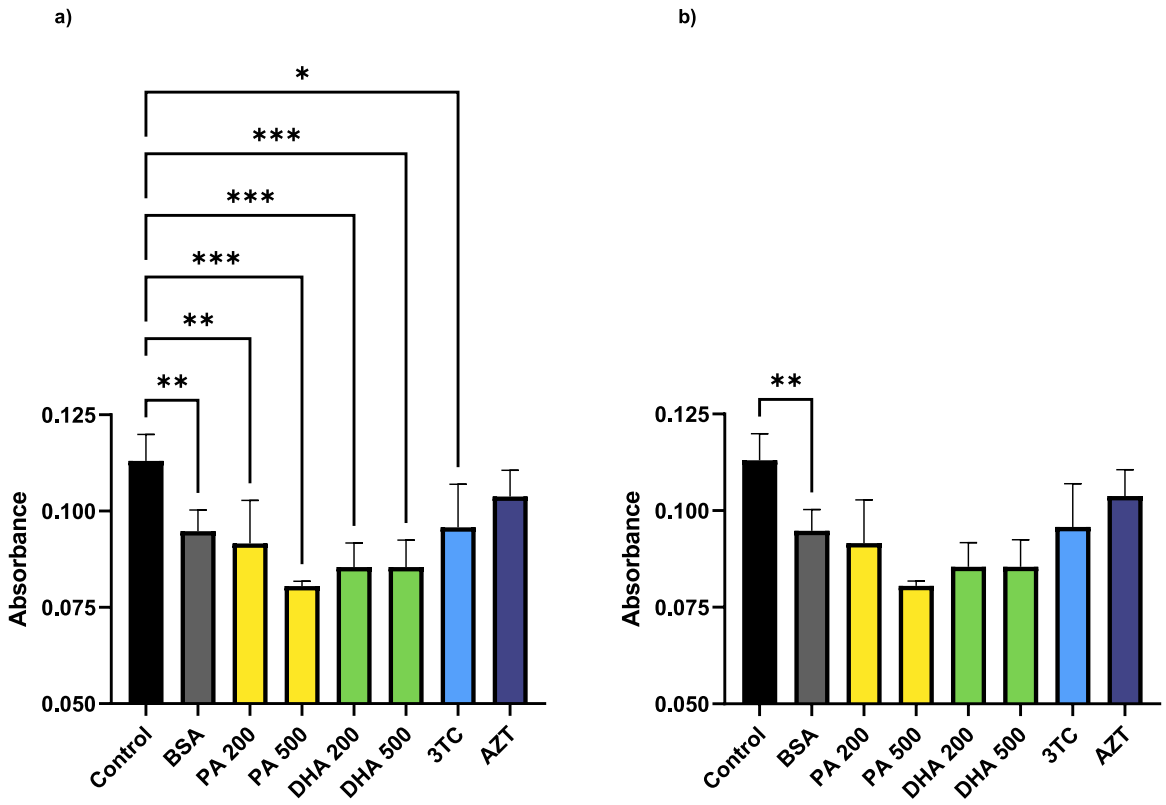

Fig. 12. The absorbances captured after a lipid staining after 24 h incubation with DHA, PA, 3TC, or AZT. Control— untreated differentiated SH-SY5Y cells; BSA—incubation with 10 % fatty acid-free BSA; DHA 200 and DHA 500— incubation with 200 μM or 500 μM DHA; PA 200 and PA 500—incubation with 200 μM or 500 μM PA; 3TC— incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine. Statistically significant differences: a) compared to the untreated SH-SY5Y cells, b) compared to the BSA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

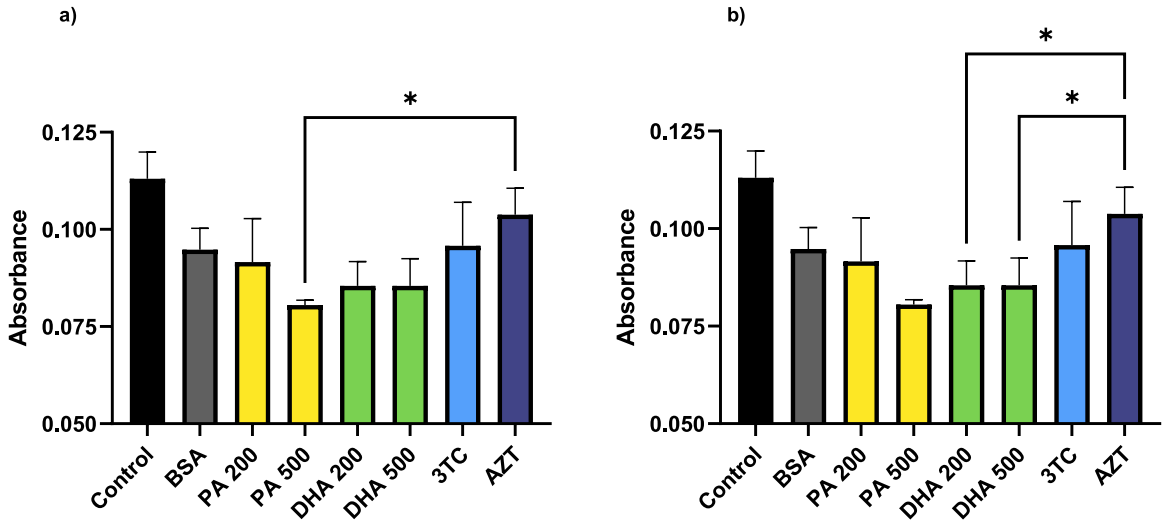

Fig. 13. The absorbances captured after a lipid staining after 24 h incubation with DHA, PA, 3TC, or AZT. Control— untreated differentiated SH-SY5Y cells; BSA—incubation with 10 % fatty acid-free BSA; DHA 200 and DHA 500— incubation with 200 μM or 500 μM DHA; PA 200 and PA 500—incubation with 200 μM or 500 μM PA; 3TC— incubation with 10 μM lamivudine; AZT—incubation with 6 μM zidovudine. Statistically significant differences: a) between PA and NRTIs, b) between DHA and NRTIs: * $p < 0.05$.

concentration under PA treatment contradicts the assumptions made, as well as literature reports showing an inverse relationship [43,44]. However, given the small number of studies performed on neuronal cells, it can be assumed that dopaminergic neurons do not respond to PA in the same way as cells in other tissues. In the case of DHA, again, the results are aligned with our expectations, underlining its potential positive influence on cell metabolism.

Of the measured phosphorylation levels of MAPK protein kinases, only the effect of 3TC on ERK, resulting in a 2.5-fold increase in phosphorylation, showed statistical significance. From a clinical point of view, the most important consequence of excessive ERK activation is cellular senescence, which is manifested by cell-cycle arrest, as well as the secretion of pro-inflammatory factors. This condition is correlated with aging diseases [45].

Regarding the rest of the measured conditions, it was impossible to achieve statistical significance when comparing these protein kinases' phosphorylation levels to the baseline condition. Without statistical significance, any assertions about the influence of these treatments remain speculative and must be interpreted with caution. As a result, the actual impact of studied treatments on selected protein kinases remains unresolved, highlighting the necessity for further studies, especially taking into account that there is limited research in this area.

PI3K is a key secondary messenger involved in intracellular signaling pathways. One of the primary pathways influenced by PI3K is the AKT/mTOR (protein kinase B/mammalian target of rapamycin) pathway. In terms of lipid metabolism, PI3K/AKT signaling influences the activity of key enzymes involved in fatty acid synthesis and storage. AKT activation leads to the activation of ATP-citrate lyase and acetyl-CoA carboxylase, which are crucial for the *de novo* synthesis of fatty acids. Moreover, PI3K/AKT signaling promotes the expression of sterol regulatory element-binding proteins, which are transcription factors that regulate the expression of genes involved in lipid biosynthesis. PI3K is also a key component of the insulin signaling pathway. The increased phosphorylation/total ratio of PI3K, AKT as well as InsR and InsRS1 has been related to potential antidiabetic activity via amelioration of IR [46,47]. Our determination of PI3K phosphorylation/total ratio under the treatment with both selected NRTIs demonstrated an amplification in relation to untreated control, suggesting a potential role in the amelioration of IR via a similar mechanism as shown in a study conducted by Tong et al. [46]. Similarly, Li et al. demonstrated that treatment with 3TC led to a significantly increased expression of formaldehyde-induced inhibition of AKT activity in the hippocampus and cortex of mice [48]. Conversely, a study by Mohan et al. presents opposing findings. They reported that 3TC decreased the activity of both PI3K and AKT in human hepatoma cells [49]. Furthermore, Shimizu et al. demonstrated that 3TC had no effect on AKT in hepatocellular models [50]. The opposing findings suggest that the effect of 3TC on PI3K may be context-dependent, varying with cell type, experimental conditions, or additional factors influencing PI3K regulation. Consistent with the premise of our study, increased concentrations of PA decreased PI3K activity, potentially leading to a lower ability of the cell to cope with excess glucose and fatty acids, and thus the development of insulin resistance.

Our assessment of the impact of NRTIs on InsR and InsRS1 did not reveal any connections between studied drugs and those key elements of insulin signaling in dopaminergic neurons.

The only statistically significant change was observed for InsR concentration after the treatment with 500 μM DHA. This effect contradicts the positive DHA influence on the dopaminergic cell. However, in light of known insulin signaling-inhibiting properties of PA, which were not demonstrated in our study, the factual influence of DHA on the InsR expression remains elusive [51]. While treatment with NRTIs led to no discernible effect on the InsRS1 expression, a minor decrease was demonstrated in the case of InsR expression. However, as the results failed to provide statistical significance, the decrease might be a product of chance and not a determined causality. Therefore, any potential influence of 3TC and AZT on InsR and InsRS1 in dopaminergic neurons remains conjectural and needs further analysis.

Following the administration of all studied compounds, the total lipid content in the dopaminergic neurons was reduced in comparison with untreated cells. The results for DHA are in line with the premise of the study, however, the opposing results for PA were not expected. Taking into account that the FABP4 concentration was also lowered in all studied conditions, lesser lipid content seems inconsistent, however, there might be a different metabolic mechanism leading to the lipid accumulation in dopaminergic neurons under the influence of NRTIs and FFAs. A comparison of the decreasing effects imposed by NRTIs and FFAs on lipid content demonstrated that lipotoxic concentration of PA affected lipid accumulation statistically significantly greater than AZT. Taking into account that both concentrations of FABP4 and lipid content were reduced by all studied compounds, a conclusion can be made that these parameters do not contribute to lipotoxic effects in dopaminergic neurons.

By relating these results to the overall picture of lipotoxicity, NRTIs might play a role by leading to the release of FFA from the cells, depleting a potential energy deposit, and releasing them into the bloodstream. Increased concentrations of free FFAs in the bloodstream might accumulate in other ectopic cell types, thus contributing to lipotoxicity. However, based on the demonstrated results, we can conclude that NRTIs do not contribute to lipotoxicity in measured parameters, as they either positively influence them, or when the effect is negative it does not align with the effect imposed by lipotoxic PA. However, to fully elude the role of NRTIs in lipotoxicity in dopaminergic neurons, more studies need to be conducted.

However, it is imperative to recognize the limitations associated with this investigation. One of the primary limitations of this study is the use of an *in vitro* cell line model of neurodegenerative disorders, which may not fully replicate the responses observed in patient-derived materials. The *in vitro* environment lacks the complexity of *in vivo* conditions, where multiple factors, including the microenvironment and interactions with other cell types, influence the outcome. Additionally, the short-term exposure of cultured cells to NRTIs and FFAs in this study contrasts with the long-term treatment regimens experienced by patients. This discrepancy may lead to differences in the cellular responses observed, underscoring the need for caution when extrapolating these results to clinical scenarios. Future investigations should include comprehensive assessments of IR, ROS concentrations, lipid peroxidation levels, and other markers of pathological states. These studies are essential for confirming the mechanisms by which NRTIs and FFAs affect lipid metabolism and for identifying potential therapeutic targets to mitigate these effects. The issue of lipid disorders within the CNS and the influence of NRTIs remains an area with many unresolved questions. Understanding the intricate mechanisms behind lipid metabolism disruption is crucial, as it has significant implications for the management of patients undergoing NRTI therapy and for the FFAs' role in the pathogenesis of neurodegeneration. Addressing these questions through ongoing research will provide valuable insights into the potential risks associated with lipotoxicity in CNS and help develop strategies to prevent or minimize these adverse effects.

5. Conclusion

In conclusion, under the set conditions, lamivudine and zidovudine do not cause the disruption of homeostasis in dopaminergic neurons involving FABP4, and PI3K or via affecting the lipid content of the cell. However, they may alter the mitochondrion function through increased superoxide production or lead to cell senescence by enhancing ERK activation. Both tested FFAs also do not induce lipotoxicity via FABP4, or FABP5, and do not enhance lipid accumulation in dopaminergic neurons. While DHA was set as a positive control and this result is consistent, such influence of PA was not expected. This suggests that the homeostasis of lipid transport and storage in such cells might be resistant to the studied conditions. PA, however, contributed to a decrease in PI3K activity, and also, in opposition to baseline assumptions, did not increase mitochondrial superoxide generation. The results show similarities between the effects of NRTIs and FFAs on dopaminergic neurons, and in these cases, the effects do not affect the development of lipotoxicity. When it comes to superoxide levels, the influence of both studied groups differed, therefore, the potential NRTI-induced dysfunction of mitochondria is probably not related to lipid homeostasis. However, the results raise new questions, making this line of research worthy of further engagement and exploration.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work the author(s) used ChatGPT in order to improve the readability and language of the manuscript. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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CRediT authorship contribution statement

Adriana Kubis-Kubiak: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Conceptualization. **Agnieszka Piwowar:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Katarzyna Lipke:** Writing – original draft, Visualization, Validation, Methodology, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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13.2. Załącznik 2. Oświadczenia współautorów publikacji.

Wrocław, 05.12.2024
miejscowość, data

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OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że w pracy:

1. Lipke Katarzyna, Kubis-Kubiak Adriana, Piwowar Agnieszka, Molecular mechanism of lipotoxicity as an interesting aspect in the development of pathological states - current view of knowledge, Cells, 2022, vol. 11, nr 5, s. art.844 [34 s.]. DOI:10.3390/cells11050844
2. Lipke Katarzyna, Kubis-Kubiak Adriana, Piwowar Agnieszka, The influence of nucleoside reverse transcriptase inhibitors on mitochondrial activity, lipid content, and fatty-acid-binding protein levels in microglial HMC3 cells, Pharmaceuticals, 2023, vol. 16, nr 12, s. art.1661 [19 s.]. DOI:10.3390/ph16121661
3. Lipke Katarzyna, Kubis-Kubiak Adriana, Piwowar Agnieszka, Investigating the role of nucleoside reverse transcriptase inhibitors in modulating lipotoxicity: Effects on lipid dynamics stress pathways, and insulin resistance on the function of dopaminergic neurons, Biomedicine & Pharmacotherapy, 2024, vol. 181, s. 117701, DOI:10.1016/j.biopha.2024.117701

mój udział polegał na ustaleniu koncepcji i założeń pracy, ocenie merytorycznej pracy, edycji i nadzorze nad manuskryptem, zatwierdzeniu ostatecznej wersji pracy.

A. Piwowar
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OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że w pracach:

1. Lipke Katarzyna, Kubis-Kubiak Adriana, Piwowar Agnieszka, Molecular mechanism of lipotoxicity as an interesting aspect in the development of pathological states - current view of knowledge, Cells, 2022, vol. 11, nr 5, s. art.844 [34 s.]. DOI:10.3390/cells11050844
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mój udział polegał na ustaleniu koncepcji i założeń pracy, ocenie merytorycznej pracy, pomocy w przeprowadzeniu części badań, edycji i nadzorze nad manuskryptem, zatwierdzeniu ostatecznej wersji pracy.

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13.3. Załącznik 3. Wykaz publikacji i abstraktów potwierdzony przez bibliotekę.

Katarzyna Lipke

1. Publikacje w czasopismach naukowych

1.1 Publikacje w czasopiśmie z IF

Lp.	Opis bibliograficzny	IF	Punkty
1	Gałęzowska Joanna, Boratyński P.J., Kowalczyk R., **Lipke K.**, Czapor-Irzabek Hanna: Copper(II) complexes of chiral 1,2,3-triazole biheterocyclic 'click' ligands equipped in Cinchona alkaloid moiety, Polyhedron, 2017, vol. 121, s. 1-8, DOI:10.1016/j.poly.2016.09.058	2,067	30
2	**Lipke Katarzyna,** Kubis-Kubiak Adriana, Piwowar Agnieszka: Molecular mechanism of lipotoxicity as an interesting aspect in the development of pathological states - current view of knowledge, Cells, 2022, vol. 11, nr 5, art.844 [34 s.], DOI:10.3390/cells11050844	6	140
3	**Lipke Katarzyna,** Kubis-Kubiak Adriana, Piwowar Agnieszka: The influence of nucleoside reverse transcriptase inhibitors on mitochondrial activity, lipid content, and fatty-acid-binding protein levels in microglial HMC3 cells, Pharmaceuticals, 2023, vol. 16, nr 12, art.1661 [19 s.], DOI:10.3390/ph16121661	4,3	140
4	**Lipke Katarzyna,** Kubis-Kubiak Adriana, Piwowar Agnieszka: Investigating the role of nucleoside reverse transcriptase inhibitors in modulating lipotoxicity: Effects on lipid dynamics stress pathways, and insulin resistance on the function of dopaminergic neurons, Biomedicine & Pharmacotherapy, 2024, vol. 181, art.117701 [14 s.], DOI:10.1016/j.biopha.2024.117701	6,9*	100
	Podsumowanie	19,267	410

**IF 2023*

2. Abstrakty

Lp.	Opis bibliograficzny
1	**Lipke Katarzyna,** Czapor-Irzabek Hanna, Chmielewska Ewa, Gałęzowska Joanna: Nowe aminobisfosfoniany - badania koordynacyjne względem biologicznie aktywnych jonów metali, W: II Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 3 kwietnia 2017 r. Książka abstraktów 2017, 32 poz.P16
2	**Lipke Katarzyna,** Piwowar Agnieszka: Xenobiotics and estrogens as potential inducers of lipotoxicity, W: 4th International Wroclaw Scientific Meetings. Wrocław, 09-10 October 2020, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Weżgowiec, Wrocław 2020, Wydawnictwo Naukowe TYGIEL sp. z o.o., s. 162-163, ISBN 978-83-66489-37-0

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