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# Antyproliferacyjne działanie inhibitora czynnika transkrypcyjnego SOX18 w niedrobnokomórkowym raku płuc

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# Spis treści

**PODZIĘKOWANIA** …………………………………………………….…………….... 3

**WYKAZ PUBLIKACJI STANOWIĄCYCH ROZPRAWĘ DOKTORSKĄ** ……..…………………….……………………………….………… 4

**STRESZCZENIE** …………..………………….………….……………….……….…… 5

**SUMMARY** ………….....……………….....………………………….….………….…… 8

**1. WSTĘP** ……………..…………….…..………………..…………..……….….….. 11

1.1. Nowotwory płuc ……………...……………..………………..………... 11

1.2. Rola czynników transkrypcyjnych w rozwoju i progresji raka płuc ........ 12

1.3. Czynniki transkrypcyjne z rodziny SOX ……..…………………...…. 13

1.3.1. Podgrupa czynników transkrypcyjnych SOXF ……………….......... 14

1.3.2. Białka SOXF w kancerogenezie nabłonka oddechowego …….….…. 14

1.3.3. SOX18 - potencjalny czynnik stymulujący proliferację komórek nowotworowych …………………………………………………….... 15

1.4. Sm4 - inhibitor homodimeryzacji SOX18……………………….….. 16

1.5 Piśmiennictwo………………………………………………………18

**2. ZAŁOŻENIA I CEL PRACY DOKTORSKIEJ** ……….………..… 22

**3. PUBLIKACJE** …………..………………...…………………….…..... 23

I. Rodak, O., Peris-Díaz, M., Olbromski, M., Podhorska-Okołów, M., Dzięgiel, P. *Current Landscape of Non-Small Cell Lung Cancer: Epidemiology, Histological Classification, Targeted Therapies, and Immunotherapy.* Cancers. 2021 Sep 20; 13(18):4705. …………………………………….. 23

II. Rodak, O., Mrozowska M., Rusak, A., Gomułkiewicz, A., Piotrowska, A., Olbromski, M., Podhorska-Okołów, M., Ugorski, M., Dzięgiel, P. *Targeting SOX18 Transcription Factor Activity by Small-Molecule Inhibitor Sm4 in Non-Small Lung Cancer Cell Lines*. Int J Mol Sci. 2023 Jul 11; 24(14):11316. ………………………..………… 57

**4. PODSUMOWANIE I WNIOSKI** ………………………………..….. 75

**5. ZAŁĄCZNIKI** …………………………………………...…..…… 77

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## STRESZCZENIE

Badanie aktywności czynników transkrypcyjnych w transformacji nowotworowej jest niezmiernie istotne. Zmiany w ich ekspresji prowadzą do przeprogramowania mechanizmów molekularnych, kierujących procesami życiowymi komórek. Między innymi, wzmożona aktywność czynników transkrypcyjnych może prowadzić do patologicznego podtrzymania aktywności cyklu komórkowego, pomimo współistniejącego stresu komórkowego. Ze względu na nie w pełni poznaną strukturę czynników transkrypcyjnych oraz ich złożone interakcje, opracowanie ich inhibitorów stanowi duże wyzwanie. Dotychczasowe badania koncentrowały się jedynie na modulacji ekspresji genów. Jednakże, w ostatnich latach opracowano inhibitory specyficzne dla poszczególnych czynników transkrypcyjnych, czego przykładem jest inhibitor Sm4, który hamuje aktywność transkrypcyjną czynnika SOX18.

SOX18, białko należące do rodziny SOX (ang. sex determining region Y-related high-mobility group box), pełni kluczową rolę w regulacji transkrypcji podczas rozwoju zarodkowego. SOX18, wraz z SOX7 i SOX17, tworzą podgrupę białek SOXF, odpowiedzialną za rozwój układu sercowo-naczyniowego, limfangiogenezę i różnicowanie komórek krwi. Ostatnie doniesienia wskazują także na ich udział w gojeniu ran, neowaskularyzacji i modulacji bariery śródbłonkowej. Białka SOXF, a zwłaszcza SOX18, zyskały uwagę ze względu na istotne zmiany ekspresji w komórkach nowotworowych, które powiązano z ich aktywnością w procesach naprawczych tkanek. W przeciwieństwie do pozostałych białek SOXF, SOX18 prezentuje unikalną zdolność do formowania homodimeru, co może przyczyniać się do jego onkogennej natury. Udowodniono, że SOX18 promuje migrację, inwazję i proliferację w wielu typach nowotworów. Z kolei badanie, dotyczące raka tarczycy wskazało, iż pełni rolę przeciwnowotworową, ujawniając złożoną aktywność białka SOX18 zależną od molekularnego kontekstu.

Choć udział białka SOX18 w transformacji nowotworowej czyni go potencjalnym celem terapeutycznym, to jego rola w patogenezie niedrobnokomórkowego raka płuc (NDRP) nie została wyjaśniona. NDRP jest najczęściej diagnozowaną chorobą nowotworową na świecie. Podczas gdy globalne tendencje w zachorowalności na NDRP zaczęły spadać, w poszczególnych rejonach świata obserwujemy znaczne zróżnicowanie wskaźników występowania, co ściśle wiąże się z poziomem edukacji i rozwoju ekonomicznego. Ponadto, NDRP cechuje się jednym z najwyższych wskaźników śmiertelności na świecie. Skutkiem czego jest rosnąca liczba badań naukowych, które umożliwiają opracowanie nowych

strategii diagnostycznych oraz terapeutycznych. Uaktualniona klasyfikacja histopatologiczna, genotypowanie komórek guza, czy wprowadzenie terapii celowanej dla zaawansowanych stadiów choroby, istotnie przyczyniły się do wzrostu wskaźników prognostycznych w NDRP. Szczególnie ważny jest trend medycyny spersonalizowanej, który skupia się na rozpoznawaniu znanych mutacji genetycznych w komórkach raka płuc, nazywanych „mutacjami kierującymi" transformacją nowotworową. Rozpoznanie mutacji pozwala na zastosowanie terapii inhibitorami, specyficznymi dla nieprawidłowo funkcjonujących białek. Rosnąca grupa zatwierdzonych leków dla terapii celowanej w NDRP umożliwia terapeutyczne modulowanie następujących białek: rodziny EGFR (ERBB-1, ERBB-2), ALK, ROS1, MET, RET, NTRK i RAF. Niemniej jednak, wciąż u znacznej części chorych nie stwierdza się obecności mutacji powyższych białek, pozostawiając ich bez możliwości skorzystania z terapii celowanej. Stąd, tak ważne jest poszukiwanie nowych celów terapeutycznych, czego przykładem są badania nad oceną antyproliferacyjnego potencjału inhibitora SOX18 w raku płuc.

W niniejszej pracy doktorskiej zbadano wpływ Sm4 - inhibitora białka SOX18, na regulację cyklu komórkowego w liniach komórkowych niedrobnokomórkowego raka płuc, tj. LXF-289 (gruczolakoraka) SK-MES-1 (raka płaskonabłokowego), oraz linii fibroblastów płucnych IMR-90. Ocenie podlegała cytotoksyczność związku Sm4, wykonana metodą MTT. Następnie przeprowadzono doświadczenia, w których komórki otrzymywały inhibitor w stężeniu 10 µM lub 20 µM przez 72h. Zmiany cyklu komórkowego zostały ocenione poprzez znakowanie DNA jodkiem propidyny oraz detekcję komórek z użyciem cytometru przepływowego. W celu potwierdzenia uzyskanych wyników, wykonano ocenę ekspresji cyklin A1, D, E na poziomie mRNA oraz białka, odpowiednio technikami q-PCR oraz Western blot. Następnie, wykorzystując tożsame techniki biologii molekularnej określono poziom ekspresji genów oraz białek grupy SOXF oraz białka p21. Dla potwierdzenia uzyskanych wyników ocenie poddano archiwalny materiał tkankowy z guzów płuc, w którym za pomocą metody immunohistochemicznej określono ekspresji białek SOX18 oraz p21.

Uzyskane wyniki potwierdziły, że Sm4 wykazuje działanie cytotoksyczne we wszystkich trzech liniach komórkowych. Inhibitor w stężeniu 20 µM wywołał akumulację komórek nowotworowych w fazie replikacji DNA (S), co zostało potwierdzone istotnie mniejszą ekspresją cyklin D oraz E. Analiza ekspresji białek SOXF wykazała, iż hamowanie aktywności białka SOX18, nie indukuje wzrostu stężenia białek SOX7 lub SOX17. Pomimo

braku znaczących zmian w ekspresji białek, Sm4 spowodował istotne zwiększenie ekspresji genu *SOX17*, co częściowo podtrzymuje tezę o silnym powiązaniu mechanizmów transkrypcyjnych tych białek. Jednakże, prawdopodobnie w wyniku procesów na poziomie epigenetycznym, zwiększony poziom mRNA *SOX17*, nie przekłada się na zwiększony poziom białka. Poszukiwania białka odpowiedzialnego za hamowanie cyklu komórkowego wywołanego inhibitorem Sm4, wykazały aktywację ekspresji genu *CDKN1A* oraz konsekwentnie, istotnie zwiększoną ekspresję kodowanego przez niego białka – p21. Inhibitor kinaz cyklinozależnych 1A (p21) jest kluczowym białkiem regulującym przejście z fazy S do G2/M. Zatem, uzyskane wyniki sugerują złożoną interakcję między SOX18 a p21 w kontekście raka płuc. Ostatecznym potwierdzeniem związku między białkami była analiza materiału klinicznego, która wykazała dodatnią korelację między nasileniem ekspresji cytoplazmatycznej SOX18, która może świadczyć o braku aktywności białka, a obecnością p21 w jądrze komórkowym.

Przedstawione w pracy wyniki wskazują na potencjał Sm4 jako inhibitora cyklu komórkowego komórek nowotworowych poprzez pośrednią aktywację białka p21. Niemniej jednak, należy podkreślić, iż konieczna jest kontynuacja badań, by w pełni zrozumieć związek między SOX18 a p21 w raku płuc, jak również zbadać potencjał terapeutyczny hamowania SOX18.

## SUMMARY

Investigating transcription factor activity is essential due to their varying expression in pathological conditions, such as cancer, where altered molecular networks sustain cell divisions under stress. Developing pharmacological TF inhibitors is challenging due to not fully discovered structure and their complex interactions. Previous studies have focused on gene expression modulation, but recent research has explored TF-specific inhibitors. For instance, the Sm4 inhibitor effectively disrupts SOX18's transcriptional activity.

SOX18, a member of the SOX family (sex determining region Y-related high-mobility group box), plays a pivotal role in transcriptional regulation during embryogenesis. SOX18, along with SOX7 and SOX17, forms the SOXF subgroup responsible for cardiovascular development, lymphangiogenesis, and blood cell differentiation. Emerging evidence indicates their involvement in wound healing, neovascularisation, and endothelial barrier modulation. In cancer research, SOXF proteins, particularly SOX18, have gained attention due to their altered expression levels in cancer cells which aligns with their activity during tissue repairment processes. Unlike other SOXF proteins, SOX18 presents unique mode of action as a homodimer, which may contribute to its tumorigenic role. SOX18 exhibits an oncogenic role in promoting migration, invasion, and proliferation across diverse cancer types. However, a study on thyroid cancer cell lines indicated its role as a tumor suppressor, revealing its complex context-dependent activity.

While the activity of SOX18 in carcinogenesis underscores it as a potential cancer therapeutic target, its transcriptional regulation mechanisms in non-small cell lung cancer (NSCLC) are not yet fully understood. NSCLC is the most frequently diagnosed cancer in the world. When the global trends in NSCLC incidence have started to decline, we can observe region-dependent differences related to the education and the economic level. Moreover, NSCLC presents one of the highest mortality worldwide. To overcome this issue, an increasing understanding of NSCLC biology provided new diagnostic and therapeutic strategies. The reorganization of histopathological classification, tumor genotyping, or introduction of targeted therapy for advanced states of the disease are relevant in increasing prognostic factors. Precision medicine is focused on the recognition of a genetic mutation in lung cancer cells called “driver mutation” to provide a variety of specific inhibitors of improperly functioning proteins. Growing group of approved drugs for targeted therapy in NSCLC currently allows the following mutated proteins to be modulated: EGFR

family (ERBB-1, ERBB-2), ALK, ROS1, MET, RET, NTRK, and RAF. Nevertheless, treatable mutations are not being detected in a substantial portion of NSCLC patients, leaving them without the possibility of targeted treatment. Therefore, it is of utmost importance to investigate novel therapeutic targets, such as study on antiproliferative potential of SOX18 inhibitor in NSCLC treatment.

In this doctoral thesis, the impact of Sm4, an inhibitor of the SOX18, on the cell cycle regulation in non-small cell lung cancer cell lines was investigated. These cell lines included LXF-289 (adenocarcinoma), SK-MES-1 (squamous cell carcinoma), and the IMR-90 lung fibroblast cell line. The cytotoxicity of the Sm4 compound was assessed using the MTT method. Subsequently, experiments were conducted in which cells were treated with the inhibitor at concentrations of 10 μM or 20 μM for 72 hours. Cell cycle disturbances were examined through DNA labeling with propidium iodide and cell detection using flow cytometry. To confirm the obtained results, the expression of cyclins A1, D, and E was evaluated at the mRNA and protein levels using respectively q-PCR and Western blot techniques. Subsequently, employing identical molecular biology techniques, the expression levels of SOXF group genes and proteins, as well as the p21, were determined. To validate the obtained results, tissue material from lung tumors was subjected to immunohistochemical methods to determine the expression of SOX18 and p21 proteins.

The obtained results confirmed that Sm4 exhibits cytotoxic activity in all three cell lines. Treatment with the Sm4 inhibitor resulted in cell accumulation in the S phase, as confirmed by the cyclin expression level results. The expression results of SOXF proteins allowed us to reject the research hypothesis that inhibiting the activity of SOX18 would induce the expression of SOX7 or SOX17 proteins, which are known for their anti-proliferative properties. Despite the lack of significant changes in the expression of SOX7 or SOX17 proteins, Sm4 led to a significant increase in the expression of the SOX17 gene, partially supporting the hypothesis of transcriptional interactions of these proteins. However, likely due to epigenetic processes, the increased level of SOX17 mRNA did not translate into an increased expression level of the protein. Further experiments revealed the activation of *CDKN1A* gene expression and consequently, a significantly increased protein expression - p21, as the protein responsible for inhibiting the cell cycle induced by the Sm4 inhibitor. The Cyclin-Dependent Kinase Inhibitor 1A (p21) is a key protein that regulates the transition from the S phase to the G2/M phase. Therefore, the obtained results suggest a complex interaction between SOX18 and p21 in the context of lung cancer. The ultimate confirmation

of the relationship between the proteins was the analysis of clinical material, which revealed a positive correlation between the intensity of cytoplasmic SOX18 expression, indicating protein inactivity, and the presence of p21 in the cell nucleus.

The results presented in this study indicate the potential of Sm4 as an inhibitor of the cell cycle and the growth of cancer cells through indirect activation of the p21 protein. However, it should be emphasized that further research is necessary to fully understand the relationship between SOX18 and p21 in lung cancer, as well as to explore the therapeutic potential of inhibiting SOX18 in lung cancer treatment.

# 1. WSTĘP

## 1.1. Nowotwory płuc

Według statystyk epidemiologicznych, rak płuc jest najczęściej diagnozowaną chorobą nowotworową na świecie [1]. Na przestrzeni ostatnich dekad obserwuje się znaczny postęp, dotyczący stanu wiedzy, możliwości diagnostycznych, oraz skuteczności terapii pacjentów z niedrobnokomórkowym rakiem płuc (NDRP, ang. NSCLC, Non Small Cell Lung Cancer). Rozwój ten w znacznym stopniu wynika ze wzrastającej specyficzności oraz przepustowości technik badawczych, które umożliwiają zrozumienie mechanizmów kierujących kancerogenezą na poziomie molekularnym. Zrozumienie biologii nowotworów płuc na poziomie molekularnym pozwala dziś na uprawianie medycyny personalizowanej, w której decyzję terapeutyczną uzależnia się od detekcji zaburzeń molekularnych w komórkach nowotworowych, obecnych u konkretnego pacjenta. Tym samym, rozpoczęła się era terapii celowanej oraz immunoterapii, które skupiają się na hamowaniu aktywności białek, wykazujących działanie onkogenne. Dziś znamy wiele kluczowych białek lub ich mutantów, których obecność w komórkach guza, ma istotny wpływ na rozwój choroby oraz skuteczność terapii [2]. Niemniej jednak, wciąż u znacznej części pacjentów nie rozpoznaje się obecności mutacji genów poznanych białek, co stanowi o potrzebie dalszych badań w celu poszukiwania nowych celów terapii personalizowanej.

Istotną „rewolucją" jest możliwość sekwencjonowania genomu komórek nowotworowych, jako stały element diagnostyczny u pacjentów z rakiem płuc [3]. Także, dotychczasowa klasyfikacja histopatologiczna raka płuc została zaktualizowana, dzięki czemu rozpoznanie podtypu histopatologicznego stało się kluczowe dla dalszej diagnostyki oraz wyborze schematu leczenia [4]. Ponadto, diagnoza NRDP oraz rozpoznanie jego podtypu stanowi również czynnik prognostyczny i predykcyjny. Oba wskaźniki mają duże znaczenie, ponieważ jedynie 2-18% chorych na NRDP przeżywa 5 lat od postawienia rozpoznania [5].

Poza późnym wykrywaniem choroby, istotnym problemem z klinicznego punktu widzenia jest dynamicznie rozwijająca się heterogenność guzów, przede wszystkim pod względem mnogości odmian genetycznych (genotypów) populacji komórek pojedynczego guza oraz ich wariancji na przestrzeni czasu [6]. Obecność subpopulacji komórek o zmiennych cechach molekularnych komplikuje wybór właściwego schematu leczenia. Co więcej, lista znanych genotypów raka płuc wzbogaca się o nowo odkrywane warianty.

Zatem, jednym z największych wyzwań dla nowoczesnej biologii nowotworów oraz medycyny stała się dynamika transformacji nowotworowej komórek budujących płuca.

## 1.2. Rola czynników transkrypcyjnych w rozwoju i progresji nowotworów płuc

Czynniki transkrypcyjne to białka posiadające domeny wiążące się z DNA, dzięki którym uczestniczą w wiązaniu promotora, regulującego ekspresją przynależnego genu [7]. Czynniki te mogą funkcjonować samodzielnie lub jako część kompleksu białkowego, niekiedy też tworząc homo- lub heterodimer. Każda z tych zmian konformacji zmienia specyficzność wiązania do genów docelowych [8]. Ekspresja poszczególnych czynników transkrypcyjnych jest zwykle specyficzna dla typu oraz stanu komórki, kierując jej funkcją w organizmie.

Czynniki transkrypcyjne stanowią unikalną klasę białek, które programują ekspresję złożonych zestawów genów, odpowiedzialnych za szereg procesów komórkowych [9]. Działają jak molekularne włączniki programów różnicowania, kontroli podziału komórki, czy inicjacji śmierci komórkowej. Oczywistym stało się, że czynniki transkrypcyjne mogą być również odpowiedzialne za patologiczne wzbudzanie ekspresji genów, wspomagających kancerogenezę [7–9]. Aktywność czynników transkrypcyjnych ulega istotnym zmianom w licznych typach nowotworów poprzez wzmożoną niestabilność genetyczną, tj.: translokacje chromosomowe, amplifikacje lub delecje genów, punktowe mutacje, lecz również w wyniku zmian podstawowych szlaków sygnałowych [9].

W ostatniej dekadzie, obserwujemy wzrost zainteresowania rolą czynników transkrypcyjnych w mechanizmach zmian ekspresji genów komórek nowotworowych, co przekłada się na rozwój interesujących opcji terapeutycznych [10,11]. Liczne badania doprowadziły do wyjaśnienia roli niektórych czynników transkrypcyjnych, w tym białek szczególnie zaangażowanych w rozwój nowotworów płuc. Do tej grupy zaliczane są między innymi rodziny białek:

1. Nuclear factor kappa B (NF-κB) - odgrywają kluczową rolę w procesie zapalnym, oraz odpowiedzi immunologicznej. W nowotworach płuc, jak również w stanach przednowotworowych, obserwuje się wzmożoną aktywność szlaków regulowanych przez NF-κB, co przyczynia się do inicjacji nowotworzenia oraz zdolności inwazji, a nawet rozwoju oporności komórek rakowych na stosowane terapie [12].
2. Signal transducer and activator of transcription (STAT) - biorą udział w przekazywaniu sygnałów od cytokin i czynników wzrostu. Podwyższony poziom

aktywnej ufosforylowanej formy białka STAT3, występujący w komórkach NDRP, stymulując ekspresję genów kluczowych w promowaniu proliferacji oraz przeżycia komórek nowotworowych, poprzez obniżenie zdolności układu immunologicznego do rozpoznawania komórek patologicznych [13].

3. Rodzina białek p53 pełni rolę w utrzymaniu stabilności genomu oraz regulacji cyklu komórkowego. Szczególnym zainteresowaniem cieszy się białko p53, nazywane „strażnikiem genomu", gdyż mutacje punktowe lub utrata heterogeniczności alleli genu *TP53* to najczęściej obserwowane zmiany w komórkach nowotworów płuc. Z kolei białko p63 stało się istotnym biomarkerem w diagnostyce płasko-nabłonkowego raka płuc, ponieważ obserwuje się zupełną utratę ekspresji p63 w tym typie histologicznym [14].
4. Hypoxia-inducible factor (HIF) jest podrodziną białek, funkcjonującą jak sensor zawartości tlenu w cytoplazmie komórek. Silna hipoksja występująca w guzach płuc aktywuje czynnik HIF-1$\alpha$, który inicjuje ekspresję czynników wzrostu naczyń (np. VEGF), jak również wykazano korelacje z ekspresją innych czynników wzrostu (np. EGFR) [15].

Zrozumienie funkcji i nieprawidłowej regulacji wspomnianych rodzin czynników transkrypcyjnych daje wgląd w mechanizmy molekularne raka płuc i może ułatwić rozwój nowych terapii skierowanych na modulację ich aktywności.

W odniesieniu do NDRP warto również wspomnieć o mniej poznanej rodzinie czynników transkrypcyjnych SOX (ang. sex-determining region Y (SRY)-related high-mobility group (HMG) box), które zaliczane są do kluczowych białek kierujących organogenezą i różnicowaniem się komórek w wyspecjalizowane tkanki, w tym nabłonka oddechowego [16]. Wykazano, że białka rodziny SOX oprócz rozwoju zarodkowego, aktywne są także w patogenezie różnych chorób [17].

### 1.3. Czynniki transkrypcyjne z rodziny SOX

Rodzinę SOX tworzy 20 strukturalnie homologicznych białek, które zostały usystematyzowane względem homologii strukturalnej w 8 podgrup (SOXA-H). Cechą wspólną, która warunkuje ich aktywność transkrypcyjną – wiązania się z promotorami genów, jest domena HMG (ang. High Mobility Group) [18].

Białka rodziny SOX odgrywają kluczową rolę na różnych etapach embriogenezy. Są to czynniki transkrypcyjne, które regulują ekspresję genów niezbędnych dla właściwego rozwoju komórek macierzystych oraz późniejszego różnicowania się komórek w wyspecjalizowane struktury tkankowe. W procesie rozwoju zarodkowego, białka SOX kontrolują wiele istotnych procesów, takich jak determinacji fenotypu płciowego (białko SRY), rozwoju układu sercowo-naczyniowego (białka podgrupy SOXF), czy układu nerwowego (podgrupy B, C, D, E) [19–21]. Choć poziom ekspresji czynników SOX w wyspecjalizowanych tkankach spada, to obserwowano ich aktywację podczas gojenia się ran, procesów zapalnych, jak również transformacji nowotworowej [18]. Stąd, poznanie mechanizmów działania białek rodziny SOX w procesach patologicznych ma kluczowe znaczenie dla rozwoju oraz implementacji nowych strategii terapeutycznych [19].

### 1.3.1. Podgrupa czynników transkrypcyjnych SOXF

Grupę białek SOXF stanowią trzy białka, tj. SOX7, SOX17 oraz SOX18. Geny kodujące białka SOXF, zostały zakwalifikowane do wspólnej grupy ze względu na wysoką konserwatywność ewolucyjną sekwencji oraz położenia intronu domeny HMG. Ponadto, domena HMG cechuje się bardzo wysoką homologicznością sekwencji pomiędzy białkami SOXF, odróżniając je od pozostałych czynników transkrypcyjnych rodziny SOX [18].

Białka SOXF należą do kluczowych czynników regulujących rozwój embrionalny [17]. Podczas gastrulacji, SOX7 indukuje różnicowanie się endodermy, wewnętrznego listka zarodkowego, z którego rozwijają się: układ pokarmowy, moczowy, endokrynny, a także układ oddechowy [22]. Co ciekawe, także w późniejszym etapie rozwoju zarodkowego, SOX7, SOX17 i SOX18 współdziałając, kierują rozwojem układu sercowo-naczyniowego, limfangiogenezą oraz hematopoezą [23].

### 1.3.2. Białka SOXF w karcynogenezie nabłonka oddechowego

Białka SOXF można podzielić na dwie podgrupy, prezentujące przeciwstawną aktywność w transformacji nowotworowej. Funkcje supresorowe przypisuje się białkom SOX7 oraz SOX17, podczas gdy białko SOX18 wykazuje aktywność promującą kancerogenezę [17]. Wyniki licznych badań, uzyskanych z wykorzystaniem nadekspresji lub wyciszenia ekspresji *SOX7* w liniach komórkowych NDRP, wykazały jego aktywność proapoptotyczną, hamowanie proliferacji oraz zdolności komórek do migracji i inwazji [24–

28]. Zbliżonym profilem aktywności supresorowej, cechuje się białko SOX17. W przypadku NDRP jego ekspresja również ulega obniżeniu, ponieważ w przebiegu transformacji nowotworowej rośnie ilość zmetylowanego DNA w sekwencji promotorowej genu *SOX17*, co zostało potwierdzone w materiale klinicznym [29–31]. Obniżona ekspresja, a co za tym idzie aktywność białka SOX17 w komórkach NDRP, skutkuje utratą prawidło funkcjonującej blokady, zastąpionej m. in. niekontrolowanym pobudzeniem do podziału komórkowego [32].

Odmienny charakter prezentuje białko SOX18, które w patogenezie raka płuc, a także innych typów nowotworów, odgrywa rolę protoonkogenu. Immunodetekcja białka SOX18 wykazała zróżnicowane nasilenie ekspresji SOX18 w guzach płuc, od słabej do wysokiej. Warto jednak zauważyć, że podwyższona ekspresja cytoplazmatyczna czynnika SOX18 okazała się markerem złego rokowania [33]. Badania na poziomie epigenetycznym, również przyczyniły się do lepszego zrozumienia funkcji czynnika transkrypcyjnego SOX18 w kancerogenezie płuc. Wykazano, m. in. że w NDRP występują: wysoki stopień zmetylowania promotora genu *SOX18,* niski poziom mRNA *SOX18* oraz wysokie stężenie microRNA hamujących translację SOX18. [34,35]. Natomiast brak dostępnych danych, dotyczących mechanizmów działania SOX18 w komórkach nowotworowych płuc, pozostawia swojego rodzaju niszę do eksploracji potencjału białka SOX18 jako celu terapeutycznego w guzach NDRP.

### 1.3.3. SOX18 - potencjalny czynnik stymulujący proliferację komórek nowotworowych

W obszarze badań aktywności białka SOX18 w kancerogenezie nieliczne prace dotyczą raka płuc. Jednakże w kontekście nowotworów innego pochodzenia, mechanizmy działania czynnika SOX18 zostały dość obszernie opisane, a wyniki badań głównie dotyczą doświadczeń przeprowadzonych w modelu *in vitro*. Nasilenie ekspresji białka SOX18, obserwowano w nowotworach różnego pochodzenia, w tym pęcherza moczowego, gruczołu piersiowego, szyjki macicy, jelita grubego, krtani, wątroby, nerek, kostniakomięsaka, trzustki oraz gruczołu krokowego [36–39]. Co więcej, badania przeprowadzone na nowotworowych liniach komórkowych sprecyzowały, iż redukcja ekspresji SOX18 hamuje ich proliferację, zdolność do migracji i inwazji [40,41]. Tym samym, SOX18 jest istotnym elementem mechanizmów molekularnych, które stymulują progresję nowotworową. Jednakże, badanie przeprowadzone na liniach komórkowych raka tarczycy wykazało, że SOX18 działa jak supresor nowotworowy, co wskazuje na złożoność jego

aktywności, która zależy od specyfiki mechanizmów molekularnych obecnych w konkretnym nowotworze [42]. Ponadto, unikalna zdolność do formowania homodimerów może być kluczowym czynnikiem odmiennej natury SOX18, w porównaniu do SOX7 i SOX17 [43]. W świetle powyższych doniesień, białko SOX18 stało się atrakcyjnym celem badań, dotyczących biologii nowotworów, a w szczególności wyjaśnienia roli embrionalnych czynników transkrypcyjnych w inicjacji i progresji nowotworów.

### 1.4 Sm4 - inhibitor homodimeryzacji SOX18

Przetrwanie komórek nowotworowych zależy od efektywności przeprogramowania mechanizmów molekularnych, tak by aktywować podział komórkowy, nawet w niekorzystnych warunkach hipoksji czy niedostatku energii [44]. Stąd, poznanie aktywności konkretnych czynników transkrypcyjnych w chorobie nowotworowej, ma tak istotne znaczenie dla rozwoju onkologii. Jednak opracowanie związków farmakologicznych mających na celu wyhamowanie aktywności SOX18 jest bardzo wymagające. Aktywne formy białka lokalizują się pod otoczką jądrową, a ze względu na budowę, ich trójwymiarowa struktura nie została w pełni zobrazowana. Ponadto, niewiele wiadomo na temat kompletnego spektrum interakcji SOX18 i wynikających z nich odpowiedzi molekularnych, co dodatkowo utrudnia wyznaczanie kierunków badań [45]. Stąd, dotychczas prowadzone doświadczenia skupiały się głównie na modulacji ekspresji genów białek grupy SOXF, takich jak wyciszanie lub nadekspresja genu *SOX18* [46,47]. Uzyskane wyniki, choć wartościowe, nie wyjaśniają powiązań pomiędzy poszczególną aktywnością transkrypcyjną (indukowaną homodimeryzacją, lub wiązaniem się z kompleksem białkowym) a wynikającym z niej efektem molekularnym (wzbudzenie szklaków sygnałowych, hamowanie cyklu komórkowego).

Jednakże, Francois i wsp. wyselekcjonowali Sm4 (ang. small molecule 4) - specyficzny drobnocząsteczkowy inhibitor, który hamuje zdolność białka SOX18 do homodimeryzacji [45]. Specyficzność inhibitora została scharakteryzowana przez szereg biofizycznych metod, jak również oceniono jego wstępną cytotoksyczność z wykorzystaniem linii komórkowej prawidłowych fibroblastów nerki [45]. Ponadto, potencjał cząsteczki Sm4 został oceniony *in vivo*, w modelu mysim, poprzez inokulację komórek (4T1.2) raka gruczołu mlekowego oraz ocenę wzrostu oraz innych cech guzów, po doustnym leczeniu inhibitorem. Pomimo braku istotnego wpływu na rozmiar guza, a zatem brak antyproliferacyjnego działania, w doświadczeniu wykazano znaczne ograniczenie neowaskularyzacji, jak również zdolności

do tworzenia przerzutów [48]. Niemniej jednak, powyżej opisane wyniki należy interpretować jako specyficzne dla danego typu nowotworu, z uwagi na różnorodność odpowiedzi biologicznych w aspekcie białka SOX18, które silnie zależą od specyfiki mechanizmów molekularnych danej populacji komórek nowotworowych. W rezultacie istnieje potrzeba prowadzenia dalszych, szeroko zakrojonych badań, dotyczących różnych typów nowotworów z wykorzystaniem opracowanego inhibitora – cząsteczki Sm4.

W odpowiedzi na przedstawione zagadnienie, powstała niniejsza praca doktorska, w ramach której przeprowadzono badania nad antyproliferacyjnym potencjałem inhibitora Sm4 oraz oceniono mechanizmy działania na poziomie molekularnym, wywołane zastosowaniem inhibitora Sm4 w hodowli linii komórkowych niedrobnokomórkowego raka płuc.

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## 2. ZAŁOŻENIA I CELE PRACY DOKTORSKIEJ

Niniejsza rozprawa doktorska zakładała opracowanie przeglądu literatury dotyczącego aktualnej wiedzy o NDRP. W związku z powyższym, powstał artykuł przeglądowy, pt. „Current Landscape of Non-Small Cell Lung Cancer: Epidemiology, Histological Classification, Targeted Therapies, and Immunotherapy", w którym szczegółowo omówiłam kwestie związane z obecną sytuacją epidemiologiczną choroby, klasyfikację histopatologiczną, biologią rozwoju oraz heterogennością raka płuc. Ponadto artykuł stanowi podsumowanie aktualnych osiągnieć badań molekularnych, tj. zatwierdzonych inhibitorów oraz przeciwciał stosowanych w terapii celowanej oraz immunoterapii NDRP.

Drugą część pracy doktorskiej stanowi praca oryginalna, pt. „Targeting SOX18 Transcription Factor Activity by Small-Molecule Inhibitor Sm4 in Non-Small Lung Cancer Cell Lines". Wyniki badań zostały uzyskane oraz statystycznie opracowanie w ramach następujących założeń oraz celów pracy doktorskiej:

1. Określenie wskaźnika IC50 (stężenie hamujące w 50 % funkcje życiowe komórek) inhibitora białka SOX18 – Sm4 w liniach komórkowych niedrobnokomórkowego raka płuc.

2. Określenie potencjału inhibitora Sm4 jako substancji antynowotworowej poprzez ocenę jego wpływu na cykl podziału komórkowego.

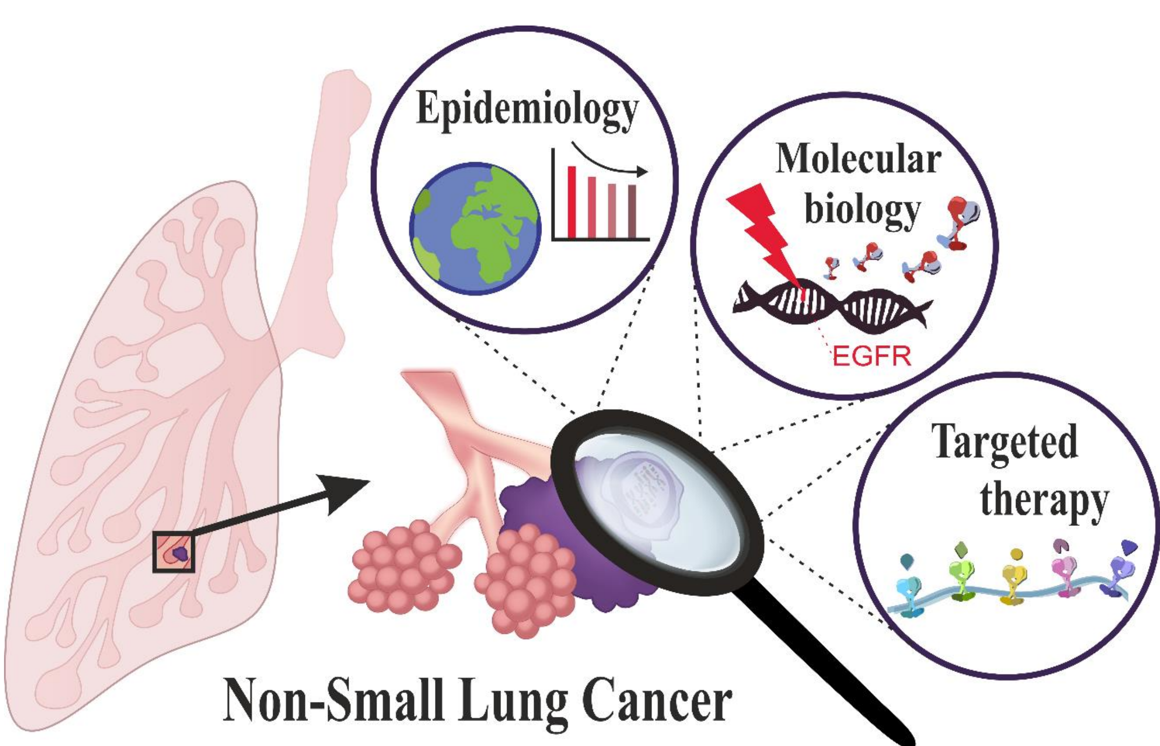
3. Określenie zmian ekspresji wybranych białek, które wynikają z zastosowania inhibitora Sm4 w liniach komórkowych, w tym białek regulujących proces podziału komórkowego.

4. Ocena odpowiedzi znanych elementów aparatu molekularnego, powiązanych z mechanizmami działania białka SOX18. Ewaluacja wpływu zahamowania aktywnego białka SOX18 na ekspresję genów i białek grupy SOXF – SOX7 oraz SOX17.

## 3. PUBLIKACJE

I. Publikacja przeglądowa:

Rodak, O., Peris-Díaz, M., Olbromski, M., Podhorska-Okołów, M., Dzięgiel, P. Current Landscape of Non-Small Cell Lung Cancer: Epidemiology, Histological Classification, Targeted Therapies, and Immunotherapy. *Cancers.* 2021 Sep 20; 13(18):4705.



*Review*

# Current Landscape of Non-Small Cell Lung Cancer: Epidemiology, Histological Classification, Targeted Therapies, and Immunotherapy

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**Simple Summary:** The abundance and the dynamic of the studies on NSCLC require frequent summaries of the current achievements in the field. In our review, we aimed to update the status of knowledge about NSCLC, combining its epidemiology, classification novelties, tumor molecular basis, and two of the most promising approaches in cancer treatment: targeted therapy and immunotherapy.

**Abstract:** Non-small cell lung cancer (NSCLC) is a subtype of the most frequently diagnosed cancer in the world. Its epidemiology depends not only on tobacco exposition but also air quality. While the global trends in NSCLC incidence have started to decline, we can observe region-dependent differences related to the education and the economic level of the patients. Due to an increasing understanding of NSCLC biology, new diagnostic and therapeutic strategies have been developed, such as the reorganization of histopathological classification or tumor genotyping. Precision medicine is focused on the recognition of a genetic mutation in lung cancer cells called "driver mutation" to provide a variety of specific inhibitors of improperly functioning proteins. A rapidly growing group of approved drugs for targeted therapy in NSCLC currently allows the following mutated proteins to be treated: EGFR family (ERBB-1, ERBB-2), ALK, ROS1, MET, RET, NTRK, and RAF. Nevertheless, one of the most frequent NSCLC molecular sub-types remains without successful treatment: the K-Ras protein. In this review, we discuss the current NSCLC landscape treatment focusing on targeted therapy and immunotherapy, including first- and second-line monotherapies, immune checkpoint inhibitors with chemotherapy treatment, and approved predictive biomarkers.

**Keywords:** lung cancer; non-small cell lung cancer; epidemiology; histopathology; cancer biology; targeted therapy; immunotherapy; predictive biomarkers

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## 1. Introduction

The status of non-small cell lung cancer (NSCLC) is a dynamically evolving landscape. Over the past decades, the advancement of knowledge, the discovery of new drugs, and the diagnostic possibilities have grown exponentially, setting new standards in oncology (Figure 1). Such improvement resulting from continuous technological development allows us to get insights into the molecular mechanisms of cancer cells. NSCLC, targeted by hundreds of research groups, is the infamous winner of the world's epidemiological statistics on cancer [1,2]. The growing number of patients over the last decade has demanded putting more effort into cancer research, which has resulted in a better understanding

of the biology of lung cancer. This knowledge allows us today to practice personalized medicine in which the therapeutic decision depends on the characteristics of the cancer of individual patients.

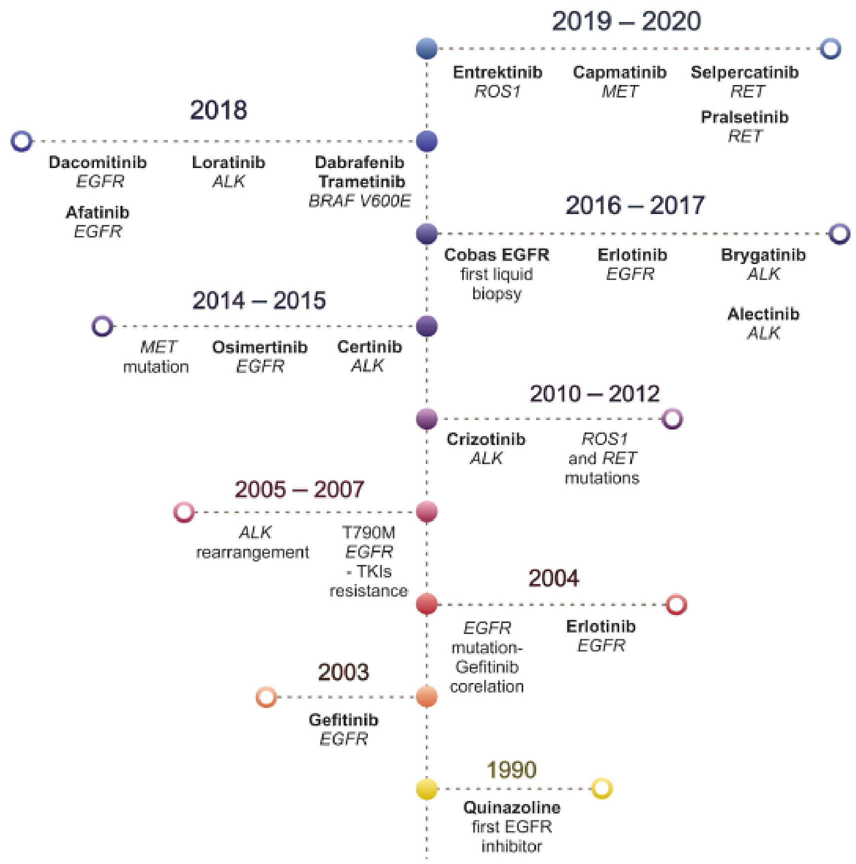


**Figure 1.** Development of targeted therapy in NSCLC. Over the last decade, there has been an acceleration in the emergence of new inhibitors approved in NSCLC targeted therapy. The approval dates of the inhibitors in the treatment of NSCLC refer to the approvals issued by the Food and Drug Administration (FDA), Silver Spring, MA, USA.

A breakthrough discovery in lung cancer pathogenesis solidly strengthened the trend of personalized medicine in the treatment algorithms. In 2004, almost simultaneously, two research groups published results confirming the correlation between the effectiveness of a drug (gefitinib) inhibiting the activity of a mutant receptor protein and the presence of mutations in the gene encoding this receptor in treated patients [3,4]. The increased knowledge on the epidemiology and the biology of key mutations has a significant impact on the effectiveness of selected therapy [5]. An important revolution in the clinical approach is the possibility of sequencing the genome of neoplastic cells as a permanent diagnostic element in patients with lung cancer [6–13]. Therefore, due to the achievements of genetics, the existing histopathological classification of lung cancer has been thoroughly rebuilt, adjusting the diagnosis to the current knowledge. Thanks to the introduced changes, a diagnosis of the histopathological subtype is a crucial step in diagnostic algorithms and selecting the treatment regimen [7,14,15]. Although the modifications changed the classification of lung cancers, the definition of NSCLC maintains its clinical significance. The diagnosis of NSCLC and the recognition of its subtype are also prognostic and predictive factors. Both

indicators are of great importance for patients since only 2–20% of patients with NSCLC survive five years from diagnosis [2,16,17]. The main issue is the tumor heterogeneity often observed. This phenomenon refers to the multitude of genetic variants occurring within a single tumor and their variance over time [18,19]. Constant differentiation of new molecular subpopulations complicates diagnostics and the choice of the appropriate treatment. Moreover, the list of known lung cancer genotypes continues to be fueled by newly discovered variants. Thus, the dynamic of lung cancer is one of the biggest challenges in modern biology and medicine [20]. A promising therapy emerged since 2010 when the clinical benefit of immunotherapy was demonstrated [21].

This review summarizes the recent advances in NSCLC and details our focus on epidemiology, the latest histopathological classification, lung cancer heterogeneity, targeted therapy, and immunotherapy. The treatment perspectives in targeted therapy of the best-known genotypes of NSCLC, the approved immunotherapies, and the predictive biomarkers are also reviewed.

## 2. Trends in Epidemiology

The most recent global report on the epidemiology of neoplastic disease states that lung cancer has the highest mortality among 36 cancer types considered, and it is the second most frequently diagnosed cancer type in the world [1,2]. In 2020, based on data from 185 countries, the approximate number of diagnosed cases was estimated at 2,206,771 (11.4% of all cancers), while mortality was 1,796,144 (18.0%) [2]. The mortality is associated with a high degree of malignancy and late diagnosis. As many as 65.33% of men diagnosed with lung cancer are in the advanced local stage (stage III) or present metastases (stage IV) [22,23]. Unfortunately, we still observe a lack of reliable markers for the early stage of the disease [24,25]. However, recent research focused on miRNAs (microRNAs), which have a potential diagnostic value. Their detection in combination with tomography shows a significant increase in the effectiveness of the diagnosis [26]. For example, a phase I/II biomarker study identified two potential miRNAs (miR-15b and miR-27b) that differentiated NSCLC patients from healthy controls with a specificity of 84%, a sensitivity of 100%, a negative predictive value of 100%, and a positive predictive value of 82% [27]. A meta-analysis showed that miR-210 and miR-21 could be used as a diagnostics tool for NSCLC [28]. A recent study generated 2588 miRNAs profiles from a large sample set. The authors identified the miR-17-3p as the best single miRNA for detecting lung cancer with a cross-validation score of 0.9087. Furthermore, the combination of miR-1268b and miR-6075 achieved the best accuracy in the discovery set (cross-validation score of 0.9904) [29].

Moreover, the age of the diagnosed patients is noteworthy. Reports consistently indicate that the incidence of lung cancer over 45 years of age increases dramatically [1,2,5,30]. Hence, specific and effective screening tests are of utmost importance for people over 50 years old.

Although trends in the global incidence continue to rise, the number of new lung cancer cases has been observed to increase or decrease depending on the region. It happens especially among men in highly developed countries such as the United Kingdom, the United States, Australia, New Zealand, Singapore, Germany, the Netherlands, Uruguay, and the Scandinavian countries. Additionally, in developing countries of Eastern Europe, a slow decline in the disease has been also observed [1,2]. During the 1980s in the United States, the lung cancer incidence level reached a plateau for men and has steadily declined [31]. However, the growing number of cases in women is alarming [22,32]. The trends in cigarette smoking shape the patterns of incidence rates observed in particular populations over the decades. In Central Europe, there is a significant gender discrepancy in the values of risk factors for developing lung cancer and mortality. The risk rates are, respectively, 6.29 and 5.67 in men, while in women, they are 1.52 and 1.20, respectively [1]. In men, the risk of disease or death is one of the highest in the world, reflecting the sensitivity to exposure to carcinogenic substances (including tobacco) in the population of

Central European men over decades of the last century. Although cigarette smoking remains the leading cause of lung cancer, statistics shows that 12% of people diagnosed with lung cancer have never smoked cigarettes [33]. Interestingly, higher frequency of EGFR mutations was genotyped in never smokers (42.5%) compared to current (4.9%) or former smokers (13.5%) [34,35]. Comparing the incidence of lung cancer in Chinese and French, the rates are 22.8 and 22.5 per 100,000 women, respectively. Although the incidence is at the same level, it is worth noting that the percentage of smokers is much lower among Chinese women than among French women [1]. In the case of China, high exposure to smoke from coal combustion is considered a factor. Thus, air pollution is a significant predisposition to lung cancer [5,36,37].

Important elements shaping the epidemiology of lung cancer are geographic and economic factors (Figure 2). The cumulative risk of death from lung cancer is not unequivocally followed by mortality value. In North America and Western Europe, the cumulative risks amount to 4.27 and 4.25, respectively, while in the rest of Europe, risk is estimated to be lower, ranging from 3.54 to 3.67 [2]. The advancement in the medical care undoubtedly affects the effectiveness of cancer treatment [38]. Hence, in North America, despite the highest risk of disease, the mortality rate is lower than in Western Europe or East Asia (2.64, 3.04, and 3.36, respectively) [2].

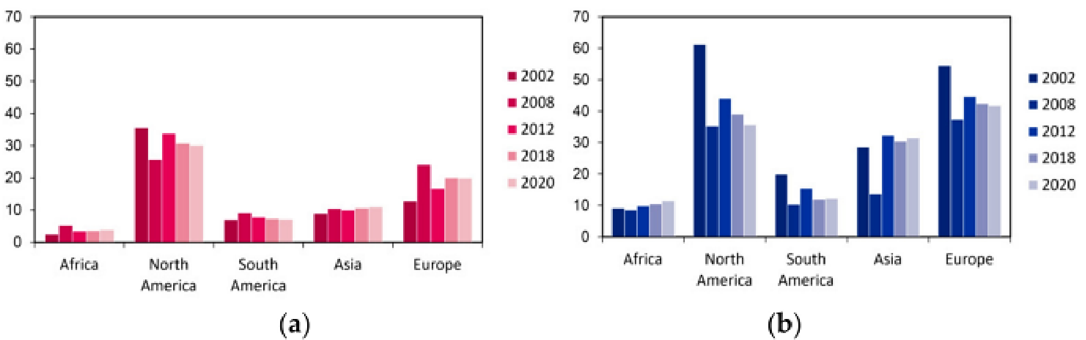


**Figure 2.** The number of people diagnosed with lung cancer per 100,000 inhabitants. (**a**) Incidence among women concerning world regions in 2002–2020; (**b**) Incidence among men concerning world regions in 2002–2020 [1,2,39–41].

To conclude, despite the local decreasing trends in morbidity and mortality, the incidence rate of lung cancer is continuously increasing worldwide (Figure 2). Therefore, there is a growing need for new solutions, starting from anti-smoking education, counteracting environmental pollution, and ending with the development of innovative diagnostic and therapeutic methods.

## 3. Advancements in Histopathological Classification

In 2015, the World Health Organization (WHO) published a new histological classification of lung cancer, which is a direct result of the achievements of molecular biology that are modeling the current clinical procedure [14]. Three main histological types were maintained: adenocarcinoma (AD), squamous cell carcinoma (SqCC), and neuroendocrine tumors. The other types distinguished were: large cell carcinoma (LCC), adenosquamous carcinoma, sarcomatoid carcinoma, and other unclassified cancers. In addition, the classification of proliferative changes in the lungs includes types of rare occurrence: salivary gland-type tumors, papillomas, adenomas, mesenchymal tumors, lymphohistiocytic tumors, tumors of ectopic origin, and metastases to the lung [42] (Table 1). The most important changes in the classification include (i) reorganization of the group of adenocarcinomas; (ii) restriction of features that classify lesions as large cell carcinoma; (iii) the distinction of a group of neuroendocrine hyperplasia; (iv) change of nomenclature of variants of squamous cell carcinoma [15].

**Table 1.** Comparison of previous and new histopathological classifications of lung cancer, published by the World Health Organization [42]. While NSCLC does not exist in histopathological classification, in general use, it comprises: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.

| Classification 2004 | | Classification 2015 | |
|---|---|---|---|
| **Adenocarcinoma** | Adenocarcinoma, mixed subtype<br>Acinar adenocarcinoma<br>Papillary adenocarcinoma<br>Bronchioloalveolar carcinoma<br>Solid adenocarcinoma with mucin production<br>Fetal adenocarcinoma<br>Mucinous cystadenocarcinoma<br>Mucinous ("colloid") carcinoma<br>Signet ring adenocarcinoma<br>Clear cell adenocarcinoma | **Adenocarcinoma** | Lepidic adenocarcinoma<br>Acinar adenocarcinoma<br>Papillary adenocarcinoma<br>Micropapillary adenocarcinoma<br>Solid adenocarcinoma<br>Invasive mucinous adenocarcinoma<br>Colloid adenocarcinoma<br>Fetal adenocarcinoma<br>Enteric adenocarcinoma<br>Minimally invasive adenocarcinoma<br>Preinvasive lesions: adenocarcinoma in situ |
| **Squamous cell carcinoma** | Papillary<br>Clear cell<br>Small cell<br>Basaloid | **Squamous cell carcinoma** | Keratinizing<br>Nonkeratinizing<br>Basaloid<br>Preinvasive lesions: Squamous cell carcinoma in situ |
| **Small cell carcinoma** | Combined small cell carcinoma | **Neuroendocrine tumors** | Small cell carcinoma<br>Large cell neuroendocrine carcinoma<br>Carcinoid tumors<br>Preinvasive lesion |
| **Large cell carcinoma** | Large cell neuroendocrine carcinoma<br>Combined large cell neuroendocrine carcinoma<br>Basaloid carcinoma | | |
| | Lymphoepithelioma-like carcinoma | **Large cell carcinoma** | |
| | Clear cell carcinoma | **Adenosquamous carcinoma** | |
| | Large cell carcinoma with rhabdoid phenotype | **Sarcomatoid carcinomas** | |
| **Adenosquamous carcinoma** | | **Other and Unclassified carcinomas** | |
| **Sarcomatoid carcinoma** | | **Salivary gland-type tumors** | |
| **Carcinoid tumor** | | **Papillomas** | |
| **Salivary gland tumors** | | **Adenomas** | |

The group of adenocarcinomas was systematized depending on the invasiveness of the lesions. They start from pre-invasive (including adenocarcinoma in situ) through minimally invasive and end with invasive lesions. Among the latter, many variants existed in the 2004 classification. Furthermore, the issue of poorly differentiated neoplastic lesions often diagnosed as large-cell carcinomas was also resolved. Currently, tumors showing a positive immunohistochemical reaction with pneumocyte markers, i.e., the thyroid transcription factor-1 (TT1) or napsin, are no longer classified as LCC. The presence of at least five spots with increased mucus production (the presence of mucin granules in the cytoplasm of more than five cells in the field of view) classifies the lesion into the group of adenocarcinomas. In the absence of such observations, the diagnosis is a squamous cell carcinoma. The modification resulted in a significant decrease in the number of diagnoses of large cell carcinoma, which went down to 1% of the total number of cases [19,43]. Another issue that was taken into account was the classification of tumors with epithelial and non-epithelial origin characterized by neuroendocrine function. Previously dispersed among many subtypes, now, they form a common histological subgroup: neuroendocrine

tumors. Interestingly, there is small cell lung cancer (SCLC) included as a subtype of neuroendocrine tumors, which previously functioned independently.

To clarify difficulties in the nomenclature and increase the usefulness of the classification, changes in the systematics of squamous cell carcinomas were introduced. Currently, we distinguish keratinizing, non-keratinizing, basal cell carcinomas, and pre-invasive lesions (squamous cell carcinomas in situ). The variant of small cell squamous cell carcinoma that was easily confused with small cell carcinoma has been abandoned. Moreover, the algorithm for classifying tumors as squamous cell carcinomas has been simplified. The condition for this to happen is the detection of squamous markers, i.e., p40, p63, or cytokeratin 5/6 [44]. These markers qualify the lesions as squamous cell carcinomas also in the absence of keratinization, which allows distinguishing them from adenocarcinomas that present a morphology similar to squamous cell carcinomas [15,42].

The scientific community positively received the updated classification [15,45]. However, pathologists indicate difficulties in the differential diagnosis of adenocarcinomas in situ and those with minimal invasion. It was shown that the assessment based on standard morphology is relatively subjective, emphasizing the need to refine the definition and introduce additional assessment markers [46].

The current diagnostic recommendations emphasize the value of immunohistochemical evaluation (IHC) [15,42]. The vast majority of diagnoses are based on a small amount of tissue, since only a biopsy can be performed in patients in advanced cancer stages [47]. IHC has become the basis for differential diagnosis. In the case of unclear morphology of the cellular component or its heterogeneity, IHC allows identifying the histological type of a lesion. It seemed that the histopathological classification of lung neoplasms had only prognostic significance. Currently, the value of histological assessment has increased significantly, becoming an indispensable element of diagnostic procedure algorithms. Strong emphasis on the molecular nature of the neoplasm dictates the necessity to perform costly tests to detect mutations. Thus, accurate histological diagnosis has become crucial, since each histological type of NSCLC is associated with a characteristic range of gene mutations [12,48,49]. IHC is facing a challenge that targets new biomarkers with higher precision and specificity in the diagnosis of key histological types.

## 4. Genetic Basis of NSCLC Heterogeneity

The heterogeneous nature of the composition and the growth of NSCLCs is the main obstacle in the therapy of patients in the advanced stage of the disease. All stages of carcinogenesis, from pre-initiation to progression, must be examined to elucidate the underlying causes of this phenomenon. Unfortunately, the multi-layered nature of NSCLC evolution is like a Gordian knot, remaining unsolved. Nevertheless, our knowledge of NSCLC biology is growing, leading to improved conclusions. The first is that genetic disorders are the basis of the neoplastic process [50,51]. Although carcinogenesis begins with a genetic mutation, it should be remembered that a single mutation is not enough for a neoplastic transformation (Figure 3). A pivotal factor in carcinogenesis is increasing genetic instability [52–55]. By genetic instability, we understand the variability in the severity of disturbances in DNA structure between generations of cells in a given population. Genome instability is, in a sense, a natural phenomenon inherent to the aging process of cells, which, in healthy tissues, manifests itself as somatic mosaicism [56]. On the other hand, tumorigenesis exacerbates it, generating variability at chromosome levels, epigenetic processes, or microsatellite structures.

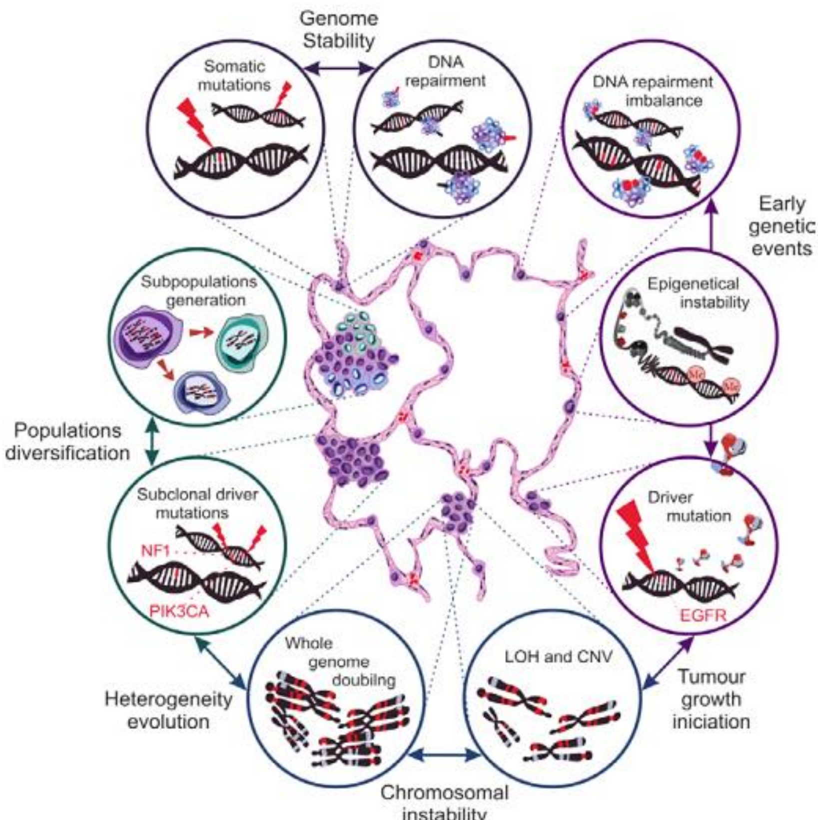


**Figure 3.** Genetic basis of neoplastic transformation of lung cells and heterogeneity of NSCLC (non-small cell lung cancer). In normal lung tissue, the cell's genome is kept in balance between mutation occurrence and repair. When endogenous or exogenous factors disrupt this balance, genetic instability occurs, which initiates the pre-initiation phase. During this time, the increasing instability of epigenetic control and the occurrence of new mutations change the activity of the molecular mechanisms. For neoplastic transformation of the cell, occurring changes need to accumulate and cause defects at the chromosome level (initiation phase). Afterward, the whole genome doubling of clonal cells leads to the development of separate populations with different genotypes. Carcinogenesis enters the progression phase, which results from the formation of cells with increased proliferation and invasiveness, triggering the metastasis formation.

The stabilization of genetic processes depends on the balance between the intensification of genetic mutations and the cell repair mechanism. In carcinogenesis, the significance of the disturbances in the DNA mismatch repair (MMR) during replication was proven. Tissue analysis of 77 primary NSCLCs showed that more than three-fourths of neoplastic lesions display impairment of the expression of the proteins responsible for MMR repair mechanisms, i.e., bMLH1, hMLH1, and hMSH2, confirming their participation in the pathogenesis of NSCLC [57].

The microsatellite instability (MSI) measurement is used to determine the level of genome instability. Microsatellites are cyclic repeats of several base pairs in the non-coding regions of DNA. By detecting specific MSI phenotypes (depending on the intensity of variance in length and the mutation of microsatellite sections), we can assess the level of dysregulation of the DNA repair processes and, indirectly, the predisposition of cells to neoplastic activation [55]. Several areas of chromatin were located as markers of MSI severity specific to lung tissue cells (on chromosomes 2, 5, 8, 10, 11, and 17). MSI was

identified in 68% of NSCLCs that correlates with the stage of disease advancement and the survival time. Moreover, MSI is a strong probability indicator of the recurrence or the initiation of new tumors growth [58,59]. In addition, analysis of MSI in neoplastic tissue allows identifying subclonal populations indicative of the level of tumor heterogeneity. The variation in the intensity of DNA methylation or the occurrence of dysfunctional histone modifications are called epigenetic instability. The global hypomethylation of DNA and the local hypermethylation of the promoters of specific genes result in the disturbance of the proper course of signaling pathways, modulating the intensity of their activity. In contrast, the importance of the acetylation of histone proteins lies in the disorganization of the chromatin packaging system. The exposure of certain regions encoding specific genes leads to a change in the intensity of their expression, which, over time, induces a higher frequency of mutations [54,60]. The epigenetic modification profiles occurring in neoplastic cells are strongly correlated with their histological lineage, creating chromatin mutation patterns characteristic of specific NSCLC subtypes [61]. In addition, the impairment of the APOBEC family enzymes that convert cytosine to uracil during RNA transcript editing leads to an increased mutation accumulation. The assessment of the areas of neoplastic transformation within the lungs and the associated lymph nodes reveals the presence of point mutations characteristic of the APOBEC enzyme [62,63]. Based on the analysis of the genomes of thousands of tumors, including NSCLCs, a specific mutation pattern was established, which is a kind of signature of the defect of the APOBEC protein. This finding confirms APOBEC participation in the development of neoplastic changes in the lungs [19,63]. Neoplastic patterns of genome distortion are also observed in tumor margins, showing normal morphology [64]. Cancerization in the area of the tumor margin tissue is called "field cancerization". The changes relate to epigenetic modifications and, consequently, the weakening or the intensification of protein expression while maintaining the correct morphology of cells. The observation of lung tissue in people subjected to long-term exposure to carcinogens showed the presence of numerous "field defect" foci in the bronchial tree [65]. Therefore, recurrences often observed in lung cancer or multifocal primary lesions result from the presence of multiple areas of "field defect" and their molecular nature [26,66,67]. The development of epigenetic instability associated with a specific pattern of protein expression dysregulation may be (but not always) a direct trigger of the neoplastic transformation process.

However, it determines its direction depending on the histological type, constituting a solid basis for the initiation of oncogenic activation [60]. The molecular basis for the evolution of neoplastic processes is best known in adenocarcinomas of the lung. Thus far, specific genetic changes responsible for the initiation, the promotion, and the disease progression have been found. The presence of the mutant EGFR (epidermal growth factor receptor) and the K-Ras (Kirsten rat sarcoma viral oncogene homolog) proteins is often observed in the majority of cells of subsequent generations of clones. Hence, they qualify as driver mutations, responsible for the initiation of neoplastic growth, which makes them important as therapy targets [68]. However, it is worth paying attention to the possibility of the simultaneous development of several primary tumors with a different driver mutation basis, which complicates the choice of targeted therapy. In such patients, later metastases originated most often from one primary tumor, precisely from one of its clonal subpopulations [69]. This confirms the importance of monitoring the molecular evolution of NSCLC over time. A prospective study of 100 NSCLC patients in the TRACERx project used the discovery of the presence of circulating tumor DNA (ctDNA) in the blood to trace genetic variation over time. It was shown that driver mutations may develop during the isolation of a subpopulation of cells, initiating the emergence of a cell line with a new genotype, or they may exist only as a "passenger mutation", not acting as a driver. Mutations of NF1, PK3CA, and KRAS genes are common driver mutations. Nevertheless, their occurrence is not observed in the whole tumor but at the level of specific cell subpopulations, suggesting their appearance at the stage of tumor growth promotion [19].

Key mutations cannot initiate the neoplastic transformation on their own. The development of a clonal cell population also depends on the level of chromosomal instability (CIN), defined as the variance of the number of chromosomal structure impairments in the cell population of a given tissue [53]. One of the subtypes that drive carcinogenesis is the loss of heterozygosity (LOH). The phenomenon consists of silencing or losing alleles encoding correct proteins, favoring alleles burdened with mutations [59]. For instance, in several lung tumors, the p53 suppressor activity was abolished due to the LOH of *TP53* alleles [70]. LOH together with unbalanced duplications of mutant alleles (copy number variations, CNVs) of genes lead to allelic imbalance (AI). The presence of AI on oncogenic genes gradually modulates the activity of proteins that control cell division, which results in the accumulation of subsequent mutations. Both the driver mutations and the CIN work together in an endless loop. Mutations appear one at a time but are fixed thanks to CIN mechanisms. In the cell, the proportion between normal and mutated protein variants changes until there is a significant predominance of impaired proteins, leading to the activation of the carcinogenesis process [71]. However, the initiation of carcinogenesis does not slow down the processes responsible for genetic instability. A comparative analysis of individual subclonal populations showed the variable occurrence of LOH in genes responsible for chromatin remodeling, histone methylation, and response to DNA damage, which led to the formation of new cells genotypes independent of the driving mutation [19]. This observation confirms that CIN is the driving force behind the initiation and the promotion of heterogeneity in lung tumors [72]. Yet, the decisive event is the phenomenon of "whole-genome doubling" (WGD). Genome duplication is considered to be of great importance in tumors with advanced LOHp; especially, it is observed most often in lung cancer. Moreover, it was shown that the mechanisms of natural selection, counteracting the increasing homozygosity of cells, function only until the genome doubles [73]. In addition, alterations in cancer genes that occurred before and after than WGD were related to tumor initiation and progression, respectively [19]. WGD is a turning point in the formation of new subclonal populations, since strengthening all genetic changes occurring in a single cell allows for the differentiation of a genotypically and a phenotypically new cell population. By overcoming one of the milestones of lung carcinogenesis, tumor growth accelerates. The appearance of polyploid cells, characterized by their invasiveness (enhanced proliferative activity acquired as a result of increasing CIN), leads to the final stage of carcinogenesis: progression.

The evolution of lung cancer is still not fully understood, and only a few publications addressed this complex subject [19,62,74–78]. As a result of cell aging, oxidative stress, exposure to mutagenic factors, or hypoxia, the replication machinery generates a higher number of errors [79–83]. Unrepaired genes mutations, decreased allelic heterozygosity, and quantitative changes in gene expression modulate the activity of the proteins responsible for cell division. When key mutations remain unattended due to a growing deficiency of the repair mechanisms, alternations of the most important signaling pathways are triggered. There is a hypothesis that formation of the neoplastic cell protoplast occurs under the natural selection, where a cell with a specific compilation of genetic aberrations survives despite environmental pressure coming from the processes controlling cell proliferation [54,84,85]. Generations of clones proliferate, and the ineffective mechanism regulating the course of cell division over time results in WGD, this being the first step towards the diversification of subclonal populations. From that point onwards, mutations lose their importance, and CIN takes control of the evolution of heterogeneity.

## 5. Overview of Targeted Therapy for NSCLC

The lack of a uniform pathomechanism of NSCLC results in the lack of a standardized treatment method. The issue arises from the wide range of driver mutations, and, hence, the number of cancer cells genotypes that initiate and maintain the process of carcinogenesis in the lungs.

Carcinogenesis is initiated when an irreversible and heritable mutation occurs in one of the key proteins that control any vital cell functions (proliferation, adhesion, DNA repair, etc.). However, later on, the promotion of neoplastic change depends on the development of oncogenic patterns of gene expression (oncogene addiction) in subsequent generations of cells. The goal of therapy is to be able to disable them effectively. One strategy called targeted therapy aims to inhibit the activity of key proteins resulting from driver mutations (Figure 4). Nevertheless, despite the growing amount of research aimed at better understanding the cancer process and finding effective inhibitors of target proteins, our capabilities remain insufficient to treat each patient effectively. One main goal of this review was to update and summarize the knowledge of available targeted therapies for different NSCLC genotypes. Current and developing treatments for selected driver mutations are discussed as well as milestones in the progress of effective targeted therapy.

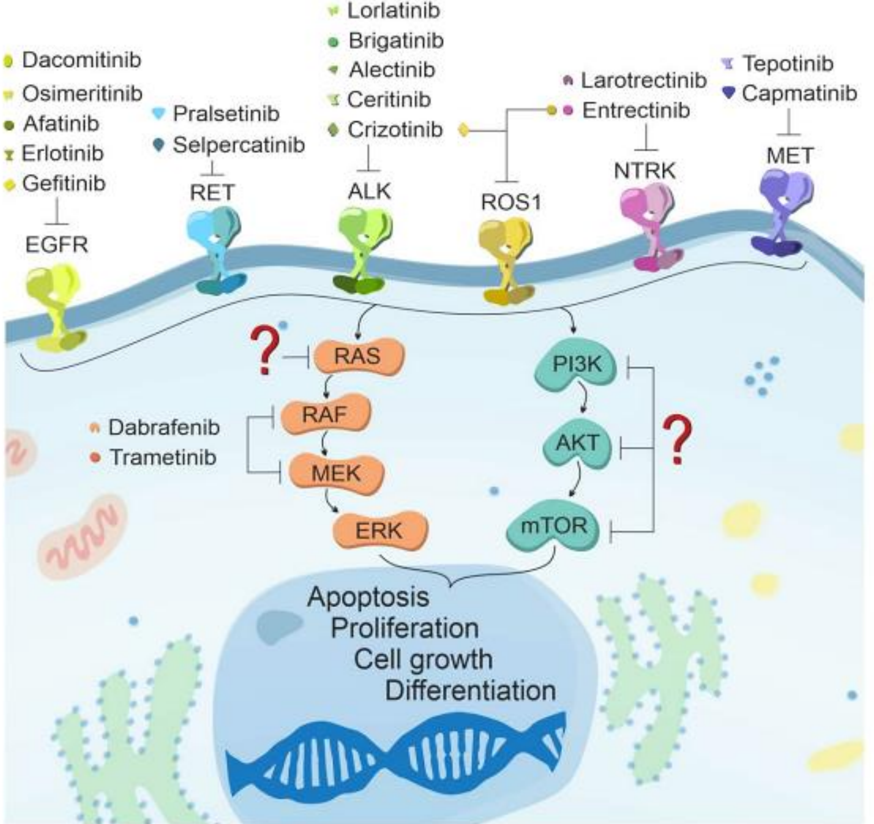


**Figure 4.** Schematic representation of the leading signaling pathways for which FDA-approved inhibitory substances were developed thus far (EGFR—epidermal growth factor receptor; HER2—human epidermal growth factor receptor 2; ALK—acute lymphoma kinase; ROS1—c-ros1 oncogene; NTRK1—neurotrophic tyrosine kinase receptor type 1; MET—tyrosine-protein kinase Met; RAS—rat sarcoma 2 viral oncogene homologs family; RAF—proto-oncogene c-RAF; MEK—mitogen-activated protein kinase; ERK—extracellular signal-regulated kinases; Pi3K—phosphoinositide 3-kinases; AKT—protein kinase B; mTOR—mechanistic target of rapamycin kinase).

### *5.1. Protein Genes from the Epidermal Growth Factor Receptor (EGFR) Family*

The *ERBB1* gene mutation was the first discovered mutation in NSCLC. Nowadays, it is the most common target of targeted therapy, since as many as 20% of patients with

lung adenocarcinoma carry it [34]. The gene codes for the EGFR protein (ERbB1, HER1), which is a membrane receptor from the group of tyrosine kinases. The binding of the ligand to the extracellular domain of the receptor becomes possible through the formation of homo- or heterodimers (with other proteins from the ERBB family, i.e., HER2, HER3, or the MET protein) [86,87]. This results in intracellular signal transduction and the autophosphorylation of tyrosine residues, which activate EGFR-dependent signaling pathways responsible for the control of the cell cycle [88]. In neoplastic cells, impaired EGFR function most often results from *ERBB1* gene overexpression, increased gene copy number, or the presence of a mutation [89]. Mutated proteins do not degrade and form dimers with high affinity, which leads to the unlimited activity and the autonomy of the receptor [86]. It triggers inhibition of apoptotic pathways, continuous proliferation, and blockade of the gene expression patterns that define cell differentiation [3,4,88]. Moreover, it contributes to the initiation of angio- and lymphangiogenesis processes in the neoplastic tumor, which is the first step towards the invasion of cancer cells and the formation of metastases [90,91]. The identification of a mutation of EGFR-encoding genes in patients, even with advanced NSCLC, is considered a favorable prognostic factor. The presence of mutated EGFR most probably means that the tumor is sensitive to tyrosine kinase inhibitors (TKIs) [92]. Unfortunately, tumor recurrence or TKI resistance usually appear. As a result, up to now, three generations of TKIs were developed. The T790M mutation in the *ERBB1* gene is responsible for TKI resistance. The mutant blocks the specific binding of first and second-generation TKIs to EGFR [93]. Two major explanations of the development of resistance were described. In one, the mutation has not been diagnosed, e.g., due to its presence in a small subpopulation of cell clones. The second results from the appearance of the mutation during treatment de novo. Osimertinib is the only third-generation TKI approved by the Food and Drug Administration (FDA) that irreversibly blocks the receptor burdened with the T790M mutation as well as most common EGFR mutations. Due to its ability to cross the blood–brain barrier, osimertinib received accelerated approval in 2015 for patients with NSCLC metastases with the T790M mutation [94]. Shortly after, the results of the AURA3 clinical trials achieved full approval of the drug, showing an overall response rate of 72% and a 10.1 month progression-free period versus 31% response and 4.4 months progression-free in patients treated with chemotherapy [95].

Moreover, the decision to introduce liquid biopsies (blood tests or tests for free circulating nucleic acids from cancer cells) for the presence of the T790M mutation—cobas®® EGFR Mutation Test v2 [95]—largely contributed to the success of these studies. Although the test accelerated the possibility of a therapeutic decision-making, later studies showed that the negative results may be false in up to 30% of the cases. [96,97]. Hence, the lack of detection of the T790M mutation in the blood requires confirmation by analyzing the tissue material taken directly from the tumor. However, nowadays, less attention is paid to identify such mutations, since osimertinib is used as a first-line therapy [98]. Its superiority was confirmed by the latest results of the FLAURA clinical trials where osimertinib was compared with first-generation TKIs. The results showed increased progression-free and overall survival (OS) when using osimertinib as a first-line therapy. The medians were 38.6 and 31.8 months for third- and first-generation drug (gefitinib or erlotinib) groups, respectively [99,100]. This led to the approval of osimertinib for first-line treatment in 2018. Moreover, the efficacy of osimertinib was tested against less common mutant EGFR variants, also obtaining satisfactory results [101]. However, there are also significant disadvantages in using third-generation TKIs. One of them is the occurrence of severe toxicity (irAE—immune-related adverse events) during osimertinib therapy. In this sense, toxicity was found in 15% of patients who had previously undergone immunotherapy [102]. With at least a year break between the above-mentioned therapies, the side effects were reduced, which proves the very long half-life of the checkpoint inhibitors used in immunotherapy [103]. More importantly, the simultaneous use of osimertinib and durvalumab (immunotherapy drug) increased the percentage of patients who developed interstitial lung

disease to 38%. Moreover, the response rate to treatment with the combination of drugs was lower than that of osimertinib alone, i.e., 64% vs. 80% [104].

Despite significant advances in the knowledge of the *ERBB1* gene mutations, new questions continue to arise, especially around the process of acquiring resistance. It is worth mentioning that recent reports indicated cancer transition into neuroendocrine subtype (from 3% to 10% of adenocarcinomas), the treatment of which is less effective [105–107]. Furthermore, this differentiation may lead to the development of resistance to third-generation TKIs [108]. TKI resistance is the "Achilles heel" of targeted therapy. However, it should be remembered that a better understanding of the molecular basis of the *ERBB1* gene mutation led to significant success, increasing the overall survival of patients with the above-mentioned mutation, which accounts for approximately 20–23% of patients with lung adenocarcinoma [109].

The *ERBB2* (HER2—human epidermal growth factor receptor 2) gene mutation encoding a receptor from the EGFR family occurs in 3% of lung adenocarcinomas [110]. Thus far, clinical trials focusing on the use of monoclonal antibodies showed a 44% response rate, achieving a maximum reduction of 69% in the mass of neoplastic lesions [111]. On the other hand, in other studies, a lack of efficacy of these antibodies in combination therapy with chemotherapy in patients with *ERBB2* gene amplification was found [112]. Hence, clinical trials are currently using inhibitors against the most common mutation of HER2. In vitro studies carried out on organoids showed the anti-tumor activity of pyrotinib resulting from the inhibition of the tumor cells growth. Therefore, pyrotinib was qualified for clinical trials, where it showed a response rate of 53.3% in a group of 15 patients [113]. On the other hand, preliminary phase I results on an inhibitor TAK-788 did not show satisfactory results. There were only 3 out of 14 patients that showed a partial response to treatment. The last compound with therapeutic potential for *ERBB2* mutation is poziotinib, which achieved an overall response rate of 42% in the second phase of clinical trials [114].

### *5.2. Anaplastic Lymphoma Kinase (ALK) Receptor Gene*

The physiological function of ALK is not yet fully understood. While many studies assessed individual components of the downstream ALK signaling pathways, their selectivity does not allow for a comprehensive understanding of the role of the ALK receptor. Although the exact molecular mechanisms are unknown, the existing knowledge shows a set of cellular processes in which the ALK receptor is involved (control of cell cycle, cell growth, cell differentiation, and anti-apoptotic signaling pathways) [115]. Thus far, three main activation mechanisms of the oncogenic receptor have been identified. These are gene amplification, fusion with a gene of another protein, or mutation in the sequence of the *ALK* gene itself. The location for *ALK* is considered very common for chromosomal translocation, leading to fusion with another gene and, thus, the production of linked proteins with altered properties. This mechanism of *ALK* genetic aberration is most often observed in lung adenocarcinomas, and its incidence is approximately 5–6% [116,117]. Until now, among the 22 fusion partners, the most frequently identified concerned the EML4 protein (echinoderm microtubule-associated protein-like 4), which leads to the development of adenocarcinoma [118]. Other variants observed in NSCLC are fusions with the following proteins: KIF5B (kinesin family member 5B), TFG (TRK-fused gene), KLC1 (kinesin light chain 1), PTPN3 (protein tyrosine phosphatase non-receptor type 3), and STRN (striatin). The oncogenic activity of the ALK protein results from the acquired autonomy during fusion [115]. A conformational structural change results in permanent phosphorylation and activation of the kinase domain, bypassing the ligand binding-induced ALK protein dimerization step [119]. In 2013, seven years after the discovery of the EML4-ALK fusion as a driver mutation in lung adenocarcinomas, the efficacy of the first targeted TKI, crizotinib, over chemotherapy was documented [120]. Subsequently, due to the emergence of acquired resistance, two therapeutics obtained accelerated approval for second-line treatment: ceritinib and alectinib, both second-generation TKIs. Finally, based on the promising results of the clinical trials ASCEND-4 and ALEX, they were introduced as first-

line drugs [121–124]. Another second-generation TKI with proven therapeutic potential for patients who developed resistance to crizotinib was brigatinib. Clinical trials using brigatinib exhibited a 53.6% overall response rate and a 73.3% response rate to the treatment of active measurable brain metastases. These results permitted the drug to be approved for the treatment of patients with disease progression [15]. Moreover, a study was performed in which patients who had not previously received TKIs crizotinib and brigatinib were compared. The new generation inhibitor showed a slightly higher overall response rate. Nevertheless, brigatinib showed 76% versus 26% response rate to treatment with crizotinib regarding intracranial metastases. Thus, obtained results proved a significant intracranial penetration and therefore promoted brigatinib as an effective first-line drug in advanced stage cancer [125]. Unfortunately, there are still no studies comparing brigatinib with other second-generation TKIs and, thus, no conclusion can be derived. Notwithstanding, the FDA approved brigatinib for first-line treatment in 2020.

The presence of second-generation TKIs as first-line drugs did not avoid acquired resistance to targeted therapy, attributed to point mutations in the *ALK* gene. It is noteworthy that some mutations are TKI-specific, and the same mutations can appear in response to different TKIs. One example is the G1202R mutation, which can be triggered by any TKI generation [126]. As the G1202R mutation is resistant to all TKIs of the first and the second generations, the FDA approved a third generation TKI to use in the second and the third lines of treatment: lorlatinib. Lorlatinib is a macrocyclic compound with a broad spectrum of inhibition of mutant variants. A clinical trial showed a high response rate (90%) in treatment-naive patients with *ALK* gene fusion [127]. In patients who had failed second generation TKI treatment, lorlatinib achieved a 69% response rate. Although the results obtained are very promising, common side effects of lorlatinib were observed. More than 80% of patients showed hypercholesterolemia, 60% of them showed hypertriglyceridemia, and 3% had to discontinue treatment due to other severe side effects [128]. Meanwhile, the availability of a wide range of TKIs for patients with *ALK* gene fusion seems to be very beneficial; the constant genetic evolution of NSCLC requires a personalized approach. A perfect example is the clinical case of a patient with the presence of the ELM4-ALK protein fusion with advanced lung cancer and liver metastases. The use of crizotinib (first generation TKI) resulted in an early improvement, but after 9 months, tumor progression and liver failure was observed. The patient did not respond to second generation TKIs and chemotherapy. Genotyping showed that resistance resulted from the C1156Y mutation, which is susceptible to lorlatinib. Surprisingly, after the treatment, the patient was re-diagnosed with acquired resistance to lorlatinib. The detection of the coexisting L1198F mutation paradoxically helped the patient, as this mutation increases the binding affinity of the first generation TKI. Re-therapy with crizotinib resulted in rapid regression of metastases and recovery of the liver function, confirmed by computed tomography 6 months after the start of treatment [129]. The success of the discussed case was conditioned by a flexible approach based on the documented properties of new therapeutic substances and the systematic use of molecular diagnostic techniques.

### *5.3. The C-Ros Oncogene 1 of the Receptor Tyrosine Kinase (ROS1) Gene*

The ROS1 membrane receptor is an enzyme with a highly homologous structure to the ALK protein and thus follows a similar oncogenic nature (i.e., fusion with another protein). The function of ROS1 proteins is related to the control of cell differentiation and growth. Hence, in NSCLC, when *ROS1* gene rearrangement (most often with CD74, SLC34A2, or FIG proteins) takes place, oncogenic hyperactivity of the protein is observed [130]. Due to the similar structure of ROS1 to the ALK protein, the efficacy of crizotinib in a cellular model was very quickly demonstrated that was subsequently confirmed in clinical trials [131–133]. However, the acquisition of resistance was also observed. One of the first described resistance pathomechanisms was a point mutation in the kinase domain G2032R. Interestingly, the mutation was present in all metastases, suggesting that this mutation was an early event in the carcinogenesis before the invasion phase [134]. An

alternative resistance mechanism is the cell adaptation to protein activity inhibition that initiates oncogenic patterns of gene expression. Cells become independent of the driver mutation by developing alternative signaling pathways to maintain their proliferation. Resistance to ROS1 inhibition can be mediated by EGFR or RAS activation. For example, in NSCLC cell line HCC78, resistance to ROS1 inhibition leads to cells sensitive to EGFR inhibition [135,136]. In this case, it is recommended to re-genotype the biopsy material for the presence of other protein mutations [137]. Unfortunately, crizotinib also has poor intracranial penetration. Approximately 34%–36% of patients with advanced NSCLC show the presence of brain metastases that is considered to be the most common cause of mortality [138]. Hence, a new drug that inhibits the ROS1, entretinib, was approved in 2019, showing significant activity against intracranial metastases.

Entretinib is an inhibitor of the tropomyosin receptor kinase (TRK), and its mechanism of action is based on the ROS1 receptor dependence on the TRK activity [139]. It is one of the first inhibitors approved under the FDA's revised policy. Thus, entretinib can be used for specific gene mutations independently of the cancer type. The acceptance of entretinib was based on the results of three independent clinical trials involving 53 patients. Despite the positive results (77% of the overall response rate), it should be mentioned that the inhibitor is also characterized by a high percentage (11%) of severe side effects, including those related to nervous system and cardiovascular disorders. Nevertheless, these effects can be regulated by lowering the drug dose [140]. A choice for patients with intracranial metastases is the previously discussed ALK inhibitor, lorlatinib, which is active against mutations acquired during treatment with crizotinib. In clinical trials, lorlatinib showed a 62% response rate in patients who had not received previous treatment and 35% in those who had received crizotinib. For the remaining ALK inhibitors, only the efficacy of ceritinib was assessed in clinical trials, with an overall response rate of 62% [141]. To conclude, it can be stated that targeted therapy for *ROS1* gene mutation is characterized by relatively low effectiveness. Accordingly, next generations of inhibitors are sought, exemplified by DS-6051b and repotretinib, which showed promising potential in preclinical studies thus far [142,143].

### *5.4. Tyrosine-Protein Kinase MET Gene—MET*

The mutation of the *MET* gene, which encodes another protein from the group of receptor tyrosine kinases, occurs in two variants: deletion of exon 14 and gene amplification. Exon 14 aberration reduces the degradability of the protein, which disables the mechanism regulating the number of active MET homodimers. As a result, there is an accumulation of proteins in the cell membrane, and their activity increases. Although crizotinib and cabozantinib are not direct inhibitors of MET, due to the receptor heterodimerization with proteins from the EGFR family, they show a partial inhibition of their oncogenic activity. The retrospective evaluation of 61 cases confirmed 24.6 and 8.1 months overall survival for patients treated with at least one of the inhibitors and non-treated patients, respectively. Such results represent a significant increase in overall survival [144]. In recent years, several MET inhibitors more selectively targeting the mutation have been selected for clinical trials (capmatinib, tepotinib, glesatinib, and savolitinib). Capmatinib received accelerated FDA approval this year. Unfortunately, the variant of the *MET* gene amplification remains without therapy treatment. Its presence was proven to reduce the survival time of patients, which is why it is considered a negative prognostic indicator [144]. Moreover, it is one of the mechanisms of acquired resistance to TKIs in patients with *ERBB1* gene mutation. Studies on a selected small group of 12 people (patients with a medium to a high degree of *MET* gene amplification) showed a 42% response to treatment with crizotinib, while another 42% of patients showed stabilization of the disease [145]. Only one study performed on a small group of 16 patients used an antibody-based inhibitor targeting the *MET* gene amplification (telisotuzumab vedotin). The results of the first phase of the clinical trials showed a low 18.8% response to treatment [146].

### 5.5. Tyrosine-Protein Kinase RET Gene—RET

The RET protein is a membrane tyrosine kinase receptor. RET mutations are most often diagnosed in medullary thyroid cancer. Nevertheless, its mutation leads to the development of NSCLC. Its oncogenic activity is caused by chromosomal rearrangement, which leads to the formation of proteins with altered receptor activity [147]. Its main partner is the KIF5B protein (kinesin family member 5B), which accounts for 62% of all *RET* gene rearrangement variants [148]. To date, there have been approved three RET inhibitors, namely cabozantinib, vandetanib, and alectinib. However, the lack of approval for use in NSCLC meant that they could only be used as a last-line treatment.

Retrospective studies evaluated 165 patients with the *RET* mutation where the overall response rates to cabozantinib, vandetanib, and sunitinib were 37%, 18%, and 22%, respectively. The studies showed a median overall survival of only 6.8 months [149]. It is worth mentioning that RET mutations account for about ~1–2% of lung adenocarcinomas, which additionally indicates the need to develop new and more effective inhibitors. Recently, two molecules—pralsetinib (BLU-667) and selpercatinib (LOXO-292)—obtained FDA approval for use in advanced metastatic NSCLC. The ARROW clinical trials conducted on 87 patients who had previously received chemotherapy and 27 previously untreated patients showed 56% and 70% response rates to pralsetinib treatment, respectively. On the other hand, the LIBRETTO-001 studies carried out on 105 patients who had previously undergone chemotherapy and 39 previously untreated patients showed 64% and 85% response rates to selpercatinib treatment, respectively [150]. Thus, these new developed drugs have shown promising results.

### 5.6. Neurotrophic Tyrosine Kinase Receptor Type 1—NTRK1

Another protein involved in the neoplastic transformation of lung cells is the tropomyosin receptor kinase (TRK), encoded by the *NTRK1* gene. Under physiological conditions, TRK regulates cell growth and differentiation processes. Their oncogenic activity most often results from fusion with *CD74* or *MPRIP* (myosin phosphatase Rho interacting protein) genes [151]. Although the mutation in the *NTRK1* gene is quite rare (estimated <1%), there are two TKIs targeted to these abnormal receptors. Entretinib and larotrectinib were approved after clinical trials performed on various types of cancer, including NSCLC. Nevertheless, the response rates were 70% and 75% for entretinib (higher than the overall study group—57%) and for larotrectinib (three of whom were complete responders), respectively [152,153]. Unfortunately, there are still no clinical trials comparing the two drugs, and there are no data on the resistance acquisition. Therefore, chemotherapy remains a frequent therapeutic choice for patients with *NTRK1* gene fusion.

### 5.7. V600E Mutation of the BRAF1 Gene (Rapidly Accelerated Fibrosarcoma Homolog B)

The mutation of the B-Raf V600E protein concerns an enzyme that is part of one of the most important signaling pathways: RAS/RAF/MEK/ERK (MAPK/ERK pathway). A meta-analysis of the available data (up to January 2016) on *BRAF1* mutation showed that it occurs in 2.6% of NSCLC patients, while other sources estimated that it accounts for about 8% of lung adenocarcinomas [48,154]. Initially, targeted therapy was based on the use of inhibitors alone (vemurafenib or dabrafenib), but their efficacy was not satisfactory. Their response rates were 42% and 33%, respectively [155,156]. Subsequent studies demonstrated a 63–64% increase in the response rate when trametinib, a MEK protein inhibitor regulated by B-Raf, was combined with dabrafenib therapy [157]. *BRAF1* mutations are often observed in melanomas, where they are divided into three subclasses due to their different influence on the signaling pathways. The first is the V600E mutation. The second class includes other mutations that result in moderate to high kinase activity, regardless of the activity of the regulatory protein RAS. Finally, the third class includes absent or disturbed kinase activity and other unclassified mutations [158]. Studies on NSCLC cell lines showed that the effectiveness of selected inhibitors of the MAPK/ERK

pathway depended on a specific class of *BARF1* mutations, suggesting the introduction of this division into clinical and preclinical studies [159].

### *5.8. KRAS Gene Mutation (Kirsten Rat Sarcoma Viral Oncogene Homolog)*

Undoubtedly, the multitude of targeted therapy regimens is a sign of significant advancement in NSCLC treatment. However, the availability of inhibitors with proven efficacy does not correspond to the frequency of given gene mutations in patients. There are no approved inhibitors for patients with confirmed *KRAS* mutation, which accounts for up to 32.7% of NSCLC and 27% of the adenocarcinoma subtype [160,161]. The *KRAS* mutation has been acknowledged as the milestone or the greatest challenge of targeted therapy, and the attempts to develop inhibitors have been compared to a "game of thrones" [162]. Thus, up to now, the inhibitors of KRAS protein remain the most desired small molecules in targeted therapy. Although research is still in the development stage, we would like to review selected therapeutic strategies.

The RAS family is composed of intracellular GTPases, G proteins. They are presented in two forms: active Ras-GTP and inactive Ras-GDP. As an early signal transmitter, RAS controls MAPK/ERK and phosphoinositide 3-kinase (PI3K) signaling cascades, and it is responsible for activating STAT transcription factors, collectively controlling proliferation and apoptosis of the cell [163]. Mutation triggers conformational changes, thus, the enzyme is trapped in its active form, resulting in a permanent transmission of the signal for proliferation. The RAS family is represented by three proteins with a strictly homologous structure: K-Ras (Kirsten rat sarcoma viral oncogene homolog), N-Ras (neuroblastoma rat sarcoma viral oncogene homolog), and H-Ras (Harvey rat sarcoma viral oncogene homolog). Although the discovery of the oncogenic activation of the K-Ras protein in a lung tumor cell line was described as early as 1984 [164], all attempts to find an effective targeted therapy for lung cancer patients were unsuccessful [165,166]. The problem of finding an effective inhibitor is related to the variety of mechanisms present in cancer cells with *KRAS* mutation [166].

First, the detection of the K-Ras mutant is not always related to its dominance (in other words, to its driving character). This means that a mutation can also occur as a co-mutation, a consequence of an oncogenic activation of another gene. However, to achieve an effective treatment, the target should be set up on a driver mutation that causes neoplastic transformation [165]. Moreover, point mutations lead to conformational changes of the protein, changing its activity. The multitude of occurring mutant variants results in a mutation-specific reprogramming of the cancer cell metabolism. This in turn gives us a wide variety of metabolic phenotypes, as seen in cancer cells burdened with the *KRAS* mutations. The discussed heterogeneity of changes in the sequence and, thus, in the spatial structure of the mutant K-Ras explains the lack of possibility to find a universal inhibitor [167]. On the other hand, there is considerable homology between the K-Ras protein and other GTPases associated with tyrosine kinase receptors. The structural similarity relates to the guanine nucleotide binding region: the G-domain. Thus, the lack of appropriate selectivity leads to the function inhibition of other key receptors and the complete disorganization of the signaling pathways also in healthy cells [166]. Another reason for the above-mentioned issue is the occurrence of co-mutations. Mutations in *TP53*, *KEAP1*, and *STR11* genes are mentioned as the most common. The proteins encoded by them influence, among others, the activity of immune system cells in the tumor microenvironment, which impacts the effectiveness of immunotherapy [168]. Moreover, the presence of certain co-mutations defines the metabolic phenotype of a neoplastic cell. An example is the deletion of the *LKB1* gene which, by affecting the expression of the KEAP1 protein, changes the metabolism of the Krebs cycle (TCA). Physiologically, for the proper course of TCA, the availability of glucose is necessary. However, as a result of *LKB1* deletion, neoplastic cells become independent of the process of glycolysis, deriving energy from glutaminolysis [169]. The phenomenon is the basis of an interesting therapeutic strategy [169]. Currently, phase 1 clinical trials have started to check the effect of the glutamine inhibitor telaglenastat

(CB-839) in advanced NSCLC [170]. However, the preliminary results of the studies in an animal model showed that the use of CB-839 as monotherapy in lung tumors with the K-Ras mutation did not give satisfactory results; for this reason, it is suggested to include selective inhibitors of glycolysis as well [171].

Another factor contributing to the difficulty of treating *KRAS*-mutated NSCLC is the high AI of the *KRAS* gene. As a result of the deletion of the wild-type allele or the amplification of the mutant variant, the balance between errors in the DNA sequence and the normal genome is lost, which changes the intensity of the transcription and the post-transcriptional modifications. The presence of mutations in most of the alleles is associated with a higher cancer malignancy and a shorter survival time [172].

Point mutations occur most frequently in the *KRAS* gene. This applies to codons 12, 13, and 61, of which the most common mutations are observed in the first ones: G12C (which changes the amino acid sequence in codon 12 from glycine to cysteine), G12V (change from glycine to valine), and G12D (change to aspartic acid), representing 41%, 19%, and 14% of all *KRAS* mutation variants, respectively [161]. Unfortunately, the drugs currently being tested are mainly at the beginning stage (preclinical) of the research. The most promising ones are the selective inhibitors of proteins with the $KRAS^{G12C}$ mutation. The mechanism of their action is based on the block of the mutant protein in an inactive form. In phase I clinical trials, with the use of AMG 510 and MRTX 849 in patients with the $KRAS^{G12C}$ mutation, four patients with NSCLC were assessed at the first checkpoint. Both compounds showed the same results. Stable disease was observed in two patients, and there was a partial response in one patient, representing 75% response to treatment [173,174]. At the end of 2020, the first assessment of clinical activity of adagrasib (MRTX 849) in the KRYSTAL-1 study was completed. Presented results demonstrated only 45% partial response. Interestingly, disease control rate was 96% from 51 patients with previously treated $KRAS^{G12C}$-mutant NSCLC. Moreover, higher overall response rate—64%—was observed in patients presenting a co-mutation in the *STR11* gene, suggesting the importance of co-mutation diagnostics [175]. In the case of the second compound AMG 510 (sotorasib), the full analysis of data collected during phase II clinical trial was published. Results showed disease control in 80.6% of patients, of which 81% had previously received at least one therapy (chemotherapy or/and immunotherapy). Objective response to the treatment was 37.1%, including 3.3% that achieved complete response. Drug tolerability was moderate, as 69.8% of patients showed treatment-related adverse effects.

Lonafarnib and tipifarnib are specific inhibitors of farnesyltransferase, the enzyme responsible for post-translational modification of the K-Ras mutant. The action of the enzyme is based on the catalysis of binding a hydrophobic farnesyl residue to the K-Ras, anchoring the protein in the cell membrane. Despite the promising results obtained in the preclinical phase, lonafarnib showed a 10% response rate, and disease stabilization was achieved in 38% of patients [176]. The use of tipifarnib also showed a negligible clinical effect despite the strong inhibiting activity of the K-Ras farnesylation. Moreover, this drug showed significant toxicity in treated patients [177]. Nevertheless, in 2018, tipifarnib returned to clinical trials as a treatment for squamous cell lung cancer patients with the H-Ras mutation.

Hence, the complex biology of the RAS mutant variants is not only an obstacle, but it also has great research potential. With the discussed diversity of oncogenic pathways induced by the K-Ras mutants, we observe a multitude of ongoing approaches for therapeutics development. These include (i) inhibition of K-Ras binding to the cell membrane by post-translational modifications, (ii) manipulation in the *KRAS* gene expression processes, (iii) control of protein degradation, (iv) inhibition of GTP binding or binding to effector proteins, (v) attempts to block key metabolic processes, and (vi) use of synergistic inhibitors of proteins related to the K-Ras signaling pathway [178,179]. Although there are many possibilities in the area of treatment of NSCLC caused by mutations in the *KRAS* gene, it seems that an effective and safe therapy is still to be discovered.

### *5.9. FDA Approved TKIs*

To date, the FDA has approved seventeen TKIs for NSCLC therapy (Table 2). One of them, crizotinib, can be incorporated in the treatment of two different targets: ALK and ROS-1. Furthermore, TKIs targeting NTRK received approval as tissue-agnostic drugs for cancer therapy.

**Table 2.** FDA-approved targeted therapy drugs for the treatment of NSCLC.

| Target | Inhibitor | Line of Treatment | Indication | Current-FDA Approval Year | Clinical Trial-Based Approval |
|---|---|---|---|---|---|
| EGFR | Gefitinib | first-line | metastatic NSCLC with exon 19 deletions or exon 21 (L858R) substitution mutations | 2015 | IFUM (NCT01203917) |
| | Erlotinib | first- or second-line | metastatic NSCLC with exon 19 deletions or exon 21 (L858R) substitution mutations | 2016 | IUNO trial (NCT01328951) |
| | Afatinib | first- or second-line treatment | metastatic NSCLC with non-resistant EGFR mutations; metastatic, squamous NSCLC progressing after platinum-based chemotherapy | 2018 | LUX-Lung 2 (NCT00525148), LUX-Lung 3 (NCT00949650), and LUX-Lung 6 (NCT01121393) |
| | Osimertinib | first-line or second-treatment | metastatic NSCLC with detected exon 19 deletions or exon 21 L858R mutations or T790M mutation-positive with disease progression on EGFR TKI therapy | 2018 | FLAURA, (NCT02296125) |
| | Dacomitinib | first-line | metastatic NSCLC with detected exon 19 deletions or exon 21 (L858R) substitution mutations | 2018 | ARCHER 1050 (NCT01774721) |
| ALK | Crizotinib | first-line | locally advanced or metastatic NSCLC | 2011 | PROFILE 1005 (NCT00932451) |
| | Ceritinib | first- or second-line | metastatic NSCLC | 2017 | ASCEND-4 (NCT01828099) |
| | Alectinib | first-line | | 2017 | ALEX (NCT02075840) |
| | Brigatinib | second-line | | 2017 | ALTA (NCT02094573) |
| | Lorlatinib | second- or third line | metastatic NSCLC after progression on other ALK TKI therapy | 2018 | Study B7461001 (NCT01970865) |
| ROS1 | Crizotinib | first-line | metastatic NSCLC | 2016 | PROFILE 1001 (NCT00585195) |
| | Entrectinib | first-line | | 2019 | STARTRK-1 (NCT02097810) STARTRK-2 (NCT02568267) |
| NTRK | Larotrectinib | first-line | solid tumors with detected *NTRK* gene fusion without a known acquired resistance mutation, independent of tumor origin | 2018 | LOXO-TRK-14001 (NCT02122913), SCOUT (NCT02637687), NAVIGATE (NCT02576431) |
| | Entrectinib | first-line | | 2019 | STARTRK-1 (NCT02097810) STARTRK-2 (NCT02568267) |
| RET | Pralsetinib | first-line | metastatic NSCLC | 2020 | ARROW (NCT03037385) |
| | Selpercatinib | first-line | metastatic NSCLC | 2020 | LIBRETTO-001 (NCT03157128) |
| MET | Capmatinib | first-line | metastatic NSCLC with specific mutations (exon 14 skipping) | 2020 | GEOMETRY (NCT02414139) |
| | Tepotinib | first-line | | 2021 | VISION (NCT02864992) |

## 6. Overview of Immunotherapy for NSCLC

Targeted therapies are no longer the only treatment option; immunotherapy (IO) has dramatically modified the NSCLC treatment landscape [180]. The acquired resistance to targeted therapies remains a major and inevitable challenge, and, therefore, new approaches must be considered. Cancer cells have multiple immunosuppressive mechanisms to escape

from the immunological response and survive [181]. Therefore, immunotherapy exploits the concept of activating or regulating the immune system to identify and kill cancer cells. To date, one of the main approaches is to develop immune checkpoint inhibitors (ICI) to target pathways used by cancer cells to escape the immune system. Particularly, inhibitors of cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death receptor (PD-1) and PD-ligand 1 (PD-L1) checkpoints, which regulate priming and effector phases of T-cell activation, respectively, were approved by the FDA (Table 3) [182]. Clinical trials using other immune checkpoint inhibitors are ongoing, targeting T cell immunoglobulin and mucin-containing protein 3 (TIM-3) (NCT03311412, NCT02817633, NCT03307785) [183,184], lymphocyte activation gene-3 (LAG-3) (NCT03311412, NCT03538028, NCT03156114) [184–186], V-domain lg suppressor of T cell activation (VISTA) (NCT02671955, CTRI/2017/12/01 1026) [187,188], human endogenous retrovirus-h long terminal repeat-associating protein 2 (HHLA2), and T cell lg and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) (NCT04746924, NCT04866017) [189]. Most of the ICIs proved a limited benefit with 10%–20% overall response rates of monotherapy [190]. One of the approaches to improve the ICIs efficiency consists in the development of better predictive biomarkers [191]. Another approach is the combination treatment strategies such as ICIs combinations with chemotherapy [192,193], radiotherapy [194], or TKI [104,195].

**Table 3.** FDA-approved immunotherapy drugs for the treatment of NSCLC.

| Checkpoint Inhibitor | Target | Line of Treatment | Indications | Clinical Trial-Based Approval | FDA Approval Year |
|---|---|---|---|---|---|
| Nivolumab | PD-1 | second-line | metastatic squamous NSCLC after chemotherapy; | CheckMate 017 (NCT01642004) | 2015 |
| | | second-line | extension to non-squamous NSCLC; | CheckMate 057 (NCT01673867) | |
| Pembrolizumab | PD-1 | first-line | metastatic NSCLC; with no EGFR or ALK mutation; TPS ≥ 50%; | KEYNOTE−024 (NCT02142738) | 2016 |
| | | second-line | progression after chemotherapy or TKI in metastatic NSCLC; with TPS ≥ 1%; | KEYNOTE-010 (NCT01905657) | |
| | | first-line | unresectable stage III or metastatic NSCLC; no possible definitive chemoradiation; with no EGFR or ALK mutation; TPS ≥ 1%; | KEYNOTE-042 (NCT02220894) | 2019 |
| Atezolizumab | PDL-1 | second-line | metastatic NSCLC with progression on/after chemotherapy or TKIs; | OAK (NCT02008227) POLAR (NCT01903993) | 2016 |
| | | first-line | combined with chemotherapy; metastatic non-squamous NSCLC; with no EGFR or ALK mutation; | IMpower150 (NCT02366143) | 2018 |
| Durvalumab | PDL-1 | second-line | unresectable Stage III NSCLC; with no progression after chemoradiation therapy; | PACIFIC (NCT02125461) | 2018 |
| Ipilimumab | CTLA-4 | first-line | only in the combination with nivolumab; metastatic NSCLC; with no EGFR or ALK mutation; TPS ≥ 1%; | CheckMate 227 (NCT02477826) | 2020 |
| Cemiplimab | PD-1 | first-line | advanced NSCLC; TPS ≥ 50% | EMPOWER-Lung 1 (NCT03088540) | 2021 |

### 6.1. Immune Checkpoint Inhibitors

ICIs are compounds that block immunosuppressive mechanisms of cancer cells. There are mainly seven stages, called cancer-immunity cycles (CIC), involving the immune system response to cancer cells [190]: (i) cancer antigens are released from cancer cells, (ii) cancer antigen presentation to T cells, (iii) T cells activation, (iv) T cells trafficking to tumors, (v) T cells infiltration to tumors, (vi) cancer cell recognition by T cells, and (vii) elimination of cancer cells. Cancer cells may evade the autoimmune response by several mechanisms. For example, immune checkpoint molecules such as PD-L1 expressed by tumor cells interact with PD-1 receptors expressed on activated T cells and inhibit T cell activation, promoting tumor immune escape [196].

CTLA-4 was the first known immune checkpoint, expressed on regulatory T cells (Tregs) and on the surface of activated T lymphocytes [21]. During the T cell activation (CIC third step), the receptor protein CTLA-4 competes with CD-28 receptors to bind to the B7-1 and the B7-2 ligands expressed on antigen-presenting cells (APCs) [197]. The higher affinity of CTLA-4 to bind B7 instead of CD-28 inhibits B7-CD-28 binding and suppresses the T cell activation. ICIs of the CTLA-4/CD-28 checkpoint pathway may suppress the CTLA-4-B7 binding, promoting the activation of immune responses [198].

In 2014, the first two ICIs (nivolumab (NCT01721772) and pembrolizumab (NCT01295 827)) targeting PD-1 were approved by the FDA for malignant melanoma [199,200], and, in 2015, nivolumab emerged as a novel second-line treatment in advanced squamous cell and NSCLC patients regardless of PD-L1 expression level (CheckMate 017, CheckMate 057) [201,202] (Table 2). Later, other anti-PD-1 antibodies, pembrolizumab and atezolizumab (OAK trial) [203], were introduced as second-line NSCLC therapies, and then pembrolizumab was approved for the first time as a first-line treatment for NSCLC without driver mutations (KEYNOTE-024) [204]. Approximately 25%–30% of NSCLC patients exhibit high PD-L1 expression (tumor proportion score, TPS $\geq$ 50%) and can benefit from a first-line therapy, for instance, with pembrolizumab [204]. In 2018, durvalumab was FDA-approved as a second-line therapy (PACIFIC) [205]. The results obtained in these studies are described in more detail elsewhere [191,197,206,207].

Recently, in 2021, cemiplimab was introduced for first-line treatment for advanced NSCLC (EMPOWER-Lung 1) and, therefore, it is a subject of review herein [208]. Cemiplimab, a human IgG4 against PD1 mAb, was first approved in 2018 for the treatment of locally advanced and metastatic cutaneous squamous cell carcinoma (CSCC) patients who are not candidates for curative radiotherapy or surgery (NCT02383212 and NCT02760498) [209]. The approval was based on the results of two clinical trials involving 108 patients, which showed approximately half of the patients responded to the treatment. The treatment of cemiplimab was extended for use after first-line hedgehog inhibitor therapy (NCT03132 636) [210]. Henceforth, cemiplimab can be used as a first-line treatment of advanced NSCLC patients with PD-L1 expression of a least 50%. A significant improvement was found in the overall survival and the progression-free survival (PFS) in comparison to chemotherapy. Among the 563 patients with PD-L1 of a least 50%, the median OS has not been yet reached (95% CI 17.9—not evaluable) with cemiplimab versus 14.2 months (95% CI, 11.2–17.5) with chemotherapy (hazard ratio (HR), 0.57; 95% CI, 0.42–0.77; $p$ = 0.0002). The OS rates at 24 months were 50% and 27% in the investigate and the control arms, respectively. Moreover, the median PFS with cemiplimab was 8.2 months (95% CI, 6.1–8.8) versus 5.7 months (95% CI, 4.5–6.2) with chemotherapy, and the estimated PFS rates at 12 months were 21% and 7% in the investigative and the control arms, respectively. Patients treated with cemiplimab who had PD-L1 expression lower than 50% responded similarly to those treated with chemotherapy. The PD-L1 levels positively correlated with the improvements in OS and PFS. Adverse effects were observed in 28% and 39% of patients treated with cemiplimab and chemotherapy, respectively. Overall, cemiplimab was demonstrated to be a potential new treatment for NSCLC. ICIs antibodies can therefore inhibit PD-1/PD-L1 interaction or CTLA-4 immune checkpoints, improving antitumor immunity.

### 6.2. Combination Treatment Strategies

#### 6.2.1. Immune Checkpoint Inhibitor Combined with Chemotherapy

Chemotherapy treatment has shown that it can induce PD-L1 expression on tumor cells and, thus, the combination of immunotherapy and chemotherapy may produce a synergized effect and confer better survival outcome [211]. Chemotherapy is the first choice for patients that lack targetable driver mutations. Pembrolizumab targeting PD-1 was combined with chemotherapy in several clinical trials (KEYNOTE-021, KEYNOTE-189, and KEYNOTE 407). In 2018, the combination of pembrolizumab with pemetrexed and carboplatin was approved as a first-line treatment for metastatic non-squamous NSCLC patients with no driver mutation, irrespective of PD-L1 expression based on the results shown by KEYNOTE-021 [95]. A subsequent phase III trial concluded that the addition of pembrolizumab to chemotherapy resulted in longer OS and PFS than chemotherapy alone (KEYNOTE-189) [212]. Later, an expanded approval was obtained for the combination of pembrolizumab with carboplatin and placitaxel/nab-placitaxel for metastatic squamous NSCLC, irrespective of PD-L1 expression (Keynote-407) [213].

Several IMpower clinical trials demonstrated that atezolizumab combined with chemotherapy also produced synergistic effects and improved the efficacy over standard chemotherapy. Its combination with chemotherapy-based drugs (carboplatin, paclitaxel and bevacizumab) was approved by the FDA in 2018 for metastatic non-squamous NSCLC based on the IMpower150 phase III trial [214]. The quadrupole treatment prolonged PFS and OS for patients lacking *EGFR/ALK* mutations, independently of PD-L1 expression. The first phase III trial (IMpower130) demonstrated that atezolizumab combined with chemotherapy better improved the PFS and the OS compared to chemotherapy alone [215]. A second phase III trial showed no OS improvement when using combined treatment (atezolizumab plus carboplatin/nab-paclitaxel) for advanced-stage non-squamous NSCLC patients with no driver mutations but prolonged PFS (IMpower131) [216]. The next trial, the IMpower132 [217], used a combination of atezolizumab with pemetrexed and carboplatin/cisplatin and obtained similar conclusions as the IMpower131 trial. Moreover, atezolizumab combined with carboplatin and etoposide showed improved PFS and OS for first-line treatment of extensive-stage small cell lung cancer (IMpower133) [218]. As commented above, in 2021, cemiplimab was approved by the FDA for the treatment of patients with advanced NSCLC with PD-L1 expression of at least 50%. Moreover, a phase III trial (EMPOWER-Lung 3) showed a significantly improved OS in advanced or metastatic NSCLC by using first-line cemiplimab in combination with platinum chemotherapy (22 vs. 13 months, respectively). Additionally, this year, results from a phase III trial (POSEIDON) were released, which showed durvalumab, tremelimumab, and platinum-based chemotherapy provided OS benefit and significant improvement in PFS as compared to chemotherapy alone in metastatic NSCLC patients.

#### 6.2.2. Combined Immune Checkpoint Inhibitors: ICI PD-1/PD-L1 Combined with Anti-CTLA-4

In 2018, the efficacy of nivolumab in combination with ipilimumab, an ICI targeting CTLA4, was first demonstrated in a phase I trial (CheckMate 012) as a first-line treatment of advanced NSCLC [219]. A subsequent phase II trial (CheckMate 568) [220] identified tumor mutational burden (TMB) as a predictive biomarker to assess the efficacy of the combined therapy. Patients with TMB of 10 or more mutations/megabase were associated with improved response and prolonged PFS, independently of the PD-L1 expression. The phase III trial (CheckMate 227) observed a continued clinical benefit after 2 years of follow-up [221]. Durvalumab (ICI targeting PD-L1) with tremelimumab (a human monoclonal antibody against CTLA-4) was observed to improve the OS and the PFS in patients with metastatic NSCLC and PD-L1 expression lower than 25% (ARTIC trial) [222]. Another phase III trial (MYSTIC) considered patients with PD-L1 expression higher than 25% to evaluate the safety and the effectiveness of a dual immunotherapy combining durvalumab plus tremelimumab. In this study, there was no observed significant OS and

PFS improvement with combined ICIs vs. chemotherapy [223]. However, patients with TMB of 20 or more mutations/megabase were identified with improved OS with the dual immunotherapy [224].

Combined Immune Checkpoint Inhibitors with EGFR-TKI

Durvalumab was also combined with osimertinib (a third generation EGFR-TKI) in a phase III trial (CAURAL), however, the clinical trial was terminated early because of increased incidence of interstitial lung disease-like events [104,225]. In the CheckMate 012 trial, nivolumab was combined with erlotinib in patients with EGFR-mutated advanced NSCLC [226]. Further studies are required to find appropriate target patients that may benefit from such combinations [227].

### *6.3. Predictive Biomarkers*

Immunotherapy demonstrates great potential in canter treatment, and ICIs exhibit remarkable efficacy administered as monotherapy. Despite these achievements, only a small proportion of patients (~30%) benefit, and some of them develop resistance to anti-PD-1/PD-L1 immunotherapy [206]. Therefore, it is of great interest to identify biomarkers that can distinguish potential candidates that may benefit any immunotherapy or can predict effective responses to ICIs. The PD-L1 expression, the tumor mutational burden, and the MSI and/or the DNA MMR deficiency have been used as predictive biomarkers [180].

PD-L1 expression was shown as a predictive biomarker to select patients that can benefit from pembrolizumab treatment [228]. Immunohistochemistry (IHC) is currently used as a companion diagnostic test to estimate the expression levels of PD-L1, and several commercial kits for different epitopes were released (i.e., 22C3, 28-8, SP142, SP263, and 73-10) [229]. Each ICI uses a different antibody to estimate PD-L1 expression levels. Pembrolizumab uses 22C3 clone antibody, atezolizumab uses SP142 clone antibody, nivolumab uses 28-8 clone antibody, and durvalumab uses SP263 clone antibody. However, PD-L1 cannot yet be considered a fully sensitive and specific biomarker in clinical practice [207]. The lack of standardization of PD-L1 IHC assays represents a major source of uncertainty for PD-L1 testing. Moreover, the temporal and the spatial heterogeneity in the PD-L1 expression levels challenge its efficacy as a predictive biomarker. To date, only the 22C3 assay is required before initiating a first-line treatment with pembrolizumab monotherapy [230].

The measurement of MSI status and/or MMR deficiency was used as a predictive marker for response to PD-1 blockade by pembrolizumab [231]. Several clinical trials demonstrated a correlation between MMR deficiency and pembrolizumab efficacy for patients with multiple tumor types [232]. Consequently, pembrolizumab was approved by the FDA for the treatment of solid tumors with MMR deficiency [233]. Similar to PD-L1 expression, ICH is used for MMR detection. In particular, MSI needs to detect the expression of four MMR proteins (MLH1, MSH2, MSH6, and PMS2) [234]. MSI can be measured by PCR or NGS, the latter offering greater advantages over PCR-MS methods such as greater sensitivity or specificity.

TMB as MSI or MMR is an indicator of the genomic stability and is defined as the total number of mutations per megabase of DNA based on DNA sequencing [232,235]. New generation sequencing (NGS) technologies, including whole exome sequencing (WES) or large NGS panels, are used to determine the TMB. Based on the results in CheckMate 227 and CheckMate 026, TMB was suggested as a predictive biomarker for immunotherapy with nivolumab alone or in combination with ipilimumab [221]. However, TMB presents several limitations, including long test cycles, high cost, and standardization of the threshold for high- and low-TMB [230]. In 2020, TMB was approved by the FDA as a companion diagnostic biomarker for pembrolizumab [236].

## 7. Summary

We reviewed the latest research in global epidemiology, classification, molecular basis, targeted therapies, and immunotherapy in NSCLC. As the declining exposure of popula-

tion to tobacco correlates with the economic development of particular countries, cigarette smoking seems to be a smaller and smaller issue to consider. Nevertheless, the exceeding levels of air particulates contamination are alarming due to their reported link to the growing incidence of respiratory track cancers. The high mortality observed in NSCLC patients indicates the need for early diagnosis. The implementation of molecular techniques allows us to understand the biology and the evolution of lung cancer as well as to find reliable biomarkers, improving its diagnosis. The application of tumor cell genotyping from the blood of patients contributes to the discovery of new or the assessment of the nature of already known key mutations of NSCLC. Unfortunately, the heterogeneous nature of lung tumors adds a level of complexity to its analysis. Deciphering cell molecular pathways and the recent technological development contributed to significant advancements in characterization, and organization, and tumor heterogeneity. The trend of personalized medicine has become a permanent feature in which the correlation between the histopathological diagnosis and the identification of driver mutations is an imperative for the individual choice of therapy for patients with NSCLC. Such treatment largely depends on the stage of the disease. However, low 5-year survival rates, even in patients treated at an early stage, are commonly found. Thus, the patients with an identified tumor molecular profile are advised to enroll in numerous clinical trials. Nowadays, a battery of drugs for targeted therapy is available for most of the mutated proteins (EGFR family, ALK, ROS1, NTRK, and RAF). Notwithstanding, the treatment for the K-Ras protein remains challenging. Fortunately, only in the past year, three inhibitors (pralsetinib, selpercatinib, and capmatinib) for targeted therapy and two antibody-based drugs (atezolizumab and combination of nivolumab and ipilimumab) were approved, giving hope for the development of effective treatment strategies for mutations such as the ones in the K-Ras protein. Immunotherapy emerged as an unexpected new weapon against NSCLC, and a new area of research was established. In 2015, nivolumab (anti PD-1 monoclonal antibody) was approved by the FDA as a second-line therapy for patients with advanced NSCLC. Then, other immune checkpoint inhibitors (ICIs) were successively introduced as first- and second-line monotherapy treatments or were combined with standard chemotherapy. Despite the clinical benefits of immunotherapy, a major challenge remains in the identification of patients that respond to ICIs or those that eventually do not respond anymore. A comprehensive summary of the current immunotherapies and the predictive biomarkers approved by the FDA and ongoing clinical trials was discussed above. To conclude, the landscape of therapies in NSCLC is rapidly evolving, and, thus, accurate and updated reviews are of utmost need.

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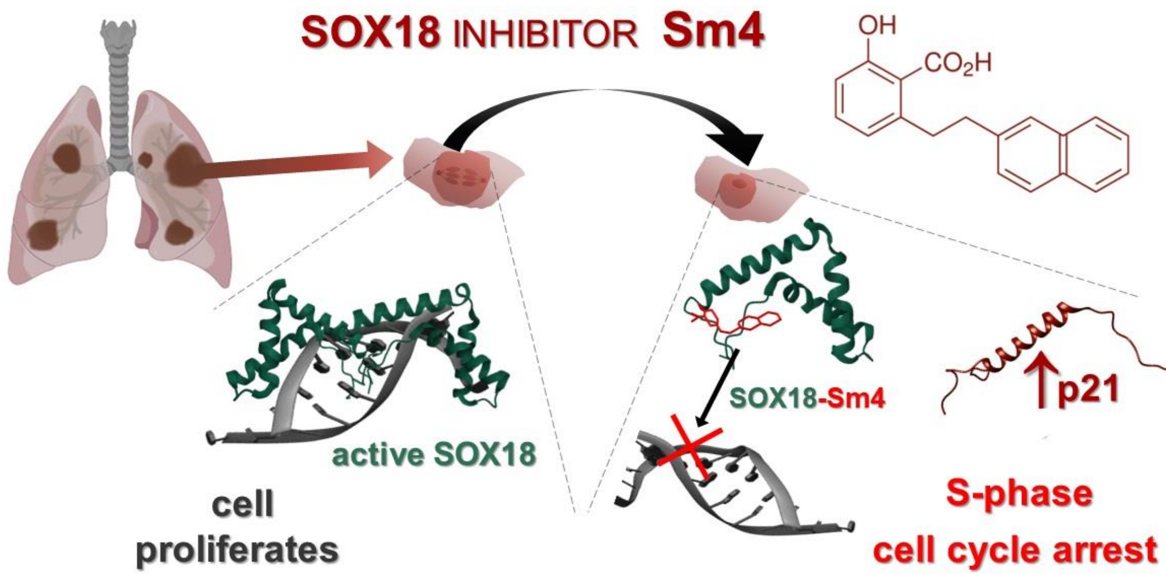

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# Targeting SOX18 Transcription Factor Activity by Small-Molecule Inhibitor Sm4 in Non-Small Lung Cancer Cell Lines

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**Abstract:** The transcription factor SOX18 has been shown to play a crucial role in lung cancer progression and metastasis. In this study, we investigated the effect of Sm4, a SOX18 inhibitor, on cell cycle regulation in non-small cell lung cancer (NSCLC) cell lines LXF-289 and SK-MES-1, as well as normal human lung fibroblast cell line IMR-90. Our results demonstrated that Sm4 treatment induced cytotoxic effects on all three cell lines, with a greater effect observed in NSCLC adenocarcinoma cells. Sm4 treatment led to S-phase cell accumulation and upregulation of p21, a key regulator of the S-to-G2/M phase transition. While no significant changes in SOX7 or SOX17 protein expression were observed, Sm4 treatment resulted in a significant upregulation of SOX17 gene expression. Furthermore, our findings suggest a complex interplay between SOX18 and p21 in the context of lung cancer, with a positive correlation observed between SOX18 expression and p21 nuclear presence in clinical tissue samples obtained from lung cancer patients. These results suggest that Sm4 has the potential to disrupt the cell cycle and target cancer cell growth by modulating SOX18 activity and p21 expression. Further investigation is necessary to fully understand the relationship between SOX18 and p21 in lung cancer and to explore the therapeutic potential of SOX18 inhibition in lung cancer.

**Keywords:** non-small lung cancer; adenocarcinoma; squamous carcinoma; transcription factors; SOX18; p21; cell cycle arrest; small-molecule inhibitor

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## 1. Introduction

SOX18 is a transcription factor (TF) belonging to the SOX family (sex determining region Y-related high-mobility group box), which is considered one of the key transcriptional regulators during embryogenesis [1]. The SOX family consists of over 20 proteins, including SOX18, SOX7, and SOX17, which constitute the SOXF subgroup responsible for the development of the cardiovascular system, lymphangiogenesis, and blood cell differentiation processes [2–5]. Moreover, their activity has been reported during wound healing, neovascularisation, or modulation of endothelial barrier integrity, while their expression is silenced in fully differentiated cells under physiological conditions [6–10]. SOXF proteins have drawn attention in cancer research due to their primarily angiogenic capabilities, and their expression levels have been found to be altered in cancer cells [11–13]. Furthermore, growing interest and published data have led to discoveries that SOX18 is involved in the cascades of the most important molecular pathways, such as Wnt/b-catenin, mTOR, or Notch1 signalling, regulating the majority of cancer-related processes [14–17]. The mode of

action of SOX18 has been described as a molecular switch for gene expression, achieved by binding to variable protein complexes or forming variable dimers, which modulate their target selectivity and/or activity [18,19]. While all SOXF TFs contain a dimerisation domain, SOX18 has the unique capability to form homodimers, which makes it stand out from other SOXF proteins as key endothelial transcription factor [20]. Despite this body of evidence, our understanding of the mechanisms of SOX18-mediated transcriptional regulation remains scattered, as every report regarding SOX18's contribution in carcinogenesis has focused on only a few specified proteins. Current evidence suggests that SOX18 has an oncogenic role in carcinogenesis, promoting migration, invasion, and proliferation in cell lines of various cancer origins, including bladder, breast, cervix, colon, larynx, liver, kidneys, osteosarcoma, pancreas, and prostate [14,16,21–35]. However, a study conducted on thyroid cancer cell lines reported that SOX18 acts as a tumour suppressor, indicating the complexity of its activity, which is likely dependent on the molecular context present in particular tumours [16]. Moreover, its unique mode of action as a homodimer may be crucial for the reported tumorigenic role of SOX18, as opposed to SOX7 and SOX17 [20]. In light of these findings, SOX18 appears to be an attractive molecular target for cancer research in studies aimed at treatment or molecular-based mechanistic investigations.

Investigating the mode of action of TFs is crucial due to their frequent differential expression in pathological conditions. In diseases, such as cancer, the survival of tumour cells requires the reprogramming of molecular network to maintain constant cell divisions during starvation or hypoxic conditions [36]. However, developing pharmacological molecules to target TF activity is very challenging since they are shielded by the nuclear membrane, and their three-dimensional structure is not fully resolved. Moreover, little is known about the complete spectrum of their interaction and resulting molecular responses [37]. Therefore, the investigation of TFs has mainly focused on gene expression modulation, such as gene silencing, knockdown, or overexpression [38–40]. However, recent studies have shown the potential of developing specific inhibitors for TFs. For instance, Francois group has reported the development of a specific inhibitor, small-molecule 4 (Sm4), that targets SOX18 TF [37]. Sm4 was derived from a natural product identified in brown alga extract and was found to be effective in disrupting homodimerisation as well as blocking other protein–protein interactions, resulting in the inhibition of transcriptional activity of SOX18 TF. Genomic, proteomic, and biophysical techniques were used to characterize Sm4's properties toward the SOX TFs family, and in vitro cytotoxic and luciferase reporter assays were performed at the COS-7 normal renal fibroblast cell line [37]. The biological responses were then evaluated in vivo, using a transgenic zebrafish reporter validated as a readout of the combinatorial activity of SOX7 and SOX18 and the implantation of 4T1.2 mammary carcinoma cells into a mouse model [41]. The study found that Sm4 significantly inhibited neovascularisation, resulting in a lower metastatic rate in the Sm4-treated group. However, the tumour size was not affected by the treatment, indicating that Sm4 has no effect on mouse carcinoma cell proliferation. Despite these findings, the biological responses dependent on SOX18 are highly influenced by the specific type of cancer. As a result, the outcomes may vary in other molecular contexts.

Non-small cell lung cancer (NSCLC) is constantly listed at the top of the world's epidemiological statistics, always presenting the highest mortality among other cancers [42]. The intricate nature of lung cancer biology hinders the effectiveness of therapeutic approaches targeting a single specific target. Although there are molecular aberrations that are frequently observed in NSCLC, targeting them alone is insufficient to fully eliminate cancerous and pre-cancerous cells from the body [43]. Additionally, NSCLC is often diagnosed in advanced or metastatic stages, and is highly metastatic, making it a significant challenge for treatment [44]. SOX18 has emerged as a potential novel target in NSCLC due to its involvement in neoangiogenesis and modulation of endothelial barrier integrity, which are important in cancer progression [13]. However, studies on SOX18 expression levels in NSCLC have yielded contradictory results. Our previous studies found variable SOX18 expression levels in NSCLC patients, with some exhibiting weak or strong

expression levels [21]. Notably, increased cytoplasmic SOX18 expression appeared to be a negative prognostic marker [21]. Recently published bioinformatic reports of public proteomic databases have revealed downregulation of SOX18 in NSCLC, with strong negative correlation of SOX family proteins expression with tumour hypoxia [17]. Epigenetic studies have also shed light on the contribution of SOX18 TFs in lung tumorigenesis. High methylation of *SOX18* promoter has been reported in few studies, which partially explains the low levels of *SOX18* mRNA found in lung cancer cohorts [45,46]. However, our research group has shown that reduced levels of certain microRNAs in NSCLC in comparison to normal lung transcriptome, increase *SOX18* transcript and activate protein synthesis [47,48]. This evidence in turn, may explain the high expression of nuclear and cytoplasmic SOX18 in NSCLC samples. Despite these findings, the full range of molecular activities of SOX18 in NSCLC remains under investigation for a better understanding of its contribution, role, and mode of action in the disease. Here, we investigated the impact of Sm4 as a SOX18 inhibitor in NSCLC cell lines.

## 2. Results

### *2.1. Sm4 Treatment Shows Comparable Cytotoxicity in NSCLC and IMR-90 Cell Lines*

To determine the cytotoxic effect of Sm4 (Figure 1A) on studied lung cancer cell lines, an MTT cell assay was performed. Post-hoc analysis revealed that Sm4 cytotoxicity was time- and concentration-dependent ($p < 0.001$) in all assayed cell lines (Figure 1B–D). The normal lung fibroblast cell line (IMR-90) was more sensitive to Sm4 treatment at 24 h than the cancer cell lines. The 50% cytotoxic concentration ($CC_{50}$) value of the fibroblast population was achieved at 74 ± 7 µM of Sm4, which was significantly lower than the values of 108 ± 5 µM and 111 ± 3 µM obtained in SK-MES-1 and LXF-289, respectively ($p < 0.001$). At longer incubation times, the IMR-90 and LXF-289 cell lines presented comparable resistance to Sm4 ($p = 0.76$). The results revealed $CC_{50}$ values to be different between all tested cell lines at the 72 h treatment period ($p < 0.05$), from which the most sensitive appeared to be the SK-MES-1 cell line. Based on the cytotoxicity results, 10 and 20 µM Sm4 treatments were selected for subsequent experiments.

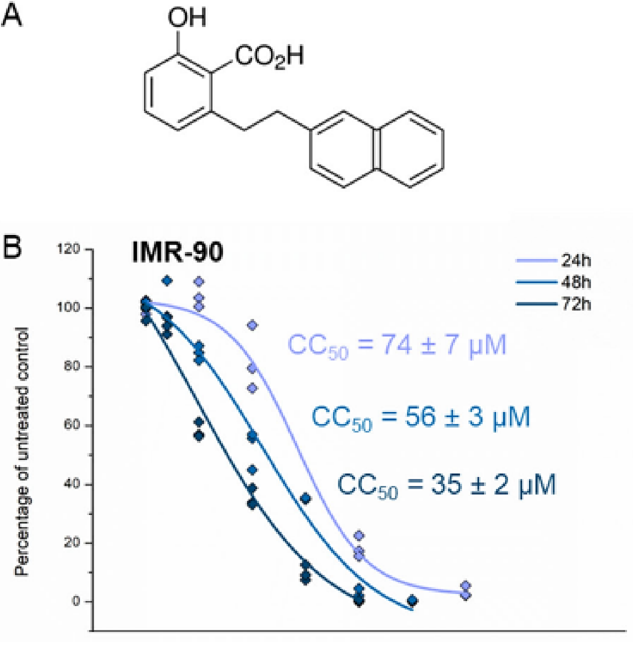


**Figure 1.** *Cont.*

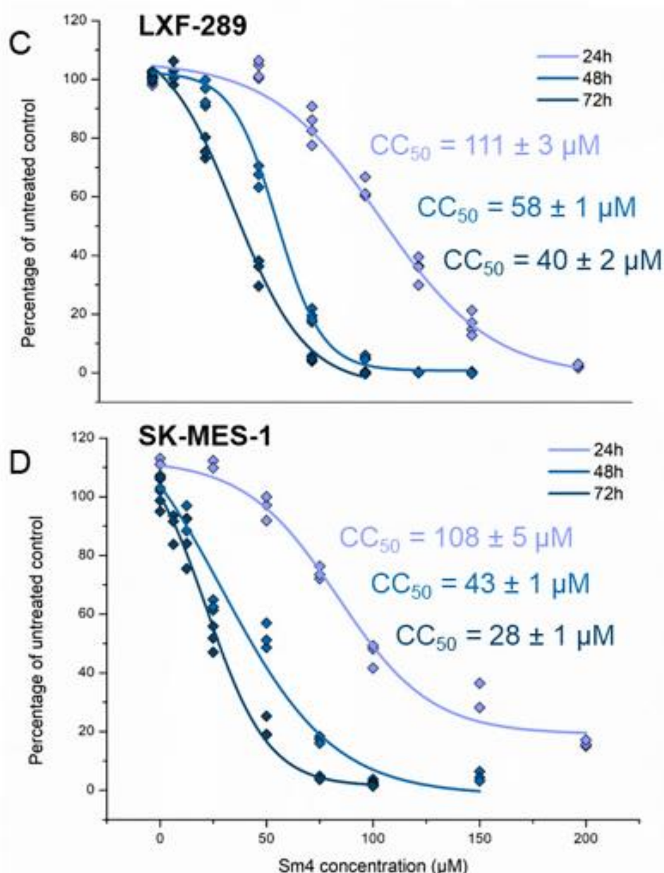


**Figure 1.** Sm4 effect on cells survival. (**A**) Sm4 chemical formula; cytotoxicity of Sm4 treatment for 24, 48, and 72 h in (**B**) lung fibroblasts IMR-90 cell line, (**C**) adenocarcinoma LXF-289 cell line, (**D**) squamous carcinoma SK-MES-1 cell line.

*2.2. Disruption of SOX18 Activity Alters Transcript Levels of SOX7 and SOX17 but Not Protein Expression*

The SOXF protein group is characterised by high homology among its members and is believed to have redundant functions in specific, not fully understood circumstances [13,17,49]. Therefore, our initial hypothesis was that Sm4 inhibition of SOX18 would trigger the expression of SOX17 or SOX7 proteins, leading to the activation of antiproliferative mechanisms.

In LXF-289 cells, addition of Sm4 induced the expression of *SOX7* and *SOX17* independently of the inhibitor treatment scheme, but *SOX18* was only increased upon one-time Sm4 treatment (Figure 2A). However, we did not detect an increase in protein levels (Figure 2B). In the SK-MES-1 cell line, SOX17 expression was increased upon Sm4 treatment, but this did not result in increased protein levels (Figure 2C). Interestingly, although *SOX18* transcription remained unaltered, we observed increased SOX18 protein levels (Figure 2C,D).

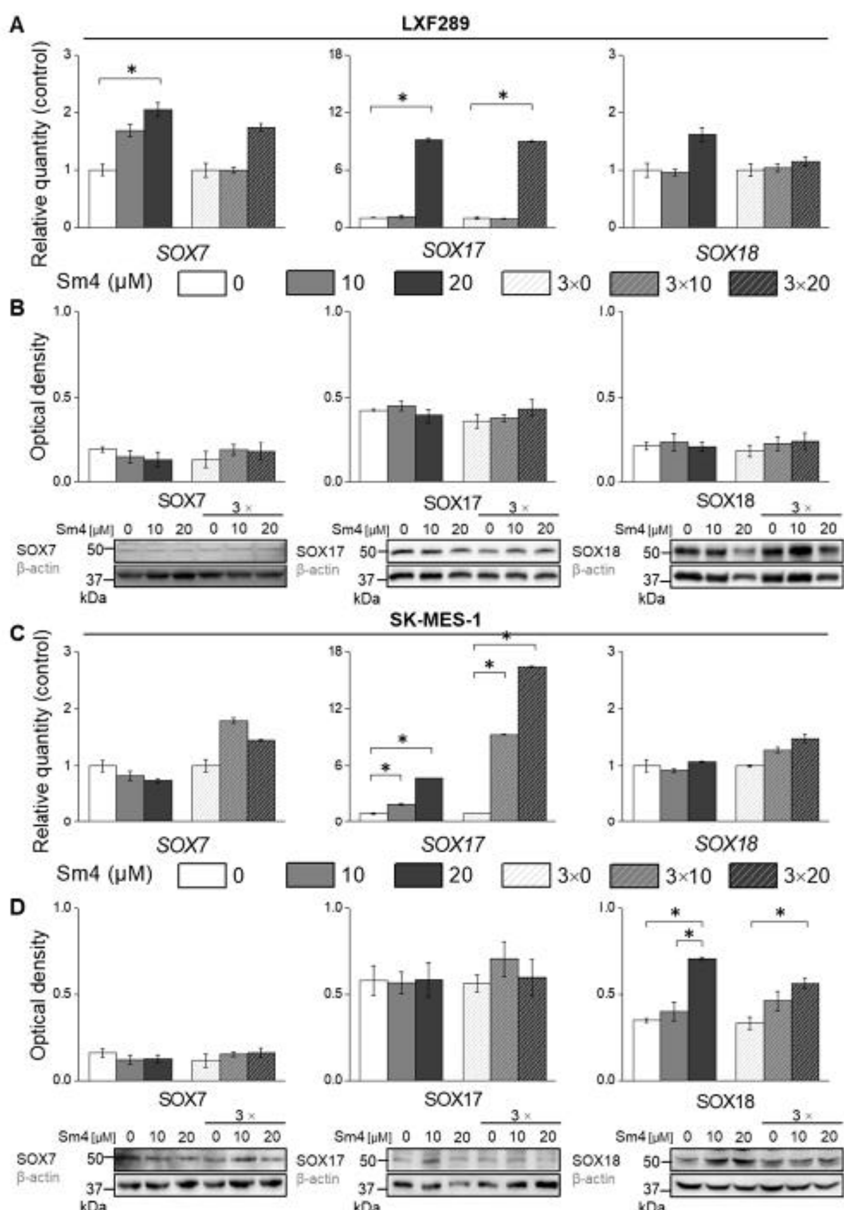


**Figure 2.** Gene expression analysis by qPCR (**A**,**D**) and protein levels by Western blot (**B**,**C**) of SOX7, SOX17, and SOX18 in LXF-289 (**A**,**B**) and SK-MES-1 cell lines (**C**,**D**). The statistical analysis of protein expression changes was based on densitometry results obtained from three independent experiments. The asterisk indicates $p < 0.05$.

*2.3. Sm4-Mediated Cell Cycle Arrest Exhibits a Prominent S-phase Cell Accumulation in LXF-289 Cell Line*

To evaluate putative cell cycle disruption triggered by Sm4, cancer cell lines were treated with 10 and 20 µM of the inhibitor for 72 h. The distribution of cell populations, in particular cell division phases, was evaluated. A significant S-phase arrest was observed in the LXF-289 adenocarcinoma cell line, with a complete disappearance of the G2/M

cell population in both treatment schemes by 20 µM Sm4 (Figure 3A,B,E,F). Similarly, treatment with 20 µM Sm4 increased the number of cells in the S-phase in SK-MES-1 cells, confirmed by a significant decrease in G1-phase population (Figure 3C,D,G,H). Results in both cell lines confirmed that the inhibitor showed significant effects on the progression of cell division phases at a concentration of 20 µM only. A one-time treatment of 10 µM Sm4 had no effect, while results from repeated treatment with 10 µM Sm4 presented a tendency toward S-phase arrest. Moreover, the cell cycle analysis performed in IMR-90 normal fibroblast cell line after 72 h from the Sm4 one-time exposure showed no differences between control and treated cells (Figure S1).

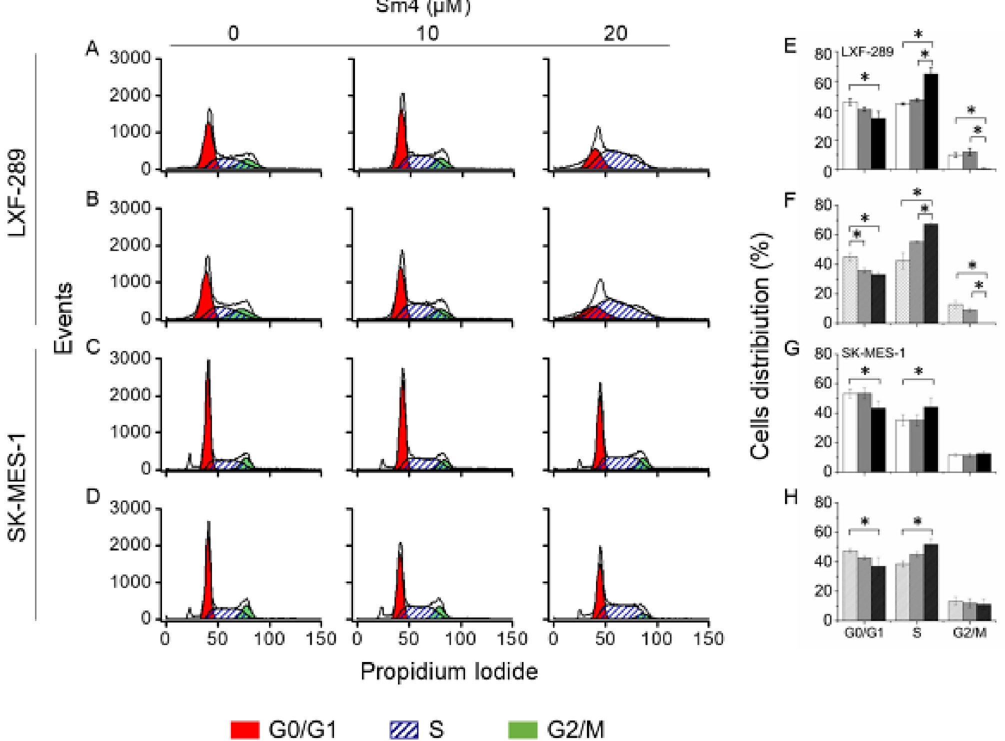


**Figure 3.** Histograms for cell cycle analysis for one- (**A**) or three-time treatment (**B**) of LXF-289 and one- (**C**) or three-time treatment (**D**) of SK-MES-1 cell lines at three Sm4 concentrations. Analysis of the cells distribution (%) are derived from the histograms (**E**–**H**). Presented results were obtained from three independent cell cycle measurements. The asterisk indicates $p < 0.05$.

### 2.4. *Differential Effects of Sm4 on Cyclin Expression in LXF-289 and SK-MES-1 Cell Lines*

The observed S-phase arrest caused alterations in the expression of proteins involved in cell cycle regulation, prompting the investigation of potentially implicated proteins due to SOX18 inhibition. As a result, the expression of cyclin D1, E, and A1 was assessed through Western blot analysis (Figure 4). In LXF-289 cells, independent of the inhibitor addition scheme, treatment with 20 µM Sm4 led to increased gene transcription of CCND1, CCNE, and CCNA1 (Figure 4A). However, despite the observed increase in gene transcription, a notable decrease in cyclin E and A1 levels was observed, as depicted in Figure 4B. Sm4 treatment resulted in the upregulation of CCNE and CCNA1 expression in the SK-MES-1 cell line, whereas CCND1 mRNA levels were not affected (Figure 4C). Furthermore, in SK-MES-1 cells, increased CCNE mRNA did not lead to a corresponding increase in protein levels (Figure 4D), while an increase in CCNA1 resulted in an increase in cyclin A1 protein levels.

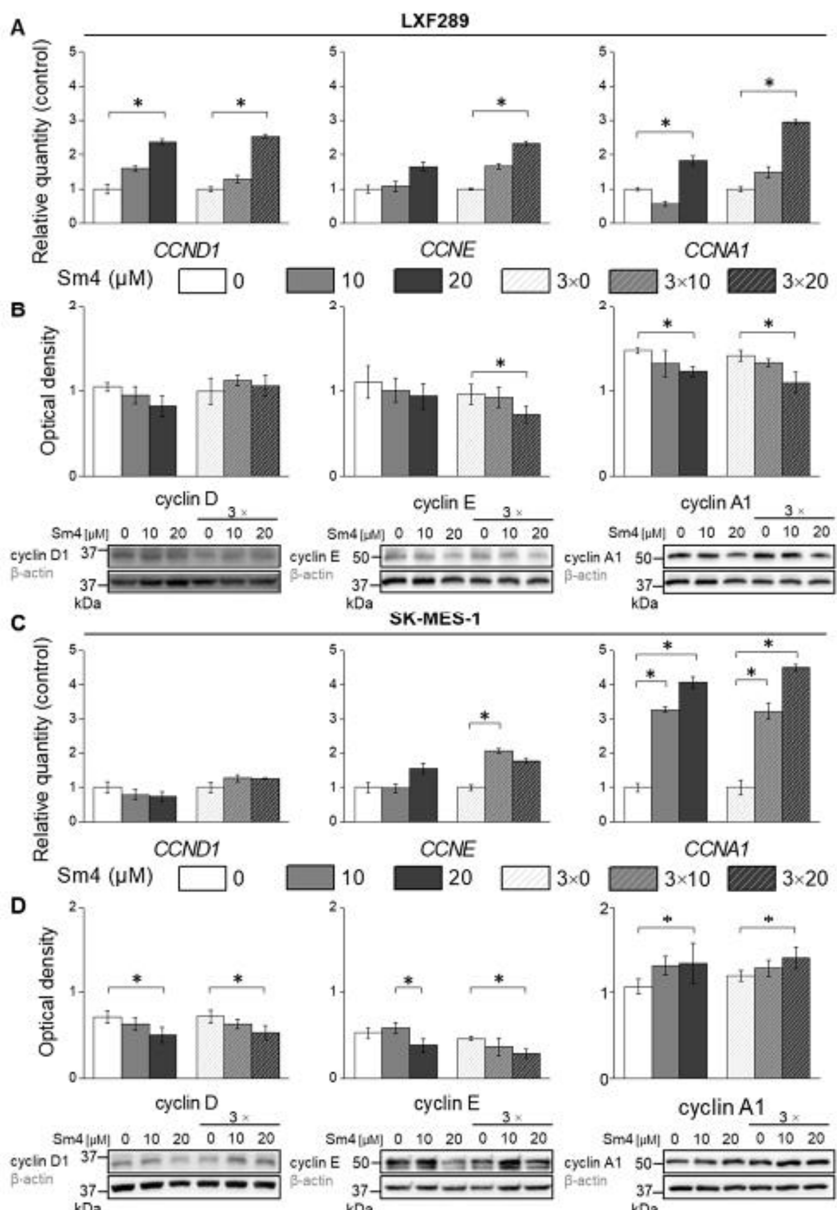


**Figure 4.** Gene expression analysis by qPCR of CCND1, CCNE, and CCNA1 and protein levels by Western blot of cyclin D, cyclin E, and cyclin A1 in LXF-289 (**A**,**B**) and SK-MES-1 cell lines (**C**,**D**). The statistical analysis of protein expression changes was based on densitometry results obtained from three independent experiments. The asterisk indicates $p < 0.05$.

*2.5. Sm4-Induced SOX18 Activity Inhibition Upregulated p21 Expression*

After observing S-phase arrest in both cell lines, we sought to investigate the p21, a key regulator of the transition from S to G2/M phase (Figure 5). The analysis of gene expression revealed a significant upregulation of CDKN1A expression, which subsequently translated into an increase in p21 protein levels upon treatment with 20 μM Sm4 in both cell lines.

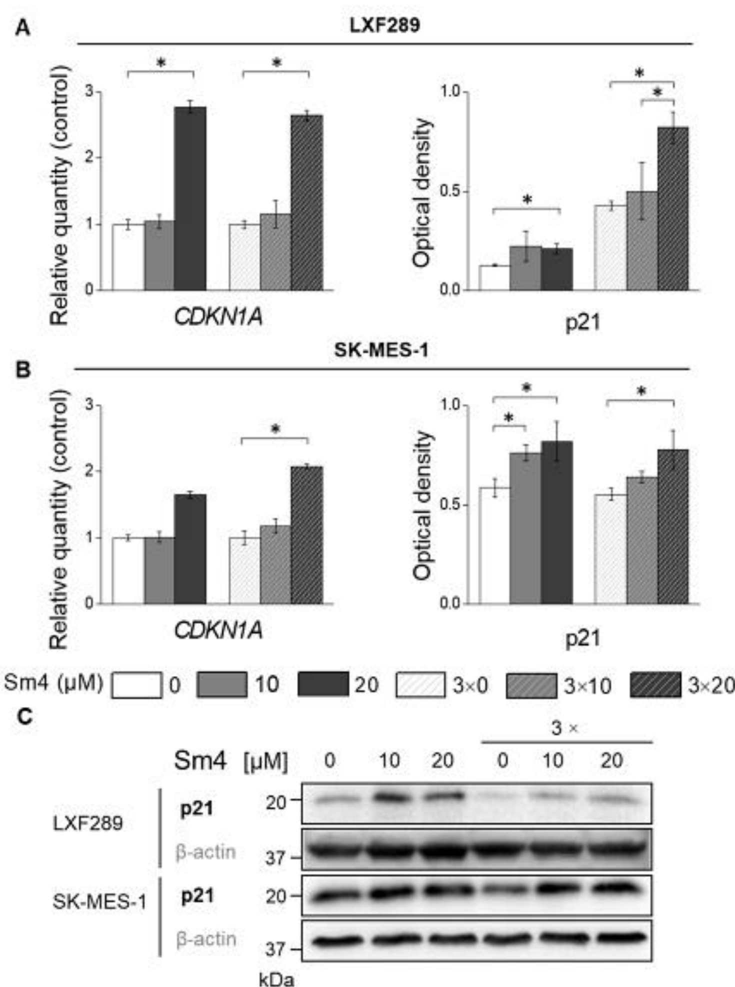


**Figure 5.** Gene expression analysis by qPCR of CDKN1A and protein levels by Western blot of p21 in LXF-289 (**A**) and SK-MES-1 cell lines (**B**). Western blot for p21 and β-actin in LXF-289 and SK-MES-1 cell lines for one- and three-time Sm4 treatment (**C**). The statistical analysis of protein expression changes was based on densitometry results obtained from three independent experiments and two technical repetitions ($n = 6$). The asterisk indicates $p < 0.05$.

### *2.6. SOX18 and p21 IHC Expression Levels Are Negatively Correlated in Lung Cancer Tissue Samples*

To check the expression patterns and intensity of SOX18 and p21 in clinical tissue samples, we performed evaluations based on IHC staining. Positive signal of SOX18 was found as nuclear or cytosolic and p21 was detected only in the nuclei (Figure 6A,C). Pearson correlation analysis of expression grading results revealed a negative correlation ($r = -0.27$) between nuclear SOX18 and nuclear p21 expression (Figure 6B), while a positive correlation ($r = 0.33$) between cytosolic SOX18 and nuclear p21 immunodetection levels (Figure 6D). Additionally, we performed a correlation analysis of *CDKN1A* and *p21* genes expression in lung using the publicly available RNA sequencing database OncoDB https://oncodb.org/ (accessed on 20 June 2023). Our analysis revealed a positive correlation between the expression of both genes, with correlation coefficients of $r = 0.22$ and $r = 0.21$ in adenocarcinoma and squamous cell lung cancer subtypes, respectively (Figure S2).

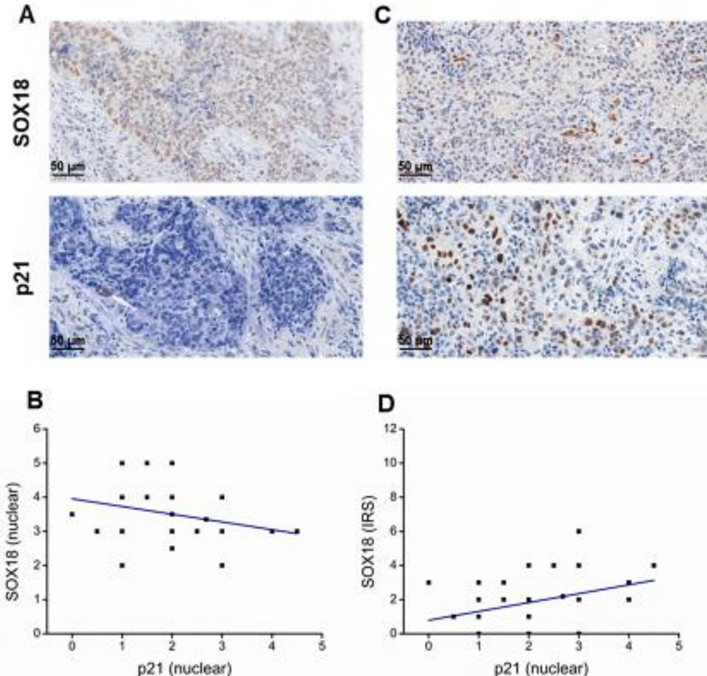


**Figure 6.** IHC of tissue samples ($n$ = 48) revealed correlations between p21 and SOX18. Representative pictures of nuclear p21 and SOX18 immunodetection (original magnification ×400). (**A**) Upper figure shows high expression of SOX18, while bottom figure shows singular immunodetection of p21 in the same sample. (**B**) Negative correlation plot between nuclear expressions of SOX18 and p21 observed in lung cancer tissue samples. (**C**) Upper figure presents high immunoreactive score of Remmele and Stegner (IRS) of SOX18, and bottom figure shows correlating high expression of nuclear p21 observed in the same sample. (**D**) Positive correlation plot between nuclear expressions of IRS SOX18 and p21 observed in lung cancer tissue samples.

## 3. Discussion

Previous studies indicated that SOX18 exhibited oncogenic activity in various types of cancers. Jethon et al. [13] indicated that expression of SOX18 correlated with poor patient outcome in non-small cell lung cancer. Sm4 is a novel compound that was identified through extensive screening for putative SOX18 inhibitors, as presented by Fontaine et al. [37]. Their investigation of the mode of action and target selectivity of Sm4 revealed that it highly depended on concentration. Cytotoxicity assays showed that Sm4 had a CC50 ranging from 50 to 100 µM in human embryonic kidney 293 cells and Hep G2 hepatocellular carcinoma cells, and a 24 h CC50 of 117 µM in COS-7 monkey kidney fibroblast-like cell line. These results suggest that Sm4 exhibits variable cytotoxicity depending on the cell line [37]. To date, there have been no studies investigating the effect of Sm4 treatment on cancer cells in vitro; therefore, this study attempted to examine this aspect.

The sensitivity of the IMR-90 cell line to Sm4 was greater than that of the cancer cell lines after 24 h of treatment. However, over time, the IMR-90 cell line developed increasing resistance to Sm4, and after 72 h, the cytotoxic effect of Sm4 was similar in all cell lines. It is noteworthy that there was a surge in the cytotoxicity of Sm4 in both cancer cell lines after 48 h, indicating that the inhibition of SOX18 activity, which affected cell function, required more than 24 h to take effect. The results of our study suggest that Sm4, a SOX18 inhibitor, has cytotoxic effect on both NSCL cell lines, LXF-289 and SK-MES-1, and IMR-90 cell lines, which could potentially limit its therapeutic value. However, in vivo studies have evaluated the efficacy of Sm4 treatment in mice with mammary gland carcinoma and have

not reported any toxicity or significant side effects [41,50]. Further research is required to better understand the mechanisms underlying the cytotoxic effects of Sm4 on lung cells and to determine its potential clinical utility.

The SOXF subgroup is composed of three closely related transcription factors that have been reported to be redundant and correlated with each other [2,12,17,51–53]. While SOX18 has been found to be overexpressed in tumours, SOX7 and SOX17 have been reported to be downregulated [9,12,54–57]. Furthermore, SOX7 and SOX17 have been demonstrated to possess antiproliferative functions in cancer cells [54–58]. Despite our initial hypothesis regarding the putative upregulation of SOX7 or SOX17 causing Sm4-mediated proliferation inhibition, our evaluation of Sm4-induced changes in gene and SOXF protein expression did not confirm this hypothesis. Although we detected a significant upregulation of *SOX17* gene expression, no changes in protein expression level were observed. These results prompted us to search for other molecular events responsible for cell cycle arrest.

The cell cycle assay revealed that LXF-289 cells underwent significant S-phase arrest, while treatment with 20 µM Sm4 increased the number of cells in S-phase and G2/M-phase in SK-MES-1. SOX18 has been studied for its role in cell cycle control, but results vary depending on the cancer type. In previous studies, *SOX18* silencing induced G0/G1 arrest in colorectal cancer, laryngeal, hepatocellular carcinoma, renal and blader cancer, while S-to-G2/M inhibition was observed in SOX18 knockdown osteosarcoma cells [14,16,21–35]. To shed more light on the observed disturbances of the cell cycle, we investigated the response of cyclins that controls the major events of cell cycle progression at the transcript and protein levels. The results showed downregulation of cyclin D and E, despite their increased gene expression, confirming the involvement of SOX18 in cell cycle progression. The difference in the observed cell cycle phases distribution between squamous cell carcinoma and lung and adenocarcinoma cell lines may be explained by the opposing results of cyclin A1 expression, which is responsible for the S-to-G2/M phase transition. This suggests that Sm4-mediated SOX18 inhibition can activate different pathways depending on the cell type, resulting in contradictory molecular behaviours. Our findings suggest that Sm4 has the potential to disrupt the cell cycle and target cancer cell growth.

Intriguingly, we observed an upregulation of SOX18 in SK-MES-1 cells following treatment with Sm4. This may be a response to the inhibited function of SOX18, resulting in the accumulation of not fully functional protein. Moreover, the observed upregulation could potentially provide an explanation for the relatively lower inhibitory effect of Sm4 on the cell cycle in SK-MES-1 cells compared to LXF-289 cells. In SK-MES-1 cells, a more notable accumulation in the S-phase was observed, while no significant differences in SOX18 concentration were detected between the two cell lines.

The S-to-G2/M phase transition in the cell division cycle is controlled by many proteins, among which p21 is considered one of the key regulators [59,60]. The p21 protein has been found to be upregulated in cancer cells as a molecular response to various small-molecule treatments that trigger S-phase arrest in different cancer cells [61–66]. Our results confirm significant Sm4-mediated p21 upregulation in both cancer cell lines, suggesting that modulation of SOX18 activity affects p21 expression and activity (Figure 7). The p21 protein is a low molecular weight macromolecule called a cyclin-dependent kinase inhibitor, presenting a variety of modes of action that orchestrate the progression of the cell division cycle [60,67]. Increased p21 expression and its nuclear accumulation overcome the strength of cyclin-kinases signalling by arresting the cell cycle at G1-phase or S-phase, as we have observed in our experiments (Figure 7) [68]. Zhu et al. reported the only study that has explored the relationship between SOX18 and p21 expression. Their study demonstrated that SOX18 silencing led to an increase in p21 expression, while SOX18 overexpression decreased p21 expression in osteosarcoma cells [35]. Notably, they observed G0/G1 cell cycle arrest, which, in conjunction with increased p53 expression, suggested that the inhibition of cell cycle progression might be mediated by the p21/p53 complex [35]. In contrast, our study was conducted on cell lines with p53 mutations, implying that the observed S-phase arrest might have occurred in a p53-independent manner. However, this pathway of cell

cycle arrest has not been further investigated in this work. The anticancer effects of p21 include the formation of a p21/PCNA complex, which successfully hinders the replication process, providing support for our findings [67–69].

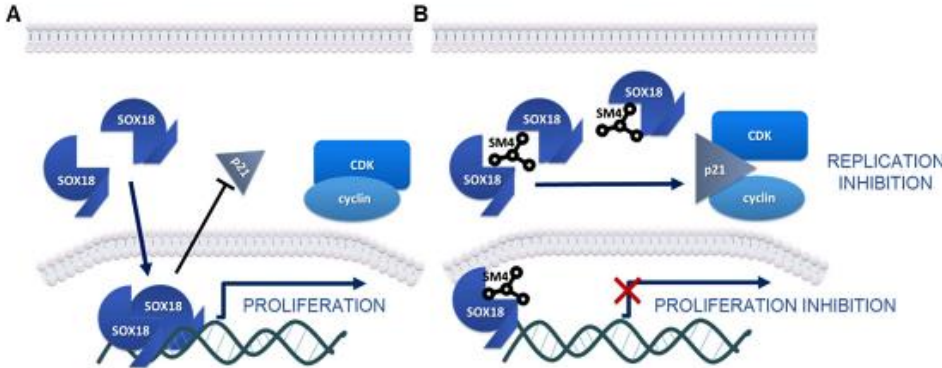


**Figure 7.** Schematic representation of the SOX18-dependent regulation of p21 expression and antiproliferative activity. (**A**) Functional SOX18 dimers control and induce the transcription of target genes that downregulate p21. (**B**) The inhibition of SOX18 homodimerisation ability mediated by Sm4 inhibits the transcription of specific genes, resulting in the upregulation of p21 and cell division arrest.

To reinforce our hypothesis that SOX18 may have a role in regulation of p21 expression in lung cancer cells, we performed an assessment of IHC staining in clinical tissue samples obtained from lung cancer patients. As a result, we demonstrated a correlation between SOX18 expression and p21 nuclear presence. It is important to note that the activity of a transcription factor, such as SOX18 depends on its location. Cytoplasmic staining indicates an inactive state of SOX18 [21,70]. This may explain the positive correlation between the IRS grading and p21 nuclear immunopositivity, as well as the negative correlation between active SOX18 found in nuclei and p21 in our assay. However, our analysis of *CDKN1A* and *p21* mRNA levels in lung tumours indicated a positive correlation. We hypothesise that the positive correlation may originate from epigenetic mechanism. Nevertheless, the observed correlation supports our results regarding their correlation. These findings suggest a complex interplay between SOX18 and p21 in the context of lung cancer (Figure 7). Further investigation is necessary to fully understand the relationship between these two proteins in the disease.

## 4. Materials and Methods

### 4.1. Materials

Inhibitor of transcription factor SOX18—small-molecule 4 (Sm4, Cat. SML1999) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and reconstituted in DMSO according to the manufacturer's recommendations as 2.5 mg/mL.

### 4.2. Experimental Design

Following the cytotoxicity assay, two concentrations of Sm4 were chosen for further experiments. The treatments were carried out for 72 h using two protocols: (i) Adding Sm4 at the desired concentration once, 24 h after plating the cells, or (ii) replacing the media with the inhibitor at 10 and 20 µM, or vehicle every 24 h. The aim of this methodology was to investigate whether a single exposure to Sm4 could inhibit SOX18 activity for 72 h or whether repeated treatments were necessary to achieve this effect.

### 4.3. Cell Culture

Two human lung cancer cell lines of two different and most frequent subtypes, i.e., LXF-289 (adenocarcinoma) and SK-MES-1 (squamous cell carcinoma) were purchased

from CLS (Cell Lines Service GmbH, Eppelheim, Germany) and normal lung fibroblast cell line was received from ATCC (Manassas, VA, USA). All cell lines were cultured in Eagle's Minimum Essential Medium (EMEM, Lonza, Basel, Switzerland), supplemented with 10% Fetal Bovine Serum, HEPES, L-glutamine, sodium purvate, non-essential amino acids, 100 units/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). All cells were maintained under 95% air and 5% $CO_2$ atmosphere at 37 °C in humidified incubator.

### 4.4. MTT Assay

Cytotoxicity of Sm4 was assessed using the MTT method. Cells were seeded into three 96-well plates at a concentration of $4 \times 10^3$/well. After 24 h, the media was replaced with media containing Sm4 at different concentrations (ranging from 5 to 200 µM). Each plate was then incubated with the treated media for different time periods: 24 h, 48 h, and 72 h. After incubation, the culture medium was removed and 100 µL of sterile MTT solution (0.5 mg/mL, Invitrogen, Thermo Fischer Scientific, Waltham, MA, USA) was added to each well. Following 4 h of incubation at 37 °C, the MTT solution was gently removed and all formed formazan crystals were dissolved in 100 µL of DMSO (Dimethyl Sulfoxide, Sigma-Aldrich, St. Louis, MO, USA) by shaking the plate for 10 min. Absorbance was measured at 460 nm using a microplate reader (Lx800, Bio-Tek, Winooski, VT, USA). The experiments were performed in triplicates.

### 4.5. Cell Cycle Assay

After a specific incubation time with the Sm4 inhibitor, cells were harvested using Trypsin-EDTA solution (0.25%, Sigma Aldrich, St. Louis, MO, USA) and then centrifuged. The cells were washed twice with cold PBS, suspended in 2 mL of 70% ice-cold ethanol solution for fixation, and subsequently centrifuged. The cells were then washed twice with cold PBS. Thereafter, the pellets were suspended and incubated in 300 µL of propidium iodide (PI)-RNAse A solution (Sigma Aldrich; Merck KGaA, St. Louis, MO, USA) for 30 min at 37 °C in the dark. DNA content was analysed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The obtained FCS files of PI spectra were assessed using ModFit LT 5.0 software (Verity Software House, Topsham, ME, USA). The experiments were performed in triplicates.

### 4.6. Gene Expression by qPCR

Cells were harvested from culture flasks, centrifuged, and washed twice with PBS. The obtained pellets were stored at −80 °C until all materials were collected. RNA isolation was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA was dissolved in RNA-pure water, and the concentration was measured using NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). For reverse transcription, 500 ng of RNA was used, and cDNA synthesis was performed using iScript cDNA synthesis kit (Bio-Rad, Marnes-la-Coquette, France) in a C1000 Touch thermal cycler (Bio-Rad, Marnes-la-Coquette, France). The TaqMan specific probes used in the experiment were purchased from Thermo Fischer Scientific (Waltham, MA, USA). The expression of the following genes was analysed: *SOX18* (Hs00746079_s1), *SOX17* (Hs00751752_s1), *SOX7* (00846731_s1), *CCNA1* (Hs00171105_m1), *CCND1* (Hs00765553_m1), *CCNE1* (Hs01026536_m1), *CDKN1A* (Hs00355782_m1), and *SDHA* (Hs00188166_m1) as a housekeeping gene. Gene expression was detected by qRT-PCR using a 7900HT Fast Real Time PCR System thermocycler with SDS 2.3 and RQ Manager 1.2 software (Applied Biosystems, Foster City, CA, USA). Data were processed into relative gene expression levels using the $2{-}\Delta\Delta Ct$ formula, where control samples (treated with control vehicle) were used as a calibrator. All reactions were performed in triplicates.

*4.7. Western Blot Analysis*

The collected cell pellets were dissolved in 100 µL of CelLytic™ MT Cell Lysis Reagent (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) supplemented with 1 µL of Proteinase Inhibitor Cocktail (100×) (Thermo-Fischer Scientific, Waltham, MA, USA) and 10 µL of 0.2 mM PMSF (Phenylmethanesulfonyl fluoride, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The lysates were then subjected to several fine needle aspirations for cell membrane mechanical disruption. Thereafter, the lysates were mixed on a vortex for 1 h at 4 °C, centrifuged, and the supernatant was collected as the final cell lysate. Protein concentration was evaluated by the Pierce BCA Protein Assay kit (Thermo Fischer Scientific, Waltham, MA, USA) according to the manufacturer's protocol, and colorimetric extinction was measured at Nanodrop (Thermo Fischer Scientific, Waltham MA, USA). The lysates were reconstituted to the target volume containing 30 µg of total protein or 50 µg in the case of particular protein detection (p21) and denatured for 10 min at 95 °C with the addition of SDS Sample Loading Buffer. Electrophoresis was performed in 6–10% polyacrylamide gels, and transferred to PVDF membrane by the wet transfer method. All membranes were blocked for 1 h in 4% BSA (Bovine Serum Albumin, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) solution at room temperature, followed by overnight incubation at 4 °C with primary antibodies, i.e., SOX7 (1:500, ab94397, Abcam, Cambridge, UK), SOX17 (1:1000, ab224637, Abcam), SOX18 (1:50, sc-166025, Santa Cruz Biotechnology, Dallas, TX, USA), cyclin D1 (1:500, AFH0082, Invitrogen, Waltham, MA, USA), cyclin E (1:1000, 32-1600, Invitrogen), cyclin A1 (1:1000, MAB7046, Novus Biologicals, Centennial, CO, USA), p21 (1:1000, 2946S, Cell Signalling, Danvers, MA, USA), β-actin (1:2000, Ab8224, Abcam).

An incubation with matching secondary antibody (Cat. 711-035-150, and 711-035-152, JacksonImmunoResearch, Bar Harbor, ME, USA) at concentration of 1:5000 was performed in room temperature for 90 min. Membrane washings were performed between every step three times for 10 min by 0.1% Tween in Tris Buffered Solution. The signal was detected using Luminata Classico or Forte chemiluminescent substrate (Merck KGaA, Darmstad, Germany). The visualisation and densitometry analysis was performed with the ChemiDoc Imaging System with the ImageLab software 4.1(Bio-Rad, Marnes-la-Coquette, France). The measurements included in the statistical analysis were obtained from three independent experiments.

*4.8. Immunohistochemistry (IHC)*

Paraffin blocks containing lung tumour tissue fragments were sectioned into 5 µm thick slices and mounted on Flex IHC Microscope Slides (K8020, Dako, Glostrup, Denmark). After deparaffinisation and rehydration, the epitopes were exposed by boiling at 97 °C for 20 min using the high-pH Target Retrieval Solution in Dako PT Link (Dako, Glostrup, Denmark). Endogenous peroxidase activity was blocked by incubating the sections in En-VisionTM FLEX Peroxidase-Blocking Reagent (Dako, Glostrup, Denmark) for 5 min at room temperature. The slides were then incubated with specific primary antibodies for 20 min at room temperature. Mouse anti-SOX18 antibody (sc-166025, Santa Cruz Biotechnology) diluted 1:25 in FLEX Antibody Diluent (Dako, Glostrup, Denmark) was used to detect SOX18 expression, and mouse anti-p21 antibody (1:1000, 2946S, Cell Signalling) was used for p21 detection. After washing with EnVision FLEX Wash Buffer (Dako, Glostrup, Denmark), the slides were incubated with a secondary antibody conjugated with EnVisionTM FLEX/horseradish peroxidase (HRP; Dako, Glostrup, Denmark) for 20 min at room temperature. Subsequently, the slides were treated with a peroxidase substrate, 3,3′-diaminobenzidine (DAB), for 10 min at room temperature and counterstained with hematoxylin (EnVisionTM FLEX Hematoxylin; Dako, Glostrup, Denmark) for 7 min at room temperature. Finally, the slides were dehydrated and coverslips were mounted. All IHC reactions were performed using the Dako Autostainer Link48 (Dako, Glostrup, Denmark). The negative control was prepared without the use of the primary antibody.

### 4.9. Evaluation of IHC Reactions

The expression of p21 and SOX18 was evaluated in 48 lung tumour tissue samples by two investigators (Mateusz Olbromski and Olga Rodak) using the BX-41 light microscope at ×200 magnification (Olympus, Tokyo, Japan). Three representative spots (1.5 mm diameter) from each tumour sample were assessed. The 12-point semi-quantitative immunoreactive score (IRS) according to Remmele and Stegner was used to evaluate the intensity of the cytoplasmic reaction [63]. This method combines two variables: The percentage of positive cells (0–4) and the intensity of the colour reaction (0–3). For the nuclear reaction, a 5-point scale was used based on the percentage of positive cells: 0 points (≤1%), 1 point (2–10%), 2 points (11–25%), 3 points (25–50%), 4 points (50–75%), and 5 points (>75% of positive cells).

### 4.10. Statistical Analysis

Results underwent statistical analysis, and their graphical presentation was performed in Origin 8.1 software. Data representing normal distribution and comparable variation were evaluated by ANOVA, followed by the post-hoc Tukey test with significance set at $p$-value < 0.05. To calculate the correlation between protein expression, the Pearson correlation coefficient method was applied.

## 5. Conclusions

Previous studies have reported that SOX18 is overexpressed in various types of cancers and is associated with poor patient outcomes. Sm4, a novel compound, has been identified as a potential inhibitor of SOX18. The observed cytotoxic concentration of Sm4 in both lung cancer and normal cell lines was consistent with previous reports, indicating a similar order of magnitude of inhibitory/cytotoxic concentration values. Contrary to our initial hypothesis, the antiproliferative effect of SOX18 inhibition was not caused by upregulation of SOX7 or SOX17. Sm4-mediated cell cycle arrest showed S-phase cell accumulation, which differed among squamous cell carcinoma and lung and adenocarcinoma cell lines due to the opposing results of cyclin A1 expression. Sm4 has the potential to disrupt the cell cycle and target cancer cell growth, potentially by modulating p21 expression. Our findings suggest a complex interplay between SOX18 and p21 in the context of lung cancer, and further investigation is necessary to fully understand the relationship between these two proteins in the disease.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms241411316/s1.

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# 4. PODSUMOWANIE I WNIOSKI

W świetle aktualnego stanu wiedzy potencjał hamowania aktywności czynnika transkrypcyjnego SOX18 w raku płuc nie został oceniony. Badania mające na celu poszukiwania nowych celów terapeutycznych dla pacjentów onkologicznych ze zdiagnozowanym rakiem płuc, w tym badania białek z grupy czynników transkrypcyjnych, są wysoce pożądane, gdyż pośród wszystkich nowotworów, te wywodzące się z tkanki płucnej cechują się najwyższą śmiertelnością na całym świecie. Epidemiologia choroby wskazuje na szereg czynników predysponujących raka płuc do bycia najczęściej diagnozowanym nowotworem, tj. palenie wyrobów tytoniowych, zanieczyszczenie powietrza, późny rozwój objawów klinicznych, oraz utrudniony dostęp do badań przesiewowych w różnych częściach świata. Z kolei sama patogeneza NRDP na poziomie molekularnym nie została wciąż w pełni wyjaśniona, skutecznie utrudniając jego wczesną diagnostykę oraz skuteczne leczenie. Stąd, tak ważne jest prowadzenie badań molekularnych z wykorzystaniem modelów *in vitro* oraz *in vivo*, których celem jest ocena potencjału terapeutycznego mechanizmów hamowania aktywności poszczególnych białek, co przekłada się również na wartość poznawczą. Istotą niniejszej pracy doktorskiej było podsumowanie dotychczasowej wiedzy, dotyczącej raka płuc oraz poznanie mechanizmów molekularnych, wywołanych hamowaniem aktywności czynnika transkrypcyjnego SOX18 w komórkach raka płuc.

Rezultatem doświadczeń przeprowadzonych na liniach komórkowych gruczolakoraka i płaskonabłonkowego raka płuc, jak również oceny immunohistochemicznej materiału tkankowego guzów płuc są następujące wnioski:

1. Działanie cytotoksyczne badanego związku jest zbliżone w obu badanych liniach komórkowych raka płuc.
2. Efekt antyproliferacyjny wnikający z zahamowania aktywności białka SOX18 nie jest spowodowany wzrostem ekspresji białek SOX7 lub SOX17.
3. Hamowanie aktywności SOX18 ma wpływ na nasilenie ekspresji genów *SOX7* oraz *SOX17*, który prawdopodobnie w wyniku mechanizmów epigenetycznych, nie przekłada się na wzrost poziomu białek SOX7 i SOX17.

4. Inhibitor Sm4 powoduje istotne zahamowanie cyklu komórkowego poprzez zatrzymanie komórek nowotworowych w fazie S (replikacji), szczególnie linii gruczolakoraka płuc.
5. Stopień zahamowania cyklu komórkowego w czasie replikacji DNA różni się między badanymi podtypami raka płuc, co może wynikać z odmiennego wpływu zahamowania białka SOX18 na ekspresję cykliny A1.
6. Hamowanie aktywności białka SOX18 inhibitorem Sm4 wykazuje potencjał antyproliferacyjny w komórkach niedrobnokomówkowych raków płuc, poprzez indukcję ekspresji białka p21.

Powyższe wnioski mogą świadczyć o terapeutycznym potencjale inhibitora Sm4 w leczeniu niedrobnokomórkowych raków płuc. Dotychczasowe doniesienia naukowe potwierdzają skuteczność badanego związku w leczeniu mięsaka Kaposiego, który wywodzi się z śródbłonka naczyń, a wywołany jest przez herpeswirus [49,50]. Dodatkowo, wykazano, iż dezaktywacja białka SOX18 inhibitorem Sm4 hamuje cykl życiowy herpeswirusa na etapie replikacji wirusowego genomu w komórce gospodarza, podobnie jak w przypadku DNA komórek nowotworowych badanych w niniejszej pracy doktorskiej [49]. Przedstawiona praca jest pierwszą, w której zastosowano drobnocząsteczkowy inhibitor Sm4 w celu hamowania proliferacji komórek linii niedrobnokomórkowych raków płuc. Stąd, w przyszłości należy poszerzyć zakres badań w celu poznania mechanizmów na poziomie epigenetycznym wywołanych działaniem inhibitora, jak również przeprowadzenie doświadczeń z zastosowaniem innych modeli badawczych, tj. hodowle komórek nowotworowych pochodzących od pacjentów (modele *ex vivo*), lub badań z użyciem zwierząt laboratoryjnych (modele *in vivo*).

## 5. ZAŁĄCZNIKI

I. OŚWIADCZENIA WSPÓŁAUTORÓW PUBLIKACJI STANOWIĄCYCH PODSTAWĘ PRACY DOKTORSKIEJ

II. OPINIA KOMISJI BIOETYCZNEJ

III. DOROBEK NAUKOWY

I.

Wrocław, 13.09.2023

dr Manuel Peris Diaz
Zakład Chemii Biologicznej
Wydział Biotechnologii
Uniwersytet Wrocławski

OŚWIADCZENIE

Oświadczam, że w pracy: **Rodak, O., Peris-Díaz, M., Olbromski, M., Podhorska-Okołów, M., Dzięgiel, P. Current Landscape of Non-Small Cell Lung Cancer: Epidemiology, Histological Classification, Targeted Therapies, and Immunotherapy. Cancers. 2021; 13(18): 4705** mój udział polegał współtworzeniu rozdziału na temat immunoterapii nowotworów płuc oraz weryfikacji językowej pracy.

Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Olgi Rodak pt. „Antyproliferacyjne działanie inhibitora czynnika transkrypcyjnego SOX18 w niedrobnokomórkowym raku płuc".

dr Manuel Peris Diaz

Manuel Peris

Wrocław 13.09.2023

dr Mateusz Olbromski
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy: **Rodak, O., Peris-Díaz, M., Olbromski, M., Podhorska-Okołów, M., Dzięgiel, P. Current Landscape of Non-Small Cell Lung Cancer: Epidemiology, Histological Classification, Targeted Therapies, and Immunotherapy. Cancers. 2021; 13(18): 4705** mój udział polegał na opracowaniu koncepcji pracy oraz korekcie pracy przed złożeniem do druku.

Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Olgi Rodak pt. „Antyproliferacyjne działanie inhibitora czynnika transkrypcyjnego SOX18 w niedrobnokomórkowym raku płuc".

Mateusz Olbromski
dr Mateusz Olbromski

Wrocław 13.09.2023

Prof. dr hab. Marzenna Podhorska-Okołów
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

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M. Podhorska Okołów

Prof. dr hab. Marzenna Podhorska-Okołów

Wrocław 13.09.2023

Prof. dr hab. Piotr Dzięgiel
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy: **Rodak, O., Peris-Díaz, M., Olbromski, M., Podhorska-Okołów, M., Dzięgiel, P. Current Landscape of Non-Small Cell Lung Cancer: Epidemiology, Histological Classification, Targeted Therapies, and Immunotherapy. Cancers. 2021; 13(18): 4705** mój udział polegał na konsultacji i opiece merytorycznej oraz korekcie pracy przed złożeniem do druku.

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Prof. dr hab. Piotr Dzięgiel

Wrocław 13.09.2023

mgr Monika Mrozowska
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy: **Rodak, O., Mrozowska M., Rusak, A., Gomułkiewicz, A., Piotrowska, A., Olbromski, M., Podhorska-Okołów, M., Ugorski, M., Dzięgiel, P. Targeting SOX18 Transcription Factor Activity by Small-Molecule Inhibitor Sm4 in Non-Small Lung Cancer Cell Lines. Int J Mol Sci. 2023 Jul 11; 24(14):11316** mój udział polegał na współudziale w doświadczeniach prowadzonych w pracowni badań molekularnych.

Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Olgi Rodak pt. „Antyproliferacyjne działanie inhibitora czynnika transkrypcyjnego SOX18 w niedrobnokomórkowym raku płuc".

mgr Monika Mrozowska

Wrocław 13.09.2023

dr Agnieszka Rusak
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

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Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Olgi Rodak pt. „Antyproliferacyjne działanie inhibitora czynnika transkrypcyjnego SOX18 w niedrobnokomórkowym raku płuc".

dr Agnieszka Rusak

A. Rusak

Wrocław 13.09.2023

dr Agnieszka Gomułkiewicz
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

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Oświadczam, że w pracy: **Rodak, O., Mrozowska M., Rusak, A., Gomułkiewicz, A., Piotrowska, A., Olbromski, M., Podhorska-Okołów, M., Ugorski, M., Dzięgiel, P. Targeting SOX18 Transcription Factor Activity by Small-Molecule Inhibitor Sm4 in Non-Small Lung Cancer Cell Lines. Int J Mol Sci. 2023 Jul 11; 24(14):11316** mój udział polegał na współudziale w doświadczeniach prowadzonych w pracowni badań molekularnych.

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dr Agnieszka Gomułkiewicz

Agnieszka Gomułkiewicz

Wrocław 13.09.2023

dr Aleksandra Piotrowska
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy: **Rodak, O., Mrozowska M., Rusak, A., Gomułkiewicz, A., Piotrowska, A., Olbromski, M., Podhorska-Okołów, M., Ugorski, M., Dzięgiel, P. Targeting SOX18 Transcription Factor Activity by Small-Molecule Inhibitor Sm4 in Non-Small Lung Cancer Cell Lines. Int J Mol Sci. 2023 Jul 11; 24(14):11316** mój udział polegał na przeprowadzeniu reakcji immunohistochemicznych oraz wsparciu merytorycznym w analizie uzyskanych wyników.

Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Olgi Rodak pt. „Antyproliferacyjne działanie inhibitora czynnika transkrypcyjnego SOX18 w niedrobnokomórkowym raku płuc".

dr Aleksandra Piotrowska

Wrocław 13.09.2023

dr Mateusz Olbromski
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

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Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Olgi Rodak pt. „Antyproliferacyjne działanie inhibitora czynnika transkrypcyjnego SOX18 w niedrobnokomórkowym raku płuc".

Mateusz Olbromski
dr Mateusz Olbromski

Wrocław 13.09.2023

Prof. dr hab. Marzenna Podhorska-Okołów
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

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M. Podhorska-Okołów

Prof. dr hab. Marzenna Podhorska-Okołów

Wrocław, 13.09.2023

prof. dr hab. Maciej Ugorski
Katedra Biochemii i Badań Molekularnych
Uniwersytet Przyrodniczy we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy: **Rodak, O., Mrozowska M., Rusak, A., Gomułkiewicz, A., Piotrowska, A., Olbromski, M., Podhorska-Okołów, M., Ugorski, M., Dzięgiel, P. Targeting SOX18 Transcription Factor Activity by Small-Molecule Inhibitor Sm4 in Non-Small Lung Cancer Cell Lines. Int J Mol Sci. 2023 Jul 11; 24(14):11316** mój udział polegał na opiece merytorycznej oraz korekcie pracy przed złożeniem do druku.

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prof. dr hab. Maciej Ugorski

Wrocław 13.09.2023

Prof. dr hab. Piotr Dzięgiel
Zakład Histologii i Embriologii
Katedra Morfologii i Embriologii Człowieka
Uniwersytet Medyczny we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy: **Rodak, O., Mrozowska M., Rusak, A., Gomułkiewicz, A., Piotrowska, A., Olbromski, M., Podhorska-Okołów, M., Ugorski, M., Dzięgiel, P. Targeting SOX18 Transcription Factor Activity by Small-Molecule Inhibitor Sm4 in Non-Small Lung Cancer Cell Lines. Int J Mol Sci. 2023 Jul 11; 24(14):11316** mój udział polegał na konsultacji i opiece merytorycznej, interpretacji wyników, zdobyciu środków finansowych oraz korekcie pracy przed złożeniem do druku.

Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Olgi Rodak pt. „Antyproliferacyjne działanie inhibitora czynnika transkrypcyjnego SOX18 w niedrobnokomórkowym raku płuc".

Prof. dr hab. Piotr Dzięgiel

II.

1

KOMISJA BIOETYCZNA
przy
Uniwersytecie Medycznym
we Wrocławiu
ul. Pasteura 1; 50-367 WROCŁAW

OPINIA KOMISJI BIOETYCZNEJ Nr KB – 752/2020

Komisja Bioetyczna przy Uniwersytecie Medycznym we Wrocławiu, powołana zarządzeniem Rektora Uniwersytetu Medycznego we Wrocławiu nr 133/XV R/2017 z dnia 21 grudnia 2017 r. oraz działająca w trybie przewidzianym rozporządzeniem Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. (Dz.U. nr 47, poz. 480) na podstawie ustawy o zawodzie lekarza z dnia 5 grudnia 1996 r. (Dz.U. nr 28 z 1997 r. poz. 152 z późniejszymi zmianami ) w składzie:

prof. dr hab. Jacek Daroszewski (choroby wewnętrzne, endokrynologia, diabetologia)
prof. dr hab. Krzysztof Grabowski (chirurgia)
dr Henryk Kaczkowski (chirurgia szczękowa, chirurgia stomatologiczna)
mgr Irena Knabel-Krzyszowska (farmacja)
prof. dr hab. Jerzy Liebhart (choroby wewnętrzne, alergologia)
ks. dr hab. Piotr Mrzygłód, prof. nadzw. (duchowny)
mgr Luiza Műller (prawo)
dr hab. Sławomir Sidorowicz (psychiatria)
prof. dr hab. Leszek Szenborn, (pediatria, choroby zakaźne)
Danuta Tarkowska (pielęgniarstwo)
prof. dr hab. Anna Wiela-Hojeńska (farmakologia kliniczna)
dr hab. Andrzej Wojnar, prof. nadzw. (histopatologia, dermatologia) przedstawiciel Dolnośląskiej Izby Lekarskiej)
dr hab. Jacek Zieliński (filozofia)

pod przewodnictwem
prof. dr hab. Jana Kornafela ( ginekologia i położnictwo, onkologia)

Przestrzegając w działalności zasad Good Clinical Practice oraz zasad Deklaracji Helsińskiej, po zapoznaniu się z projektem badawczym pt.

„Określenie epigenetycznych mechanizmów modulacji wybranych genów z rodziny *SOX* oraz ich transkryptów jako potencjalnych markerów diagnostycznych i predykcyjnych w niedrobnokomórkowych rakach płuc"

zgłoszonym przez **prof. dr hab. Piotra Dzięgiela** zatrudnionego w Katedrze Morfologii i Embriologii Człowieka Uniwersytetu Medycznego we Wrocławiu oraz złożonymi wraz z wnioskiem dokumentami, w tajnym głosowaniu postanowiła wyrazić zgodę na przeprowadzenie badania w Zakładzie Histologii i Embriologii Katedry Morfologii i Embriologii Człowieka Uniwersytetu Medycznego we Wrocławiu **pod warunkiem zachowania anonimowości uzyskanych danych**.

Uwaga: Badanie to zostało objęte ubezpieczeniem odpowiedzialności cywilnej Uniwersytetu Medycznego we Wrocławiu z tytułu prowadzonej działalności:

Pouczenie: W ciągu 14 dni od otrzymania decyzji wnioskodawcy przysługuje prawo odwołania do Komisji Odwoławczej za pośrednictwem Komisji Bioetycznej UM we Wrocławiu

Opinia powyższa dotyczy: projektu badawczego finansowanego z grantu Narodowego Centrum Nauki

Numer rejestrowy CWN UMW: OPUS.A350.19.002

Wrocław, dnia 26 listopada 2020 r.

BW

Uniwersytet Medyczny we Wrocławiu
KOMISJA BIOETYCZNA
przewodniczący
prof. dr hab. Jan Kornafel

III.

## A. Publikacje naukowe:

1. Rodak, O., Mrozowska, M., Rusak, A., Gomułkiewicz, A., Piotrowska, A., Olbromski, M., Podhorska-Okołów, M., Ugorski, M., & Dzięgiel, P. (2023). Targeting SOX18 Transcription Factor Activity by Small-Molecule Inhibitor Sm4 in Non-Small Lung Cancer Cell Lines. *International Journal of Molecular Sciences 2023, Vol. 24, Page 11316*, *24*(14), 11316. https://doi.org/10.3390/IJMS241411316
2. Rodak, O., Peris-Díaz, M. D., Olbromski, M., Podhorska-Okołów, M., & Dzięgiel, P. (2021). Current Landscape of Non-Small Cell Lung Cancer: Epidemiology, Histological Classification, Targeted Therapies, and Immunotherapy. *Cancers 2021, Vol. 13, Page 4705*, *13*(18), 4705. https://doi.org/10.3390/CANCERS13184705
3. Peris-Díaz, M. D., Rodak, O., Sweeney, S. R., Krężel, A., & Sentandreu, E. (2019). Chemometrics-assisted optimization of liquid chromatography-quadrupole-time-of-flight mass spectrometry analysis for targeted metabolomics. *Talanta*, *199*. https://doi.org/10.1016/j.talanta.2019.02.075
4. Peris-Díaz, M. D., Sweeney, S. R., Rodak, O., Sentandreu, E., & Tiziani, S. (2019). R-metabolist 2: A flexible tool for metabolite annotation from high-resolution data-independent acquisition mass spectrometry analysis. *Metabolites*, *9*(9). https://doi.org/10.3390/metabo9090187
5. Przadka, P., Liszka, B., Piatek, A., Skrzypczak, P., Dzimira, S., Nizanski, W., Rodak, O., Kinda, W., Biezynski, J., & Kielbowicz, Z. (2019). Total prostatectomy combined with urethral anastomosis in a dog: A case report. *Veterinarni Medicina*, *64*(6). https://doi.org/10.17221/134/2018-VETMED
6. Rodak, O., Dzimira, S., Podolak, A., Płóciennik, M., & Niżański, W. (2018). Accuracy of ultrasonography and fine-needle aspiration cytology in the diagnosis of prostate diseases in dogs. *Reproduction in Domestic Animals*, *53*. https://doi.org/10.1111/rda.13341
7. Nowak, A., Kochan, J., Niżański, W., Partykab, A., Kozdrowski, R., Rodak, O., Tarnowska, M., Młodawska, W., Migdał, A., & Witkowski, M. (2018). Influence of Dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin) on the In Vitro Characteristics of Equine Gametes. *Journal of Equine Veterinary Science*, *61*. https://doi.org/10.1016/j.jevs.2017.11.002
8. Partyka, A., Rodak, O., Bajzert, J., Kochan, J., & Nizański, W. (2017). The Effect of L-Carnitine, Hypotaurine, and Taurine Supplementation on the Quality of Cryopreserved Chicken Semen. *BioMed Research International*, https://doi.org/10.1155/2017/7279341

## B. Doniesienia konferencyjne:

1. Rodak, O., Olbromski, M., Mrozowska, M., Rusak, A., Gomułkiewicz, A., Dzięgiel P., „Inhibition of SOX18 homodimerization properties of by Small-Molecule 4

induces S-phase cell cycle arrest triggering p21 upregulation" EACR Conference 2023, 12-15.06.2023, Turyn, Włochy, sesja plakatowa

2. Rodak, O., Dzimira, S., Pieczewska, B., Niżański, W. „Retrospective study of mammary gland tumours diagnosed by cytology and histopathology in female dogs" 21st EVSSAR Congress, 22-23.06.2018, Wenecja, Włochy, sesja plakatowa
3. Rodak, O., Niżański, W., Partyka, A., Prochowska, S., Wojtasik, B. „Kriokonserwacja kory jajnika – jako innowacyjna metoda zachowania potencjału reprodukcyjnego dzikich kotowatych" XIII Kongres „Problemy w rozrodzie małych zwierząt – płodność, ciąża, noworodek", 14 -15.10.2017, Wrocław, sesja plakatowa
4. Rodak. O., Partyka, A., Niżański, W. „Effectiveness of collagenase type IA in domestic cat ovarian follicles enzymatic isolation protocol" VIII Reproduction Biology Society Congress, 7-9.09 2017, Olsztyn, sesja plakatowa
5. Rodak, O., Niżański, W., Dzimira, S., Podolak, A., Płóciennik, M., Vasetska, A. „Assessment of reliability of ultrasound and fine needle aspiration biopsy cytological examination in diagnosis of prostate diseases in dogs" 20th EVSSAR Congress, 29.06 - 01.07.2017, Wiedeń, Austria, prezentacja ustna
6. Rodak. O., Partyka, A., Niżański, W. „Comparison of domestic cat ovarian follicles viability evaluation using different staining methods" III International Conference of Cell Biology, 26-27.05.2017, Kraków, prezentacja ustna
7. Rodak. O., Kochan, J., Niżański, W., Partyka, A., Prochowska, S., Nowak, A., Skotnicki, J., Grega, T., Pałys, M. „Ocena jajników i płodów kota domowego w różnych okresach rozwoju prenatalnego – badania wstępne" XII Kongres „Problemy w rozrodzie małych zwierząt – płodność, ciąża, noworodek", 1-2.10.2016, Wrocław, sesja plakatowa
8. Rodak, O., Małecka, M., Dzimira, S., Niżański, W. „Evaluation of the reliability of mammary tumors' cytology in dogs in comparison to histopathology" 18th. EVSSAR Congress, 17-18.09.2015, Hanover, Niemcy, prezentacja ustna

## C. Projekty badawcze:

1. Kierownik grantu nr NCN 2019/33/N/NZ9/02999 – „Wgląd w krio-stres pęcherzyków jajnikowych oraz wpływ parametrów fizykochemicznych na efektywność kriokonserwacji tkanki jajnikowej u kotowatych"
2. Stypendystka w projekcie nr POWR.03.02.00-00-I008 – „ProHum – Interdyscyplinarna Szkoła Doktorska – planowanie badań eksperymentalnych, tworzenie i optymalizacja zwierzęcych modeli doświadczalnych z umiejętnościami transferowania ich do badań klinicznych w medycynie człowieka"
3. Stypendystka w grancie nr NCN 2018/31/B/NZ5/02238 – „Określenie epigenetycznych mechanizmów modulacji wybranych genów z rodziny SOX oraz ich transkryptów jako potencjalnych markerów diagnostycznych i predykcyjnych w niedrobnokomórkowych rakach płuc NSCLC"