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Insulina, chemioterapeutyki i pochodne glukozy w terapii nowotworów litych

ROZPRAWA DOKTORSKA

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Abbreviations

INS – insulin

CRC – colorectal cancer

BC – breast cancer

FU – 5-fluorouracil

CPA – cyclophosphamide

OXA - oxaliplatin

IRI – irinotecan

DOC – docetaxel

INSR - insulin receptor

GLUT-1 – glucose transporter 1

GLUT-3 - glucose transporter 3

PIK3CA - phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

IRS 1 - insulin receptor substrate 1

PIK3R1 - phosphatidylinositol 3-kinase regulatory subunit alpha

AKT1/AKT2 - AKT Serine/Threonine Kinase 1 and 2

MAPK1 - Mitogen-Activated Protein Kinase 1

MAP2K2 - Mitogen-Activated Protein Kinase Kinase 2

SREBP-1c - sterol regulatory element-binding protein-1c

GSK3B - Glycogen synthase kinase 3 beta

CTC - circulating tumor cells

List of publications included in the dissertation

Full text

1. **Siddarth Agrawal**, Marta Woźniak, Mateusz Łuc, Sebastian Makuch, Ewa Pielka, Anil Kumar Agrawal, Joanna Wietrzyk, Joanna Banach, Andrzej Gamian, Monika Pizon, Piotr Ziółkowski.: Insulin enhancement of the antitumor activity of chemotherapeutic agents in colorectal cancer is linked with downregulating PIK3CA and GRB2
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2. **Siddarth Agrawal**, Marta Woźniak, Mateusz Łuc, Kinga Walaszek, Ewa Pielka, Wiesław Szeja, Gabriela Pastuch-Gawołek, Andrzej Gamian, Piotr Ziółkowski.: Insulin and novel thioglycosides exert suppressive effect on human breast and colon carcinoma cells
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3. **Siddarth Agrawal**, Mateusz Łuc, Piotr Ziółkowski, Anil Kumar Agrawal, Ewa Pielka, Kinga Walaszek, Krzysztof Zduniak, Marta Woźniak.: Insulin-induced enhancement of MCF-7 breast cancer cell response to 5-fluorouracil and cyclophosphamide
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Introduction

Colorectal cancer (CRC) and breast cancer (BC) are among the most frequent malignancies and leading causes of cancer-related deaths in the world. The treatment outcome of patients diagnosed with advanced diseases is poor; it is estimated that only one out of two patients will respond to classical chemotherapy [1,2]. Classical chemotherapy, which exerts its anticancer action by causing damage and inducing programmed cell death, particularly in rapidly growing tumors with high-growth fractions cells, has been a foundation in standard cancer treatment for many years. The rationale for using standard chemotherapy is to kill cancer cells in order to reduce tumor size. However, many solid tumors, including CRC, are slowly growing malignancies and have relatively more cells with a low-growth fraction and therefore are less susceptible to chemotherapy [3].

It has been reported that insulin as a pharmacological agent induces the switch from a noncycling to a cycling status and, therefore, strongly modifies the metabolism of malignant cells [4,5]. Moreover, as a classic hormone, it affects lipid synthesis, carbohydrate metabolism, cell proliferation, motility, and survival [6]. Several *in vitro* [7-12] and clinical studies [13,14] implicated insulin pretreatment may play a previously unknown significant role in increased drug uptake and cell susceptibility to cytotoxic therapy.

Malignancies of diverse origins deviate from healthy tissues in their high consumption of glucose. This phenomenon, recognized as one of the hallmarks of cancer, has attracted a great deal of interest in anticancer therapies. Conjugation of glucose with metabolic agents to selectively target cancer cells was inspired by the widespread use of radiolabeled glucose analog to visualize tumors and their metastases. The field of synthesis and evaluation of sugar-conjugated anticancer agents has grown significantly in recent years, with certain compounds in advanced clinical trials [15].

Therefore, this study was carried out to examine the effect of insulin on the sensitivity of breast and colon cancer to commonly applied chemotherapeutic agents *in vitro* and *in vivo* as well as investigate and establish the possible mechanisms of this phenomenon. Moreover, we have determined the antitumor effect of novel compounds, glucose derivatives, on breast and colon cancer, and assessed the underlying mechanisms.

Materials and methods

Insulin-induced sensitivity of MCF-7 human breast cancer cells and human colon adenocarcinoma cancer cell lines Caco-2 and SW480 to chemotherapeutic agents 5-fluorouracil (FU), cyclophosphamide (CPA), as well as oxaliplatin (OXA), irinotecan (IRI), and docetaxel (DOC) was evaluated. To investigate and establish the possible mechanisms of this phenomenon, we assessed cell proliferation, induction of apoptosis, activation of apoptotic and autophagic pathways, expression of glucose transporters 1 and 3, formation of reactive oxygen species, and wound-healing assay. To assess the underlying genetic mechanism of the therapy, we examined the mRNA expression of pathways related to the signaling downstream of insulin receptors (INSR). Moreover, we performed Western blotting to confirm expression patterns derived from the genetic analysis. To verify the results, we performed *in vivo* analysis on mice bearing MC38 colon tumors. For the quantification of circulating tumor cells in the peripheral blood, we used the maintrac method.

To analyze the antitumor effect of novel glucose derivatives: (5-nitro-2-pyridyl) 1-thio-β-D-glucopyranoside labelled as thioglycoside A, and (3-nitro-2-pyridyl) 1-thio-β-D-glucopyranoside labelled as thioglycoside B, we have performed cell viability analysis on three cancer cell lines: MCF-7 human breast cancer cell line and human colon adenocarcinoma cancer cell lines: Caco-2, SW480. We further assessed whether insulin can enhance the antitumor effect of these compounds. To investigate and establish the effectiveness and possible mechanisms of this phenomenon, we assessed cell proliferation, cell migration and motility, expression of glucose transporter 1 (GLUT-1) and proapoptotic proteins (caspase-3, BAX).

Results

Insulin pretreatment enhances cytotoxicity

The MTT viability assay showed that colon cancer cells sensitized by exogenous insulin are more susceptible to chemotherapeutic drugs. In CRC, we found that a combination of FU with insulin led to a statistically significant decrease in viability in Caco-2 and SW480 colon cancer cells compared with FU alone. A similar effect was reported in IRI-treated cells. The prior administration of insulin resulted in a significant enhancement of the cytotoxic effect of the drug. We found a two-fold decrease in the viability of cells pretreated with insulin. Interestingly, we found that OXA and DOC (in higher concentration 198 μM and 4000 μM, respectively) displayed a significantly enhanced cytotoxicity in the presence of insulin only in SW480 cells, which have a higher metastatic potential than Caco-2 cells. In BC, a combination of 500 μM FU with previous insulin sensitization led to significant decrease of viability of MCF-7 compared with 500 μM FU alone. Evidently, 100 μM FU had an inhibitory effect only in the presence of insulin. However, FU alone at high concentration resulted in an effect similar to that in the presence of additional insulin. Combination of 4000 μg/mL CPA with INS produced a significant inhibition in viability when compared with CPA alone.

Insulin enhances apoptosis

In all samples treated with INS, including INS-treated control, a significantly higher apoptotic cell percentages and higher apoptosis/necrosis ratios were detected in comparison to samples treated without INS in flow cytometry analysis using Annaexin V and propidium iodide detection method.

Insulin induces expression of GLUT-1 and GLUT-3

The immunocytochemical results showed that treatment with insulin caused an elevated expression of GLUT-1 and GLUT-3 proteins when compared with the control. The findings were confirmed in western blot analysis.

Insulin pretreatment inhibits cell proliferation and cell motility

To further examine how insulin pretreatment affected cell proliferation along with cell motility, “wound-healing” assays were performed. The results indicated that control- and INS-treated MCF-7 cells nearly completely filled the “wound” by 48 h. In stark contrast, combination of 4000 μg CPA as well as 200 μM FU with INS slightly hindered the motility/proliferation of these cells comparing to CPA or FU alone. These results are a further indication that combination of CPA and FU with INS impairs ability of breast cancer cell proliferation and motility.

ROS formation

To establish the effect of combined insulin/FU or insulin/CPA on the intracellular redox status, we determined the intracellular ROS formation. We observed that FU-treated cells exhibited decreased ROS formation. The addition of insulin to FU did not influence ROS generation. Conversely, CPA-treated cells significantly enhanced ROS generation. Insulin-/CPA-treated cells exhibited decreased levels of ROS compared with CPA-treated alone group.

Insulin and FU/IRI alter the cellular metabolism through regulating the mRNA expression of PIK3-related genes and proteins

To examine the effect of the therapy on cell metabolism, we examined the mRNA expression of pathways related to the signaling downstream of insulin receptors. We have found that FU and IRI caused significant downregulation of the key signaling substrates. The administration of drugs to Caco-2 colon cancer cells and SW480 cells resulted in a significant inhibition of INSR (insulin receptor), IRS 1 (insulin receptor substrate 1), PIK3R1 (phosphatidylinositol 3-kinase regulatory subunit alpha), AKT1 and AKT2 (AKT Serine/Threonine Kinase 1 and 2), MAPK1 (Mitogen-Activated Protein Kinase 1), MAP2K2 (Mitogen-Activated Protein Kinase Kinase 2), SREBP-1c (sterol regulatory element-binding protein-1c) and GSK3B (Glycogen synthase kinase 3 beta). Moreover, we have found significant downregulation of glucose transporters

(GLUT-1, GLUT-3, GLUT-4) and anti-apoptotic protein BCL-2. Interestingly, additional pretreatment with insulin resulted in a significantly lower expression of PIK3CA (phosphatidylinositol 3-kinase catalytic subunit alpha), which plays a critical role in cell signaling and GRB2 (growth factor receptor-bound protein 2), a regulator of cell proliferation and differentiation. These findings were confirmed for both cell lines at protein levels by Western blotting.

In vivo activity against colon cancer tumors

After our observations of *in vitro* effects, insulin and FU were evaluated in a mouse allograft model of colon cancer. There was a significant promotion in tumor weight in control as well as insulin and FU-only treated animals ($p < 0.05$ in groups 1 to 3). When the animals were treated or pretreated with insulin (groups 4 and 5, respectively) combined with FU, no raise in the tumor weight was observed. We found that the average tumor weight after the 3-weeks therapy was significantly lower in group 5 compared to other groups ($p = 0.037$). Insulin added to FU therapy was statistically different in the effects of FU alone ($P < 0.05$). Histopathological analyses of the tumors excised from control mice showed groups of large cells with different degrees of cellular and nuclear pleomorphism. Mitosis, muscle invasion, and coagulation necrosis were also noticed. In the tumors excised from treated animals, extensive areas of coagulative necrosis were observed. Thus, the results indicate that insulin combined with FU significantly inhibited tumor growth when compared to FU or insulin alone. This indicated that insulin might increase the antitumor activity of FU. Interestingly, we found that there was a statistically significant difference in the number of CTC between control (group 1) and FU combined with insulin (groups 4 and 5) (1,400 vs. 100; $p = 0.013$ and 1,400 vs. 250; $p = 0.043$).

Toxicological aspects of chemotherapeutic treatment with and without insulin

There was no significant loss of body weight in insulin and/or FU treated mice. Hematological parameters were measured after the experiment. We found that the percentage of lymphocytes was significantly lower in control animals (group 1) and

those treated with insulin alone (group 2), compared with mice treated with FU combined with insulin (group 5) (86% vs. 97%; $p = 0.041$ and 87% vs. 97%; $p = 0.045$, respectively). Moreover, we found that the percentage of monocytes was also statistically significant between groups 1 and 5 (5% vs. 1%; $p = 0.045$). No other significant changes in hematological parameters were seen in any animals. The organs removed from the treated animals were weighed. No significant changes in the liver and pancreas were seen in any animals treated with insulin alone or in combination with FU or FU alone.

Glucose derivatives exhibit antitumor effect

To identify the optimal concentration of the compounds, various doses were tested by MTT assay. The thioglycosides in concentrations 10 μg/ml and 1 μg/ml exhibited significant inhibition in viability of breast and colon cancer cells. The effect of both thioglycosides on MCF-7 and Caco-2 cell viability was similar. However, by statistical analysis we found that compound B is more cytotoxic to SW480 than compound A. The impact of non-conjugated glucose and other sugars on cell viability of breast cancer cells were assessed during preliminary studies. We found no significant changes in viability of the cells.

Insulin enhances the inhibitory effect of glucose derivatives

MCF-7 cancer cells were pretreated with 40 μg/ml insulin (INS), while colon cancer cells with 100 μg/ml. After incubation with insulin for 8 hours, cells were treated with thioglycosides A and B at concentration 10 μg/ml. Insulin alone had no significant effect on cell growth. We found that the combination of INS and thioglycosides produced a significant inhibition in growth of both breast and colon cancer cells.

Combination of insulin and glucose derivatives inhibits cell motility

Wound-healing assay was performed to assess the combined effect of insulin and thioglycosides on cell proliferation and cell motility. The results indicate that control and INS-treated cells almost completely filled the “wound” in MCF-7 and Caco-2 cells

by 24h. The wound was filled completely in SW480 cells. In case of cells treated only with thioglycosides, a slightly hindered cell was observed. In sharp contrast, an addition of INS with thioglycosides significantly inhibited wound healing. This effect was observed in all cancer cell lines.

Impact of insulin and glucose derivatives on GLUT-1 expression and apoptosis

The effect of insulin and compounds A and B on the expression of glucose transporter was analysed by immunocytochemistry. We found that treatment with insulin caused an elevated cytoplasmic expression of GLUT-1 protein when compared with the control. Comparing to the insulin alone, the combinations of thioglycosides with insulin, produced similar effects. The findings were confirmed in Western blotting analysis.

The expression of proapoptotic proteins – caspase 3 and BAX was analysed by Western blotting. We detected high levels of proapoptotic proteins in cells treated with combination of insulin and thioglycosides as well as thioglycosides only. The flow cytometry analysis showed that insulin had no significant impact on the ratio of apoptosis in all tested cancer cell lines. Over 20% of the MCF-7 breast cancer cells underwent apoptosis when treated with compound A or B. The addition of insulin did not result in significant changes in the ratio of apoptotic cells. The level of apoptosis in SW480 cells treated with thioglycoside A was slightly over 10%. The addition of insulin to compound A resulted in an increased ratio of apoptotic cells, which was slightly over 26%. Compound B with and without insulin produced a similar effect (over 20% of cells undergoing apoptosis). The apoptotic action of compound A on colon cancer cell line Caco-2 was similar to that of SW480 (nearly 10%). The addition of insulin to compound A caused an enhancement of apoptosis by slightly over 6%. The highest level of apoptosis was detected in Caco-2 cells treated with compound B (nearly 34%). Interestingly, the combination of insulin and compound B produced 23,33% of apoptosis.

Conclusion

The results of our study show that insulin pretreated colon and breast cancer cells are significantly more susceptible to commonly used chemotherapeutics. The apoptosis ratio was also enhanced when insulin was administered together with the examined drugs. The *in vivo* analysis confirmed that insulin could enhance the effect of the tested chemotherapeutic while showing no toxicity. The underlying mechanism can be related to the downregulation of PIK3CA and GRB2 expression, which are crucial for growth, proliferation, survival, and migration of cancer cells. Further experiments are warranted to determine the clinical utility of the approach. The combined use of insulin with cytotoxic agents may lead to the development of new and inexpensive strategies for the clinical chemotherapy of tumors.

Moreover, we report the discovery of novel compounds in which sugar is linked to aglycone in an efficient manner through S-glycosidic bond. This is the first study that investigated the combined use of insulin and glucose linked to anticancer agent. Our research conducted on breast and colon cancer cell lines demonstrated potent cytotoxic activity, which was significantly enhanced in the presence of insulin. We found that insulin can increase the cytotoxic action of glucose-conjugates up to two-fold. These potential candidates for future anticancer therapy displayed antiproliferative as well as proapoptotic action *in vitro*. Further *in vivo* studies are required to assess its application.

The approach based on the combination of insulin, chemotherapeutics, and/or glucose derivatives has a great deal of potential and a considerable opportunity for growth.

Summary

The present state of cancer chemotherapy is unsatisfactory. New anticancer drugs that marginally improve the survival of patients continue to be developed at an unsustainably high cost. The study aimed to elucidate the effects of insulin (INS), an inexpensive drug with a convincing safety profile, on the susceptibility of breast and colon cancer to chemotherapeutic agents: 5-fluorouracil (FU), oxaliplatin (OXA), irinotecan (IRI), cyclophosphamide (CPA) and docetaxel (DOC). To examine the effects of insulin on cell viability and apoptosis, we performed an *in vitro* analysis on cancer cell lines Caco-2, SW480 and MCF-7. To verify the results, we performed *in vivo* analysis on mice bearing tumors. To investigate and establish the possible mechanisms of this phenomenon, we assessed cell proliferation, induction of apoptosis, activation of apoptotic and autophagic pathways, expression of glucose transporters 1 and 3, formation of reactive oxygen species, wound-healing assay, and mRNA expression of pathways related to the signaling downstream of insulin receptors (INSR). Moreover, we performed Western blotting to confirm expression patterns derived from the genetic analysis. For the quantification of circulating tumor cells in the peripheral blood, we used the maintrac method. To analyze the antitumor effect of novel glucose derivatives we have performed cell viability analysis on three cancer cell lines: MCF-7 human breast cancer cell line and human colon cancer cell lines: Caco-2, SW480. We further assessed whether insulin can enhance the antitumor effect of these compounds. To investigate and establish the possible mechanisms of this phenomenon, we assessed cell proliferation, cell migration and motility, expression of glucose transporter 1 (GLUT-1) and apoptotic proteins (caspase-3, BAX).

The results of our study show that insulin pretreated colon and breast cancer cells are significantly more susceptible to commonly used chemotherapeutics. The *in vivo* analysis confirmed that insulin could enhance the effect of the tested chemotherapeutic while showing no toxicity. The underlying mechanism can be related to the downregulation of PIK3CA and GRB2, which are crucial for growth, proliferation,

survival, and migration of cancer cells. Moreover, we report the discovery of novel compounds in which sugar is linked to aglycone in an efficient manner through S-glycosidic bond. Our research conducted on breast and colon cancer cell lines demonstrated potent cytotoxic activity, which was significantly enhanced in the presence of insulin. We found that insulin can increase the cytotoxic action of glucose-conjugates up to two-fold.

The approach based on the combination of insulin, chemotherapeutics, and/or glucose derivatives has a great deal of potential and a considerable opportunity for growth. Further preclinical experiments are warranted to determine the clinical utility of the strategy.

Streszczenie

Obecne wyniki leczenia pacjentów z nowotworami litymi z wykorzystaniem klasycznej chemioterapii są niezadowalające. Koszty rozwoju i rejestracji nowych leków przeciwnowotworowych, które marginalnie poprawiają przeżycie pacjentów, stale wzrastają. Badanie miało na celu wyjaśnienie wpływu insuliny (INS), niedrogiego leku o przekonującym profilu bezpieczeństwa, na wrażliwość komórek raka piersi (BC) i raka jelita grubego (CRC) na chemioterapeutyki: 5-fluorouracyl (FU), oksaliplatyna (OXA), irynotekan (IRI), cyklofosfamid (CPA) i docetaksel (DOC). W celu oceny wpływu insuliny na przeżywalność komórek i apoptozę, przeprowadzono badania *in vitro* na liniach komórek nowotworowych Caco-2, SW480 i MCF-7. Ocena działania na modelu zwierzęcym została przeprowadzona na myszach Balb/c z guzem CRC. W celu wyjaśnienia mechanizmów tego zjawiska oceniono wskaźnik żywotności komórek, indukcję apoptozy, aktywację szlaków apoptotycznych i autofagicznych, ekspresję transporterów glukozy 1 i 3, tworzenie reaktywnych form tlenu, test proliferacji i migracji komórek „wound-healing assay" i ekspresję mRNA genów zaangażowanych w szlaki komórkowe aktywowane przez insulinę. Ponadto przeprowadzono Western blotting, aby potwierdzić wzorce ekspresji pochodzące z analizy genetycznej z ekspresją białek. Do oceny ilościowej krążących komórek nowotworowych we krwi obwodowej

zastosowano metodę maintrac. Ponadto przeprowadzono ocenę aktywności przeciwnowotworowej nowych pochodnych glukozy za pomocą testu MTT. Następnie oceniono, czy insulina może wzmagać działanie przeciwnowotworowe tych związków. Aby zbadać i ustalić możliwe mechanizmy tego zjawiska, oceniono wskaźnik żywotności komórek, proliferację i migrację komórek, ekspresję transportera glukozy 1 (GLUT-1) i białek apoptotycznych (kaspaza-3, BAX). Wyniki badań wskazują, że komórki BC i CRC poddane wcześniej działaniu insuliny są znacznie bardziej podatne na powszechnie stosowane chemioterapeutyki. Analiza in vivo potwierdziła, że insulina wzmaga działanie badanego chemioterapeutyku, nie wykazując jednocześnie toksyczności. Podstawowy mechanizm może być związany z hamowaniem ekspresji PIK3CA i GRB2, które są kluczowe dla wzrostu, proliferacji, przeżycia i migracji komórek nowotworowych. Badania przeprowadzone na liniach komórkowych raka piersi i jelita grubego wykazały, że nowe pochodne glukozy, w których cukier jest skutecznie połączony z aglikonem poprzez wiązanie S-glikozydowe, charakteryzują się wysoką aktywnością cytotoksyczną, która ulega wzmożeniu w obecności insuliny. INS może zwiększyć działanie cytotoksyczne koniugatów glukozy nawet dwukrotnie poprzez nadekspresję receptorów GLUT.

Podejście oparte na połączeniu egzogennej insuliny, chemioterapeutyków i/lub pochodnych glukozy posiada olbrzymi potencjał terapeutyczny oraz może stanowić skuteczną i bezpieczną metodę leczenia nowotworów piersi i jelita grubego.

References

1. Hu, T., Li, Z., Gao, C. & Cho, C. Mechanisms of drug resistance in colon cancer and its therapeutic strategies. *World Journal of Gastroenterology* **22**, 6876 (2016).

2. Hammond, W. A., Swaika, A. & Mody, K. Pharmacologic resistance in colorectal cancer: a review. *Therapeutic Advances in Medical Oncology* **8**, 57–84 (2015).

3. Shackney, S. E., McCormack, G. W. & Cuchural, G. J. Jr. Growth Rate Patterns of Solid Tumors and Their Relation to Responsiveness to Therapy. *Annals of Internal Medicine* **89**, 107 (1978).

4. Gross, G. E., Boldt, D. H. & Osborne, C. K. Perturbation by insulin of human breast cancer cell cycle kinetics. *Cancer Res.* **44**, 3570 (1984).

5. Pollak, M. Insulin and insulin-like growth factor signalling in neoplasia. *Nature Reviews Cancer* **8**, 915–928 (2008).

6. De Meyts, P. Insulin and its receptor: structure, function and evolution. *Bioessays* **26**, 1351–1362 (2004).

7. Agrawal, S. *et al.* Insulin-induced enhancement of MCF-7 breast cancer cell response to 5-fluorouracil and cyclophosphamide. *Tumor Biology* **39**, 101042831770290 (2017).

8. Agrawal, S. *et al.* Insulin and novel thioglycosides exert suppressive effect on human breast and colon carcinoma cells. *Oncotarget* **8** (2017).

9. Yang, Y., Wen, F. & Dang, L. Insulin enhances apoptosis induced by cisplatin in human esophageal squamous cell carcinoma EC9706 cells related to inhibition of autophagy. *Chin Med J* **127**, 353–358 (2014).

10. Zou, K., Ju, J. H. & Xie, H. Pretreatment with insulin enhances anticancer functions of 5-fluorouracil in human esophageal and colonic cancer cells. *Acta Pharmacol Sin* **28**, 721–730 (2007).

11. Miglietta, A., Panno, M. L. & Bozzo, F. Insulin can modulate MCF 7 cell response to paclitaxel. *Cancer Lett* **209**, 139–145 (2004).

12. Alabaster, O., Vonderhaar, B. & Shafie, S. Metabolic modification by insulin enhances methotrexate cytotoxicity in MCF-7 human breast cancer cells. *Eur J Cancer Clin Oncol* **17**, 1223–1228 (1981).

13. Lasalvia-Prisco, E. *et al.* Insulin induced enhancement of antitumoral response to methotrexate in breast cancer patients. *Cancer Chemother Pharmacol* **53**, 220–224 (2004).

14. Damyanov, C., Gerasimova, D., Maslev, I. & Gavrilov, V. Low-dose chemotherapy with insulin (insulin potentiation therapy) in combination with hormone therapy for treatment of castration-resistant prostate cancer. *ISRN Urol* **2012**, 140182 (2012).

15. Calvaresi EC, Hergenrother PJ. Glucose conjugation for the specific targeting and treatment of cancer. Chem Sci. 2013; 4: 2319-2333.

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Insulin enhancement of the antitumor activity of chemotherapeutic agents in colorectal cancer is linked with downregulating PIK3CA and GRB2

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The present state of cancer chemotherapy is unsatisfactory. New anticancer drugs that marginally improve the survival of patients continue to be developed at an unsustainably high cost. The study aimed to elucidate the effects of insulin (INS), an inexpensive drug with a convincing safety profile, on the susceptibility of colon cancer to chemotherapeutic agents: 5-fluorouracil (FU), oxaliplatin (OXA), irinotecan (IRI), cyclophosphamide (CPA) and docetaxel (DOC). To examine the effects of insulin on cell viability and apoptosis, we performed an *in vitro* analysis on colon cancer cell lines Caco-2 and SW480. To verify the results, we performed *in vivo* analysis on mice bearing MC38 colon tumors. To assess the underlying mechanism of the therapy, we examined the mRNA expression of pathways related to the signaling downstream of insulin receptors (INSR). Moreover, we performed Western blotting to confirm expression patterns derived from the genetic analysis. For the quantification of circulating tumor cells in the peripheral blood, we used the maintrac method. The results of our study show that insulin-pretreated colon cancer cells are significantly more susceptible to commonly used chemotherapeutics. The apoptosis ratio was also enhanced when INS was administered complementary to the examined drugs. The *in vivo* study showed that the combination of INS and FU resulted in significant inhibition of tumor growth and reduction of the number of circulating tumor cells. This combination caused a significant downregulation of the key signaling substrates downstream of INSR. The results indicate that the downregulation of PIK3CA (phosphatidylinositol 3-kinase catalytic subunit alpha), which plays a critical role in cell signaling and GRB2 (growth factor receptor-bound protein 2), a regulator of cell proliferation and differentiation may be responsible for the sensitizing effect of INS. These findings were confirmed at protein levels by Western blotting. In conclusion, these results suggest that INS might be potentially applied to clinical use to enhance the therapeutic effectiveness of chemotherapeutic drugs. The findings may become a platform for the future development of new and inexpensive strategies for the clinical chemotherapy of tumors.

Colorectal cancer (CRC) is one of the most frequent malignancies and one of the leading causes of cancer-related deaths in the world[1]. The treatment outcome of patients diagnosed with unresectable CRC is poor; it is estimated that only one out of two patients will respond to the classical chemotherapy[2,3]. The present state of systemic cancer therapy is unsatisfactory, as new anticancer drugs continue to be developed and approved by marginal

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improvements in survival at an unsustainably high financial cost[4]. As a result, it would seem more rational to attempt to improve the treatment outcome by implementing inexpensive treatments with a convincing safety profile.

Classical chemotherapy, which exerts its anticancer action by causing damage and inducing programmed cell death, particularly in rapidly growing tumors with high-growth fractions cells, has been a foundation in standard cancer treatment for many years. The rationale for using standard chemotherapy is to kill cancer cells in order to reduce tumor size. However, many solid tumors including CRC are slowly growing malignancies and have relatively more cells with a low-growth fraction and therefore are less susceptible to chemotherapy[5].

It has been reported that insulin as a pharmacological agent induces the switch from a noncycling to a cycling status and therefore strongly modifies the metabolism of malignant cells[6,7]. Moreover, as a classic hormone, it affects lipid synthesis, carbohydrate metabolism, cell proliferation, motility and survival[8]. Several *in vitro*[9–14] and clinical studies[15,16] implicated insulin pretreatment may play a previously unknown significant role in increased drug uptake and cell susceptibility to cytotoxic therapy. Therefore, this study was carried out to validate the insulin-induced enhancement of the antitumor effect of widely incorporated cytotoxic agents 5-fluorouracil (FU), oxaliplatin (OXA), irinotecan (IRI), cyclophosphamide (CPA) and docetaxel (DOC). Moreover, to establish the mechanisms underlying this phenomenon, we assessed the mRNA expression of pathways related to the signaling downstream of insulin receptors.

Results

Insulin pretreatment enhances cytotoxicity. The MTT viability assay showed that colon cancer cells sensitized by insulin are more susceptible to chemotherapeutic drugs (Fig. 1). We found that a combination of FU with insulin led to a significant decrease in viability in Caco-2 and SW480 colon cancer cells compared with FU alone. The effect was observed both in higher (500 µM) and a lower (100 µM) concentration of FU. A similar effect was reported in IRI-treated cells. The prior administration of insulin resulted in a significant enhancement of the cytotoxic effect of the drug. We found a two-fold decrease in the viability of cells pretreated with insulin. Interestingly, we found that OXA and DOC (in higher concentration 198 µM and 4000 µM, respectively) displayed a significantly enhanced cytotoxicity in the presence of insulin only in SW480 cells, which have a higher metastatic potential than Caco-2 cells[17].

Insulin and FU/IRI increase apoptosis. The flow cytometry analysis revealed a greater percentage of apoptosis after treating cells with chemotherapeutics (Fig. 2). This effect was enhanced 2-fold when SW480 cells were also pretreated with insulin before using FU. Caco-2 cells exhibited up to 50% greater apoptotic percentage when IRI treated cells were additionally pretreated with insulin. These results indicate that even though the effect can vary depending on tested cell lines and drugs, the influence of insulin on colon cancer cells remain.

Insulin and FU/IRI alter the cellular metabolism through regulating the mRNA expression of PIK3-related genes and proteins. To examine the effect of the therapy on cell metabolism, we examined the mRNA expression of pathways related to the signaling downstream of insulin receptors. We have found that FU and IRI caused significant downregulation of the key signaling substrates (Fig. 3A). The administration of drugs to Caco-2 colon cancer cells and SW480 cells resulted in a significant inhibition of INSR (insulin receptor), IRS 1 (insulin receptor substrate 1), PIK3R1 (phosphatidylinositol 3-kinase regulatory subunit alpha), AKT1 and AKT2 (AKT Serine/Threonine Kinase 1 and 2), MAPK1 (Mitogen-Activated Protein Kinase 1), MAP2K2 (Mitogen-Activated Protein Kinase Kinase 2), SREBP-1c (sterol regulatory element-binding protein-1c) and GSK3B (Glycogen synthase kinase 3 beta). Moreover, we have found significant downregulation of glucose transporters (GLUT-1, GLUT-3, GLUT-4) and anti-apoptotic protein BCL-2. Interestingly, additional pretreatment with insulin resulted in a significantly lower expression of PIK3CA (phosphatidylinositol 3-kinase catalytic subunit alpha), which plays a critical role in cell signaling and GRB2 (growth factor receptor-bound protein 2), a regulator of cell proliferation and differentiation. These findings were confirmed for both cell lines at protein levels by Western blotting (Fig. 3B).

***In vivo* activity against colon cancer tumors.** After our observations of *in vitro* effects, insulin and FU were evaluated in a mouse allograft model of colon cancer. There was a significant promotion in tumor weight in control as well as insulin and FU-only treated animals ($p < 0.05$ in groups 1 to 3, Table 1, and Fig. 4A). When the animals were treated or pretreated with insulin (groups 4 and 5, respectively) combined with FU, no raise in the tumor weight was observed. We found that the average tumor weight after the 3-weeks therapy was significantly lower in group 5 compared to other groups ($p = 0.037$, Fig. 4B). Insulin added to FU therapy was statistically different in the effects of FU alone ($P < 0.05$). Histopathological analyses of the tumors excised from control mice showed groups of large cells with different degrees of cellular and nuclear pleomorphism. Mitosis, muscle invasion, and coagulation necrosis were also noticed. In the tumors excised from treated animals, extensive areas of coagulative necrosis were observed (Fig. 5). Thus, the results indicate that insulin combined with FU significantly inhibited tumor growth when compared to FU or insulin alone. This indicated that insulin might increase the antitumor activity of FU. Interestingly, we found that there was a statistically significant difference in the number of CTC between control (group 1) and FU combined with insulin (groups 4 and 5) (1,400 vs. 100; $p = 0.013$ and 1,400 vs. 250; $p = 0.043$; Table 2 and Fig. 4C).

Toxicological aspects of FU treatment with and without insulin. There was no significant loss of body weight in insulin and/or FU treated mice (Fig. 4D). Hematological parameters were measured after the experiment (Table 3). We found that the percentage of lymphocytes was significantly lower in control animals (group 1) and those treated with insulin alone (group 2), compared with mice treated with FU combined with insulin (group 5) (86% vs. 97%; $p = 0.041$ and 87% vs. 97%; $p = 0.045$, respectively) (Table 3 and Fig. 4E1).

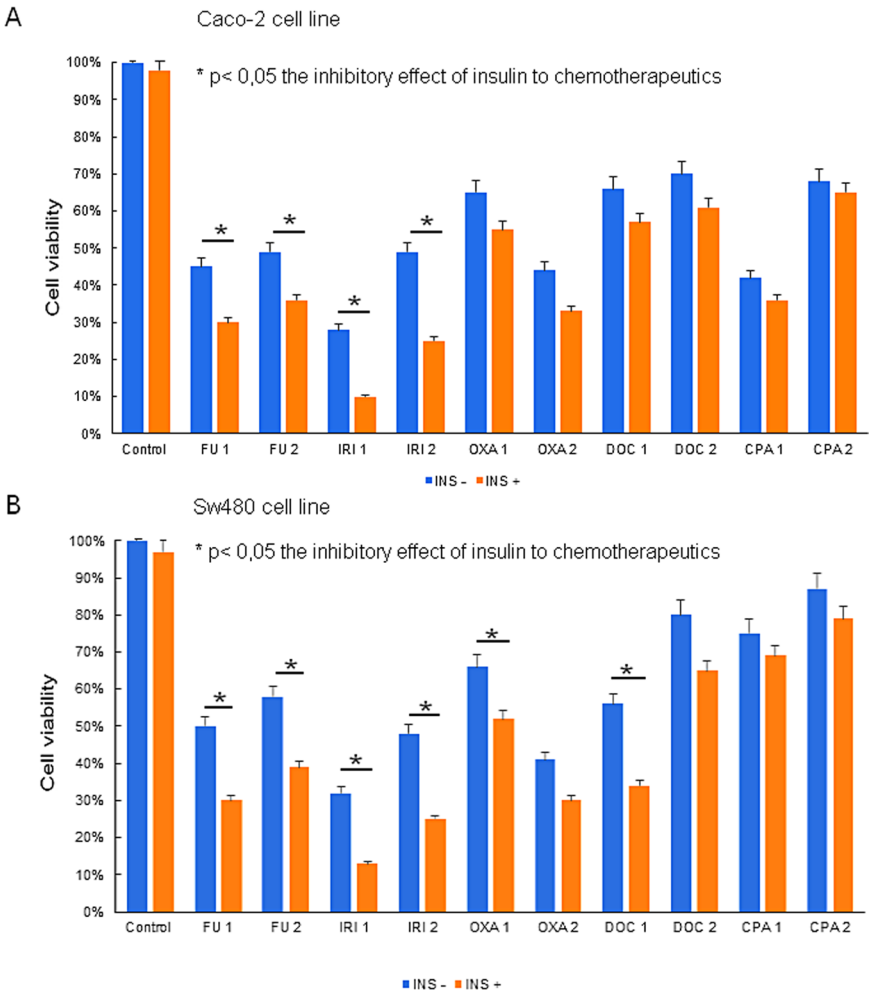

Figure 1. After an 8-hour insulin (INS) pretreatment (100 µg/ml) Caco-2 cells (**A**) were exposed to 5-fluorouracil (FU) (1) 500 µM, (2) 100 µM; irinotecan (IRI) (1) 150 µM, (2) 50 µM; oxaliplatin (OXA) (1) 50 µM, (2) 15 µM; docetaxel (DOC) (1) 4000 nM, (2) 1000 nM; cyclophosphamide (CPA) (1) 15 µM, (2) 4 µM and SW480 cells (**B**) were treated with FU (1) 500 µM, (2) 250 µM; IRI (1) 200 µM, (2) 100 µM; OXA (1) 198 µM, (2) 96 µM; DOC (1) 4000 nM, (2) 100 nM; CPA (1) 12 µM, (2) 6 µM for 48 hours. The inhibitory effect was measured by MTT assay. The results are shown as mean ± SD from three individual experiments. Statistically significant variables were marked with *($p < 0.05$).

Moreover, we found that the percentage of monocytes was also statistically significant between groups 1 and 5 (5% vs. 1%; $p = 0.045$; Fig. 4E2). No other significant changes in hematological parameters were seen in any animals. The organs removed from the treated animals were weighed. No significant changes in the liver and pancreas were seen in any animals treated with insulin alone or in combination with FU or FU alone (Fig. 5).

Discussion

In clinical oncology, anticancer drugs are often used in combination. The discovery of useful combination chemotherapy is expected to increase the response rate and the frequency of long-term survival[18]. The results of our study show that insulin pretreated colon cancer cells are significantly more susceptible to commonly used chemotherapeutics: FU, IRI, OXA, DOC. The apoptosis ratio was also enhanced when insulin was administered together with the examined drugs. The *in vivo* analysis confirmed that insulin could enhance the effect of FU while showing no toxicity. Insulin production is limited to β-cells of the pancreas, and under the normal condition, it is strictly regulated by the concentration of serum glucose. Contrary to the epidermal growth factor and other tissue growth factors that are significant for growth promotion of malignancies, insulin acts as a classic hormone, affecting cells and tissues distant from its site of release. Aberrant autocrine production of insulin by cancer tissues is rare[19]. Insulin binds to the membrane receptors of the insulin-responsive cells, which express high levels of INSR. The insulin receptor is a representative of the tyrosine kinase class of membrane receptors and is homologous to oncogenes of the tyrosine kinase class. The insulin receptors possess the ability to autophosphorylate and transphosphorylate intracellular substrates, and in turn, initiate a cascade of complex cellular reactions. The activated INSR tyrosine kinase initiates several substrates including insulin receptor substrate proteins (IRS1-4), Phosphatidyl Inositol 3-Kinase (PIK3), Akt, MAPK, and signal regulatory protein family[18,20]. Our

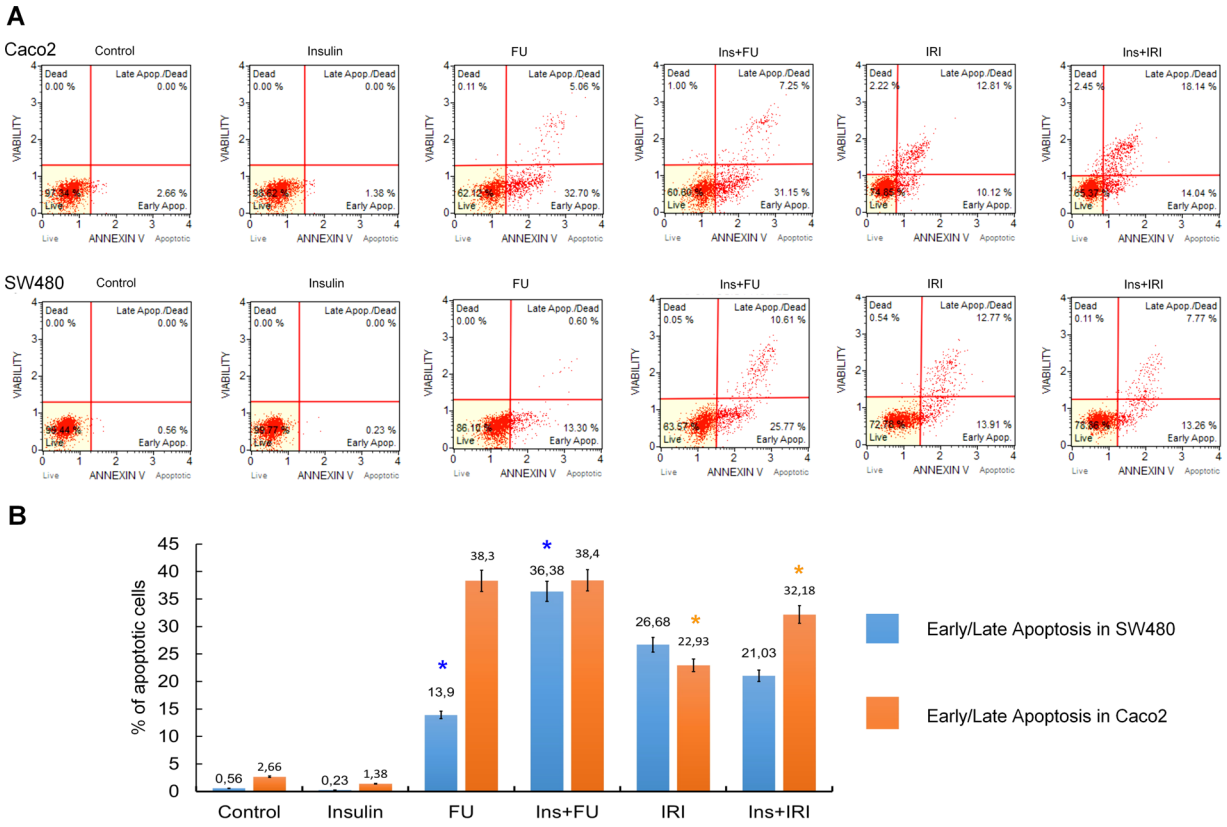

Figure 2. Effects of 5-fluorouracil (FU) and irinotecan (IRI) with and without additional insulin (INS) pretreatment on apoptosis of Caco-2 and SW480 cancer cells. Caco-2 and SW480 carcinoma cells were treated with 5-fluorouracil (FU) and irinotecan (IRI) in the concentration of 500 µM and IRI 50 µM, respectively for 48 h. (**A**) Original histogram plots include a percentage of live, early apoptotic, late apoptotic, total apoptotic, and dead cells differentiated using Muse® Annexin V and Dead Cell Assay Kit. (**B**) The graph bar presents a statistical analysis of the early/late apoptosis in different samples. The results are shown as mean ± SD from two individual experiments. Asterisks indicate significant differences between the groups (FU vs. Ins + FU in SW480 and IRI vs. Ins + IRI in Caco-2; $p < 0.05$).

genetic and protein analysis revealed that cancer cells exposed to drug treatment exhibited significantly lowered mRNA concentration of the key substrates. The results indicate that additional pretreatment with insulin resulted in a significantly lower expression of PIK3CA and GRB2 mRNA and protein when compared with the drug-only treated cells. Genetic research indicates that the PIK3 pathway is the most frequently altered pathway in malignancies, with PIK3CA being the second most frequently mutated oncogene[21,22]. This common oncogenic driver that is central to all malignant cells is recognized as a key target for novel cancer compounds[23]. Upregulation in the PIK3 signaling network provides tumor cells with enhanced capacities for growth, proliferation, survival, and migration. Several ongoing clinical trials in cancer with small-molecule inhibitors against PIK3 report promising results[24].

Research supports the role of insulin and IGF-1 as important growth factors, acting through the tyrosine kinase growth factor cascade in enhancing abnormal tumor growth[25]. Increased insulin levels have been regarded as a crucial factor for the poor prognosis of obesity-associated cancer. Moreover, aberrant insulin and IGF signaling axis have been associated with numerous malignancies including breast cancer, colorectal cancer, prostate cancer, pancreatic cancer, melanoma, osteosarcoma, and childhood malignancies[26]. Constitutively high level of insulin present in the environment initiates a cascade of phosphorylation events leading to activation of several pathways including the PI3K pathway and ultimately results in the highly proliferative and invasive cancer phenotypes[25–27]. However, in our study, insulin over 48 hours in culture did not upregulate the PI3K pathway. The discrepancy in the inhibitory effect of insulin may be due to the temporal pattern of action. During our study, as well as previously reported *in vitro* and clinical studies[9–16], the concentration of insulin is elevated only for a limited time. The conflicting results regarding the effect of insulin on the PI3K pathway may be explained due to the relatively short duration of action. Moreover, the concentration of insulin used in the *in vitro* experiment was higher than circulating concentrations observed in a clinical setting or concentrations used in the previous studies[9–16]. Higher doses of insulin are known to activate IGF1R-related signaling and affect the propensity for invasion and metastasis. These apparently conflicting results on insulin and IGF1R signaling pathway can result from differences in doses and duration of action of insulin on the cells. High doses of insulin and a short duration of action may account for the effect observed in our experiments. Notably, the *in vitro* findings may not be accountable for a sensitizing effect of acute administration *in vivo*. The results of our study indicate that the downregulation

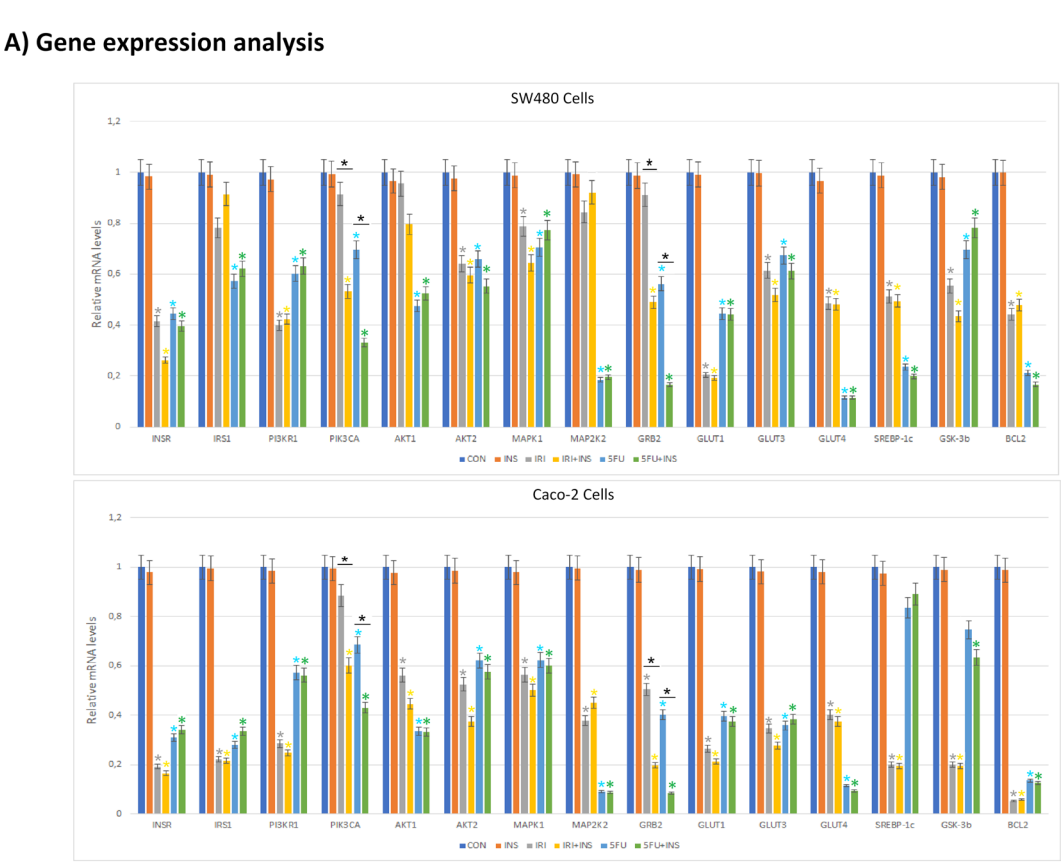

Figure 3. (**A**) Gene expression analysis of substrates involved in the pathways related to the signaling downstream of insulin (INS) receptors by quantitative RT-PCR. Caco-2 and SW480 carcinoma cells were treated with 5-fluorouracil (FU) and irinotecan (IRI) in the concentration of 500 µM and IRI 50 µM, respectively for 48 h. The mRNA expression of INSR (insulin receptor), IRS 1 (insulin receptor substrate 1), PIK3CA (phosphatidylinositol 3-kinase catalytic subunit alpha), PIK3R1 (phosphatidylinositol 3-kinase regulatory subunit alpha), AKT1 and AKT2 (AKT Serine/Threonine Kinase 1 and 2), MAPK1 (Mitogen-Activated Protein Kinase 1), MAP2K2 (Mitogen-Activated Protein Kinase Kinase 2), GRB2 (Growth factor receptor-bound protein 2), glucose transporters (GLUT-1, GLUT-3, GLUT-4), SREBP-1c (sterol regulatory element-binding protein-1c), GSK3B (Glycogen synthase kinase 3 beta), antiapoptotic protein BCL-2, caspase 3 (CASP3) was determined by quantitative RT-PCR using gene-specific primers. Data are presented as mean ± SD. Experiments were run in triplicate and carried out once. *$P < 0.05$ compared with control group. (**B**) Western blotting analysis of expression of PIK3CA and GRB2 in Caco-2 and SW480 cancer cell lines.

of this key substrate may be responsible for the sensitizing effect of insulin. GRB2 signaling acts as a controlling stage and is crucial for cell cycle advancement and cell motility, and therefore, more intricate processes such as morphogenesis, angiogenesis, and vasculogenesis[28]. Research shows that many types of malignancies, such as breast cancer, are characterized by upregulated GRB2-MAPK pathway, especially since GRB2 is coded in a chromosome region duplicated in many types of neoplasms[15,29,30]. Upregulated GRB2 is also correlated with the likelihood of metastasis in colon cancer[31]. Changes of the effect of the chemotherapeutics on cells can also be explained by the fact that GRB2 is a well-known modifier of cell endocytosis[32]. Stopping GRB2-MAPK pathway

Day	Groups of mice					*p*
	Group 1	Group 2	Group 3	Group 4	Group 5	
1	59 [32; 225]	40 [39; 237]	108 [17; 124]	106 [23; 192]	81 [27; 206]	0.997
6	166 [62; 512]	117 [70; 382]	182 [34; 211]	127 [26; 433]	200 [46; 581]	0.976
13	453 [163; 2169]	269 [257; 1494]	397 [95; 586]	268 [140; 768]	198 [0; 602]	0.738
15	737 [357; 2624]	376 [374; 1743]	834 [168; 911]	273 [150; 1358]	282 [0; 804]	0.515
20	1062 [602; 1679]	620 [461; 817]	1430 [278; 1662]	644 [493; 1006]	85 [0; 1182]	**0.037**
p	**0.043**	**0.035**	**0.044**	0.227	0.975	×

Table 1. Overview of the tumor volume measurement results (Med and Q1 and Q3) in the following days of the treatment in five groups of mice and the result of comparisons (non-parametric Kruskal-Wallis test).

at GRB2 point is also a proven way to introduce cells to the apoptotic pathway[33]. Hence, insulin affecting cancer cell metabolism by downregulating the GRB2 may have key meaning for the planning of novel treatments. The combination of insulin with FU/IRI did not alter the expression of other mRNA substrates when compared to the drug only. No significant changes were observed in insulin-only treated cells when compared with control cells. These results implicate that insulin alone does not promote growth and proliferation-related processes in cancer cells. In all cells, the expression of INSR was significantly downregulated. The downregulation of INSR resulted in a lower expression of the insulin receptor substrate 1, which controls a variety of downstream responses, including cell cycle advancement, cell motility, fatty acid biosynthesis, glucose uptake, glycogenesis, glycolysis, gluconeogenesis and antiapoptosis. The pathways that control most hallmarks of cancer were downregulated. The results are presented in Fig. 6. Circulating tumor cells (CTCs) are cancer cells that emerge from solid neoplasms, travel into the blood system, and can form distant metastases. They have been described to be a surrogate biomarker for cancer treatment response in several cancers, i.e., primary breast malignancies[34], and their appearance has been associated with shorter survival in cases with advanced breast, prostate, colorectal and lung cancer[35]. CTCs may enable more sensitive monitoring of treatment efficacy and thereby guide drug selection[36]. Also in the preclinical setting, the enumeration of CTCs is useful for tracking metastasis, developing biomarkers and testing new drugs[37]. Interestingly, in this study, we found that the addition of insulin to FU resulted in a significantly lowered number of CTCs. The results indicate that the addition of insulin may improve the outcome of systemic chemotherapy by limiting the number of CTCs. The relationship between insulin and cancer has been of interest among scientists and physicians for decades. Conflicting results regarding the relationship between the role of insulin in cancer progression and cancer treatment have been reported. Much of the controversy surrounding this issue may be ascribed to differing definitions and methodologies that make it difficult to draw satisfactory conclusions.

Our data suggest that insulin enhances the effect of chemotherapeutic agents in colorectal cancer while showing no toxicity. The underlying mechanism can be related to the downregulation of PIK3CA and GRB2, which are crucial for growth, proliferation, survival, and migration of cancer cells. Further *in vivo* and clinical experiments are required to unequivocally resolve the issue, which, in turn, may lead to the development of new and inexpensive strategies for the clinical chemotherapy of tumors.

Methods

Cell culture and experiment conditions. The human colon adenocarcinoma cell lines Caco-2 and SW480 were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. SW480 cells were maintained in RPMI 1640-GlutaMax supplemented with heat-inactivated 10% fetal bovine serum and 1% penicillin-streptomycin. Caco-2 cells were maintained in 80% MEM- Glutamax supplemented with 20% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin. Cells were incubated at 37 °C in a 5% CO2 and 95% humidified atmosphere to 80% confluence. Cell culture reagents were purchased in Life Technologies (Thermo Fisher Scientific, USA). For all experiments, cells were detached with 0.25% trypsin-EDTA, centrifuged, and seeded for experiments in an appropriate amount. The following cells were exposed to insulin (Insulin solution human, Sigma Aldrich, Merck KGaA, Darmstadt, Germany) for 8 hours in dose 100 µg/ml and then treated for a further 48 hours by tested chemotherapeutics.

Drugs preparation. 5-fluorouracil (FU) and cyclophosphamide (CPA) were purchased in Sigma Aldrich (Merck KGaA, Darmstadt, Germany) and oxaliplatin (OXA), irinotecan (IRI) and docetaxel (DOC) in Selleckchem (Munich, Germany). Aliquots of drugs were prepared according to the manufacturer's instructions and stored at −80 °C. For each experiment, compounds were freshly diluted to the desired concentrations in the culture medium. Control cells were grown in culture medium or medium with 0,05% DMSO (BioShop Canada Inc., Ontario, Canada) concentrations maximally.

Cell viability and MTT assay. The viability of Caco-2 and SW480 cells in response to the insulin and chemotherapeutics was determined by the MTT reduction assay (Sigma Aldrich, Germany). Cells were cultured in 96-well culture ($7–10 \times 10^3$/well) and treated as described above with different chemotherapeutics concentrations for 48 hours. The concentration of a drug that is required for 50% inhibition (IC50) was established during preliminary studies and used in the study. Caco-2 cells were treated with: FU (1) 500 µM, (2) 100 µM; IRI (1) 150 µM, (2) 50 µM; OXA (1) 50 µM, (2) 15 µM; DOC (1) 4000 nM, (2) 1000 nM; CPA (1) 15 µM, (2) 4 µM, while SW480 cells were treated with FU (1) 500 µM, (2) 250 µM; IRI (1) 200 µM, (2) 100 µM; OXA (1) 198 µM, (2) 96 µM; DOC (1) 4000 nM, 92) 100 nM; CPA (1) 12 µM, (2) 6 µM. Freshly prepared MTT solution in culture medium was added

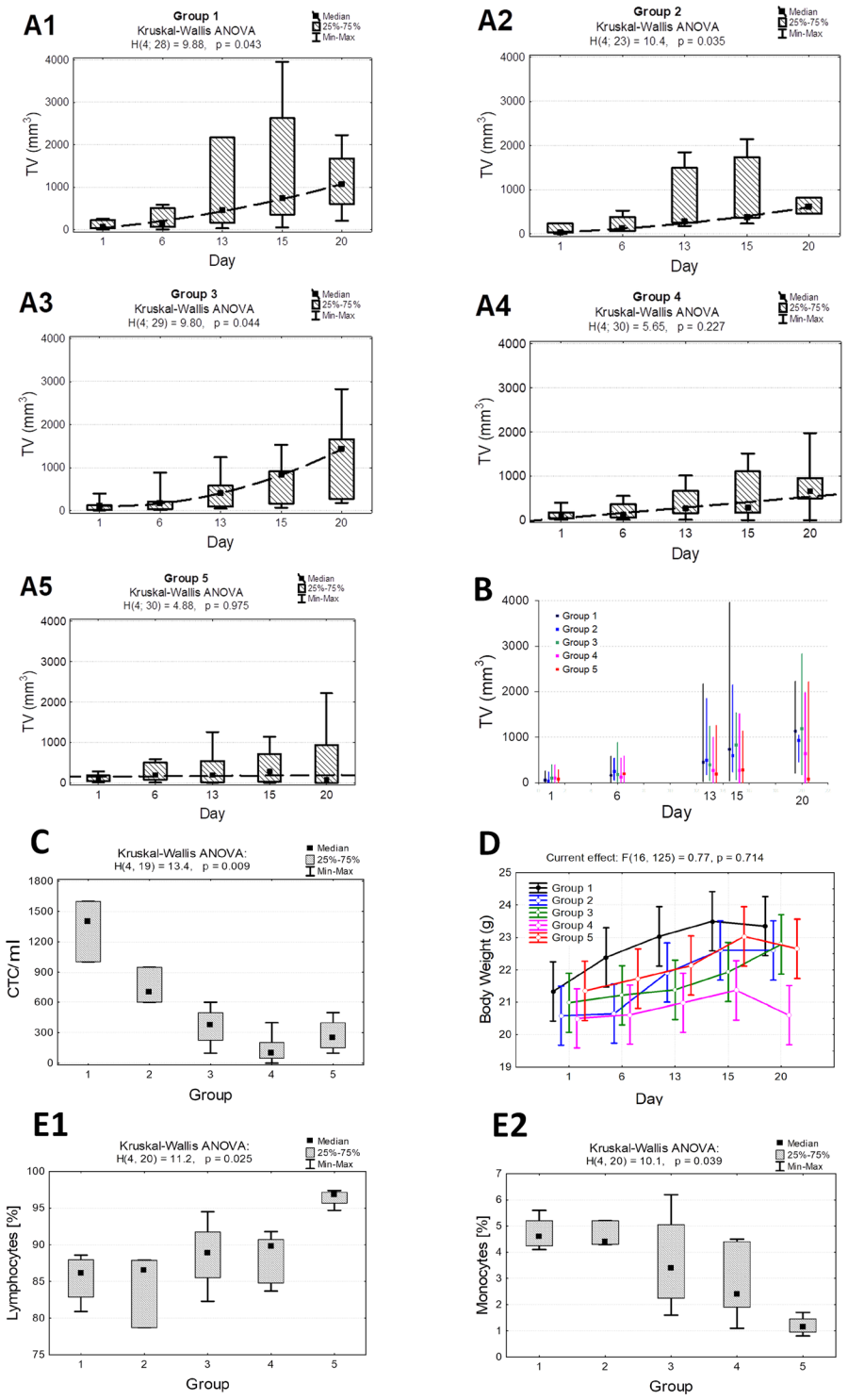

Figure 4. Results of the *in vivo* study. The animals bearing mouse colon tumor (MC38 cell line) were divided into five groups (10 mice per group): 1- control, 2- insulin (INS) only, 3- FU only, 4- insulin with FU administered together, 5- insulin administered 60 minutes before FU. (**A**) Non-parametric Kruskal-Wallis ANOVA analysis of the tumor volume in the following days of the experiment. Presented three groups (control, insulin, FU) show statistically significant tumor increase in comparison to groups 4 and 5 (combination insulin and FU) where slower tumor growth was observed. (**B**) The average tumor volume after the 3-weeks therapy in 5 groups. In group 5, the tumor volume was significantly lower compared to other groups ($p = 0.037$). (**C**) Kruskal-Wallis ANOVA for the comparison of the amount of the circulating epithelial tumor cells/ml of blood in 5 groups. Post-hoc comparisons for the CTCs analysis show that the number of CTC between control (group 1) and FU combined with insulin (group 4 and 5) was statistically significant (1,400 vs. 100; $p = 0.013$ and 1,400 vs. 250; $p = 0.043$, respectively). (**D**) The effect of the therapy on body weight in the following days of the experiment in mice differing in the treatment method (mean values and 95% confidence intervals). There was

no significant loss of body weight in insulin and/or FU treated mice. (**E**) The percentage of lymphocytes was significantly lower in control animals (group 1) and those treated with insulin alone (group 2) compared with mice treated with FU combined with insulin (group 5) (86% vs. 97%; $p = 0.041$ and 87% vs. 97%; $p = 0.045$, respectively). The percentage of monocytes was also statistically significant between groups 1 and 5 (5% vs. 1%; $p = 0.045$).

to the wells to a final concentration of 0.5 mg/ml and incubated at 37 °C for 4 h. The violet formazan crystals were solubilized with 100 µl DMSO (Sigma Aldrich, Germany) for 15 minutes. The optical absorbance (OA) was measured at 490 nm by a BioTek ELX800 multiwell reader (BioTek, Winooski, VT, USA). The 100% viability of cells was determined as absorbance of the untreated control groups, and treated groups were calculated according to the formula:

$$\text{Viable cells (\%)} = (\text{OA of experimental group/OA of control group}) \times 100.$$

For further experiments, FU and IRI were used in a concentration of 500 µM and IRI 50 µM, respectively. The dose of insulin was specified to determine the level that did not cause any significant effect in cell viability according to the control group. All assays were repeated 3 times.

Flow cytometry analysis. Caco-2 and SW480 apoptotic cells ratio following fluorouracil (500 µM) and irinotecan (50 µM) alone and with 8 h insulin pretreatment were measured using Muse® Annexin V and Dead Cell Assay Kit (Merck KGaA, Darmstadt, Germany). Cells were seeded in 6-well culture plates ($1{,}5 \times 10^5$/well) and treated as described in experimental conditions. First, cells were washed with PBS, incubated with 100 ul/well Gibco™ Trypsin-EDTA, and after 5 minutes, 300 µl of culture medium was added to each well. Next, cells were transferred to Eppendorf® tubes and gently mixed on vortex. 100 µl of each cell sample and 100 µl of Muse™ Annexin V & Dead Cell reagent were incubated for 20 minutes at room temperature in the dark. Samples were analyzed by Muse™ Cell Analyzer (Merck KGaA, Darmstadt, Germany), where the apoptotic cells were examined. The results include the percentage of live, early apoptotic, late apoptotic, total apoptotic, and dead cells in dot plots showing the binding of Annexin V-FITC and PI uptake.

RNA isolation and quantitative real-time PCR. Total RNA was isolated from cell lines, including DNase treatment using an RNeasy Mini Kit and RNase-Free DNase Set from Qiagen (Hilden, Germany) according to the manufacturer's instructions. The concentration of mRNA was measured by the Qubit RNA BR Assay for the Qubit Fluorometer (Invitrogen, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). 2 µg of RNA samples were subsequently reverse transcribed to generate complementary cDNA with Qiagen RT2 First Strand Kit using MJ Research PTC-100 PCR Programmable Thermal Controller. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was performed with an RT^2 SYBR Green qPCR Mastermix and Custom RT2 Profiler PCR Arrays from Qiagen (Hilden, Germany), using Light Cycler 480 96-well block (Basel, Switzerland). The template includes eight controls: 5 Housekeeping Genes (ACTB, HPRT1, TBP, B2M, GAPDH), 1 Genomic DNA Control (GDC), 1 Reverse Transcription Control (RTC) and 1 PCR Positive Control (PPC). Ct values were analyzed in the Qiagen web portal at GeneGlobe. Ct values were normalized based on reference genes and fold change using the $\Delta\Delta$Ct method, in which ΔCt is calculated between the gene of interest (GOI) and an average of housekeeping genes (HKG). Fold Change is then calculated using $2^{-\Delta\Delta Ct}$ formula.

Western blotting analysis. Caco-2 and SW480 cells were seeded in 6-well culture plates (9×10^4/well). The cells were washed with PBS, then the appropriate amount of RIPA buffer containing 1% protease and phosphatase inhibitors (Sigma Aldrich, Germany) was added to each well. The cells were scraped and transferred to Eppendor® tubes for low agitation for 30 min at 4 °C and then centrifuged at 16000 × g for 20 minutes. The protein level was measured at 280 nm using the Qubit Protein Assay for the Qubit Fluorometer (Invitrogen, Thermo Fisher Scientific Inc., Carlsbad, CA, USA). 50 ug protein extracts were separated on NuPAGE gels 4–12% in NuPage MES SDS Running Buffer. After electrophoresis, proteins were transferred to the nitrocellulose membrane in NuPAGE Transfer Buffer (Invitrogen, Thermo Fischer Scientific, USA). Then the nitrocellulose membrane was blocked with 10% goat serum (Sigma Aldrich, Germany) in PBST for 1 hour at room temperature and incubated overnight at 4 °C with the first antibodies: polyclonal anti-β-actin for protein normalization (dilution 1:1000, Abcam, Cambridge, UK), polyclonal anti-PI3K (dilution 1:100, Boster, USA), polyclonal anti-GRB2 (dilution 1:100, Sigma Aldrich, Germany). The next day, the nitrocellulose membrane was washed three times for 10 minutes in PBST, incubated with a secondary anti-rabbit antibody in dilution 1:1000 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) labeled with horseradish peroxidase for 1 h at room temperature. After washing 3 × 10 min in PBST, protein bands were visualized by the 1-Step Ultra TMB Blotting Solution (Thermo Fisher Scientific, USA). The membrane documentation was performed by Molecular Imager Gel Doc TMXR+ (BioRad, Hercules, CA, USA). Unprocessed original scans for the blots presented in Figure 3 are presented in the supplementary information.

Mouse allograft model of colon cancer. C57BL/6 female, 12–16-week-old mice, weighing 20–25 g were obtained from the Medical University of Bialystok (Bialystok, Poland) and maintained under specific pathogen-free (SPF) conditions. All experiments were performed according to the EU Directive 2010/63/EU for animal experiments and were approved by the 1st Local Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland (Permission No. 51/2018 issued on 16/05/2018). The mouse colon adenocarcinoma MC38 cells were obtained from Tumor Bank of Radiobiology Institute TNO, Rijswijk, Holland, and established

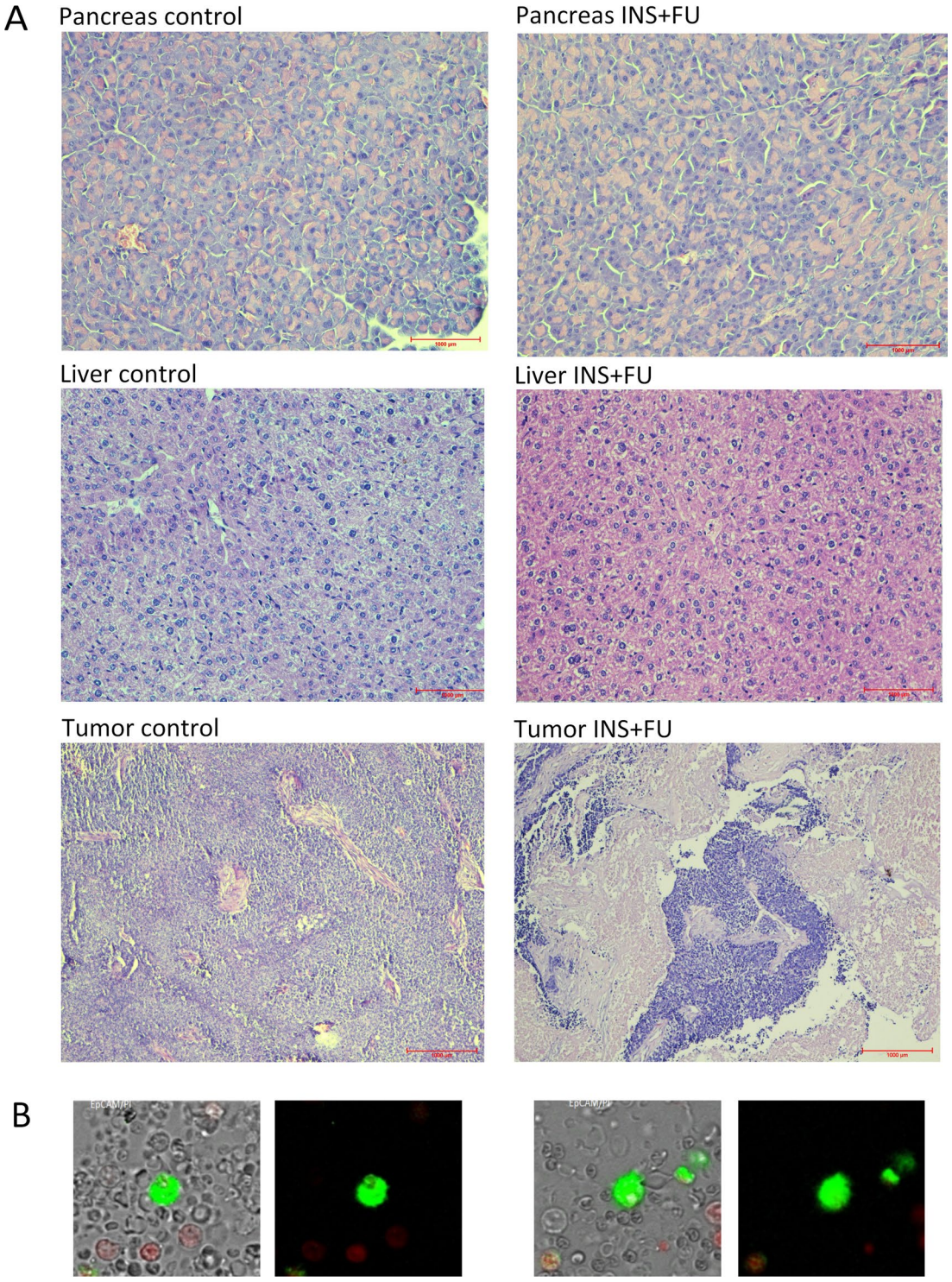

Figure 5. (**A**) Photographs of pancreas, liver, and tumor sections stained with hematoxylin-eosin (HE) to evaluate the impact of the insulin and chemotherapeutics on the tissues. The figure presents an organ from a control mouse and mouse treated with insulin and FU together (group 5). There are no significant changes in the morphology in the pancreas and liver. The figure of the tumor presents control, insulin, fluorouracil, and insulin together with fluorouracil (group 5) conditions. Sections of fluorouracil and fluorouracil with insulin reveals parts of the necrotic area in comparison to the control. Optical magnification: 200x (pancreas, liver) and 100x (tumor). (**B**) Illustrative images of CTCs detected in mouse blood. CTCs with a green EpCAM surface staining, a well-preserved morphology, but no red nuclear staining by PI is shown. The figure presents an image from a control mouse and mouse treated with insulin and FU together.

Parameter	Group					*p*
	Group 1	Group 2	Group 3	Group 4	Group 5	
CTC/μL						
Me ± *SD*	1333 ± 306	750 ± 180	363 ± 206	150 ± 158	275 ± 171	**0.009**
Med [*Q1*; *Q3*]	1400 [1000; 1600]	700 [600; 950]	375 [225; 500]	100 [50; 200]	250 [150; 400]	
Min–Max	1000–1600	600–950	100–600	0–400	100–500	

Table 2. Basic statistical analysis of the number of circulating tumor cells after therapy in five groups of mice differing in the treatment method and the result of comparisons (non-parametric Kruskal-Wallis test).

for *in vitro* culture at the Institute of Immunology and Experimental Therapy. MC38 cells were cultured *in vitro* in RPMI-1640 medium (IIET, Wroclaw, Poland) supplemented with 1 mg/mL geneticin (Gibco, UK), 2 mM L-glutamine, 1 mM sodium pyruvate (both from Sigma-Aldrich, Germany), 5% fetal bovine serum (HyClone, Thermo Fisher Scientific Inc., UK). Cells were trypsinized (IIET, Poland), centrifuged (200 g, 4 °C, 5 min $TV = \frac{1}{2} \times a^2 \times b$ min), and counted prior to transplantation. Subcutaneous transplantation: mice were subcutaneously (s.c.) inoculated in the right flank region with 1×10^6 cells suspended in 0.2 mL saline per mouse. After the tumor inoculation (day 0), 50 mice were randomly divided into 5 different groups (10 per group). All animals were monitored for activity, physical condition, body weight, and tumor growth. Tumor size was determined every other day by caliper measurement of two perpendicular diameters of the implant. Tumor volume (TV, in mm^3) was calculated with the formula: in which a is the long diameter and b is the short diameter (in mm). Mice with a tumor over 2000 mm^3 and locomotor disorders or poor condition diagnosed by a veterinary physician were euthanized. Mice lost during the experiment: 2 in group 1; 2 in group 2; 1 in group 3; 1 in group 4; 2 in group 5.

***In vivo* chemotherapy.** The animals bearing tumors were randomly divided into five groups (5–10 mice per group): 1- control, 2- insulin only, 3- FU only, 4- insulin with FU administered together, 5- insulin administered 60 minutes prior to FU. The untreated control group received the vehicle only. The dose of insulin was established during preliminary studies. Insulin was administered s.c. at a dose of 2.5 U/kg/d 1 d/wk for 3 wks. FU was dissolved in the vehicle, 0.9% saline, and given intraperitoneally (i.p.) at doses of 100 mg/kg/d, 1 d/wk for 1 wk; 150 mg/kg/d, 1 d/wk for 1 wk and 100 mg/kg/d, 1 d/wk for 1 wk. The insulin was administered either together with FU or 60 minutes prior to the injection. At the end of the experiments, tumors, pancreas, and livers were removed and processed to formalin-fixed and paraffin-embedded (FFPE) blocks for histological analysis. The blood from mice was collected to ethylenediaminetetraacetic acid (EDTA) tubes and used separately for the analysis of complete blood count and circulating epithelial tumor cells.

Complete blood count. Blood samples (500 µl–1000 µl) were drawn into normal blood count tubes with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and processed within 48 hours of collection. The level of leukocytes, lymphocytes, monocytes, granulocytes, erythrocytes, hemoglobin, hematocrit, MCV, MCH, MCHC, RDW, platelets, MPV, PDW, PCT, was measured in each blood sample (Sysmex K4500SL, serial number F2872, Japan).

Circulating tumor cells (CTCs). Circulating tumor cells (CTC) were successfully enumerated from peripheral blood using maintrac® approach, as reported previously[38]. In short, 500 ul blood was incubated with lysis buffer (Qiagen, Hilden, Germany) to remove red blood cells, and all nucleated cells were resuspended in 500 ul PBS-EDTA. Subsequently, CTC were stained with 4 µl fluorescently labeled antibodies directed against EpCAM (clone caa7-9G8, Miltenyi Biotec GmbH, Germany) and incubated for 15 min in the cold. Then, the samples were diluted with PBS-EDTA, and a defined volume of the cell suspension and propidium iodide (PI) (Sigma-Aldrich, USA) was transferred to 96 well plates (Greiner Bio-one, USA). Finally, we imaged and enumerated CTC using a fluorescence scanning microscopy (ScanR, Olympus, Tokyo, Japan), enabling visual examination of vital tumor cells. Vital CTCs were defined as EpCAM-positive cells, lacking in nuclear PI staining and with intact morphology, and only these cells were counted. Quality control regarding reagents, instrument standardization, and operator technique was assessed.

Histological evaluation of toxicity. Formalin-fixed and paraffin-embedded tissue sections of tumors, livers, and pancreas were stained with hematoxylin-eosin (HE) to evaluate the impact of the insulin and chemotherapeutics on the tissues.

Statistical analysis. The conformity of the distribution of analyzed parameters with the normal distribution was examined. The conformity was evaluated by the Shapiro-Wilk test. The homogeneity of the variance was tested with Bartlett's test. The significance of differences in mean values (Me) in 5 groups, for parameters with a non-normal distribution, was checked using the nonparametric Kruskal-Wallis test. In case of rejection of the null hypothesis with equality of average values in groups (median), to verify the differences between average values in pairs posthoc tests (Dunn test multiple comparisons) were carried out. The significance of differences in mean values (Me) in more than two populations for parameters of normal distribution and homogeneous variances was evaluated with analysis of variance (ANOVA). In case of rejection of the null hypothesis of homogeneity of variance, to verify the differences between the mean values in pairs, posthoc tests were conducted (Scheffe's test). The level $p = 0.05$ was considered as the critical significance level. Data were represented as mean ± standard deviation (Me ± SD). The analysis of the obtained results was carried out using the STATISTICA v.13 program (StatSoft, Inc. Tulsa, OK. the USA).

Parameter	Group 1	Group 2	Group 3	Group 4	Group 5	*P value*
Leukocytes ($\times 10^3/\mu L$)						
Me ± *SD*	5,85 ± 2,12	6,07 ± 1,15	4,96 ± 3,36	4,65 ± 3,24	14,95 ± 14,43	0.762
Med [*Q1*; *Q3*]	5,3 [4,6; 7,2]	6,1 [4,9; 7,2]	5,5 [3,2; 7,2]	4,3 [2,9; 8,0]	10,1 [4,7; 25,2]	
Min–Max	4,0–8,9	4,9–7,2	0,2–8,7	0,1–8,4	4,4–35,3	
Lymphocytes ($\times 10^3/\mu L$)						
Me ± *SD*	5,03 ± 1,85	5,17 ± 1,17	5,45 ± 2,22	4,84 ± 2,11	14,45 ± 14,07	0.804
Med [*Q1*; *Q3*]	4,7 [3,9; 6,2]	5,4 [3,9; 6,2]	5,7 [3,7; 7,3]	5,0 [2,8; 6,7]	9,6 [4,6; 24,4]	
Min–Max	3,2–7,6	3,9–6,2	2,8–7,7	2,6–7,1	4,3–34,4	
Monocytes ($\times 10^3/\mu L$)						
Me ± *SD*	0,25 ± 0,10	0,30 ± 0,00	0,20 ± 0,12	0,20 ± 0,19	0,18 ± 0,15	0.763
Med [*Q1*; *Q3*]	0,2 [0,2; 0,3]	0,3 [0,3; 0,3]	0,2 [0,1; 0,3]	0,1 [0,1; 0,4]	0,2 [0,1; 0,3]	
Min–Max	0,2–0,4	0,3–0,3	0,1–0,3	0,0–0,4	0,0–0,3	
Granulocytes ($\times 10^3/\mu L$)						
Me ± *SD*	0,58 ± 0,29	0,67 ± 0,15	0,45 ± 0,17	0,54 ± 0,34	0,33 ± 0,26	0.521
Med [*Q1*; *Q3*]	0,5 [0,4; 0,8]	0,7 [0,5; 0,8]	0,5 [0,3; 0,6]	0,4 [0,3; 0,9]	0,3 [0,1; 0,6]	
Min–Max	0,4–1,0	0,5–0,8	0,3–0,6	0,2–0,9	0,1–0,6	
Lymphocytes (%)						
Me ± *SD*	85,4 ± 3,4	84,4 ± 5,0	88,6 ± 5,0	88,2 ± 3,7	96,4 ± 1,2	**0.025**
Med [*Q1*; *Q3*]	86 [83; 88]	87 [79; 88]	89 [86; 92]	90 [85; 91]	97 [96; 97]	
Min–Max	81–89	79–88	82–95	84–92	95–97	
Monocytes (%)						
Me ± *SD*	4,7 ± 0,7	4,6 ± 0,5	3,7 ± 1,9	2,9 ± 1,5	1,2 ± 0,4	**0.039**
Med [*Q1*; *Q3*]	5 [4; 5]	4 [4; 5]	3 [2; 5]	2 [2; 4]	1 [1; 1]	
Min–Max	4–6	4–5	2–6	1–5	1–2	
Granulocytes (%)						
Me ± *SD*	9,9 ± 2,8	11,0 ± 4,5	7,7 ± 3,1	9,0 ± 2,4	2,4 ± 0,8	0.063
Med [*Q1*; *Q3*]	9 [8; 12]	9 [8; 16]	8 [6; 10]	9 [7; 11]	2 [2; 3]	
Min–Max	7–14	8–16	4–12	6–12	2–4	
Erythrocytes ($\times 10^6/\mu L$)						
Me ± *SD*	6,55 ± 2,26	7,42 ± 0,91	5,11 ± 2,69	5,61 ± 2,96	6,23 ± 0,90	0.497
Med [*Q1*; *Q3*]	7,0 [5,1; 8,0]	7,0 [6,8; 8,5]	5,9 [5,9; 6,0]	6,2 [5,1; 7,8]	6,1 [5,5; 7,0]	
Min–Max	3,4–8,8	6,8–8,5	0,4–7,3	0,1–8,3	5,3–7,3	
Hemoglobin (g/dL)						
Me ± *SD*	10,7 ± 2,9	11,2 ± 0,3	7,8 ± 4,2	8,9 ± 4,6	10,1 ± 1,2	0.427
Med [*Q1*; *Q3*]	11 [9; 13]	11 [11; 11]	9 [9; 10]	10 [9; 12]	10 [9; 11]	
Min–Max	7–14	11–11	0–11	0–13	9–12	
Hematocrit (%)						
Me ± *SD*	28,3 ± 8,7	31,2 ± 3,6	21,9 ± 11,4	28,7 ± 4,8	27,8 ± 3,2	0.428
Med [*Q1*; *Q3*]	30 [23; 34]	29 [29; 35]	25 [25; 27]	26 [26; 32]	28 [26; 30]	
Min–Max	16–37	29–35	2–31	24–35	23–31	
MCV (fL)						
Me ± *SD*	43,8 ± 2,5	42,1 ± 0,4	43,3 ± 2,3	43,0 ± 2,3	45,1 ± 6,0	0.804
Med [*Q1*; *Q3*]	43 [42; 45]	42 [42; 43]	43 [42; 45]	42 [42; 43]	42 [42; 48]	
Min–Max	42–48	42–43	41–46	41–47	42–54	
MCH (pg)						
Me ± *SD*	16,8 ± 1,8	15,2 ± 1,6	14,2 ± 2,7	16,0 ± 0,7	16,3 ± 1,4	0.335
Med [*Q1*; *Q3*]	16 [16; 18]	16 [13; 16]	15 [15; 15]	16 [16; 16]	16 [15; 17]	
Min–Max	16–20	13–16	10–17	16–17	15–18	
MCHC (g/dL)						
Me ± *SD*	38,3 ± 1,9	36,1 ± 3,6	32,9 ± 6,6	37,3 ± 0,7	36,2 ± 1,6	0.129
Med [*Q1*; *Q3*]	38 [37; 40]	37 [32; 39]	35 [35; 36]	37 [37; 38]	37 [35; 37]	
Min–Max	37–41	32–39	21–37	37–38	34–37	
RDW (%)						
Me ± *SD*	19,9 ± 1,0	19,6 ± 0,2	20,8 ± 1,9	21,0 ± 2,0	23,6 ± 8,7	0.618
Med [*Q1*; *Q3*]	20 [19; 21]	20 [20; 20]	21 [20; 21]	20 [20; 20]	19 [19; 28]	
Min–Max	19–21	20–20	19–24	20–25	19–37	

Continued

Parameter	Group 1	Group 2	Group 3	Group 4	Group 5	*P value*
PLT (tys./μL)						
Me ± SD	207 ± 53	305 ± 73	410 ± 201	490 ± 434	652 ± 281	0.072
Med [*Q1; Q3*]	223 [169; 245]	271 [255; 389]	453 [447; 511]	413 [264; 535]	563 [462; 843]	
Min–Max	133–249	255–389	63–578	19–1296	429–1054	
MPV (fL)						
Me ± SD	6,7 ± 1,2	5,9 ± 0,2	6,2 ± 0,4	6,9 ± 1,1	6,1 ± 0,2	0.488
Med [*Q1; Q3*]	6 [6; 7]	6 [6; 6]	7 [6; 7]	7 [6; 8]	6 [6; 6]	
Min–Max	6–9	6–6	6–7	6–8	6–6	
PDW (fL)						
Me ± SD	47,4 ± 7,4	47,8 ± 1,8	34,1 ± 7,9	31,0 ± 12,9	31,6 ± 10,5	0.080
Med [*Q1; Q3*]	50 [43; 52]	47 [46; 50]	37 [27; 41]	34 [23; 40]	36 [25; 38]	
Min–Max	37–53	46–50	25–42	10–45	16–38	
PCT (%)						
Me ± SD	0,133 ± 0,016	0,179 ± 0,041	0,262 ± 0,133	0,311 ± 0,241	0,397 ± 0,169	0.073
Med [*Q1; Q3*]	0,14 [0,12; 0,15]	0,16 [0,15; 0,23]	0,29 [0,27; 0,34]	0,28 [0,18; 0,39]	0,33 [0,29; 0,51]	
Min–Max	0,11–0,15	0,15–0,23	0,04–0,38	0,02–0,73	0,28–0,64	

Table 3. Basic statistical analysis of the hematologic test after the therapy in five groups of mice differing in the treatment method and the result of comparison (non- parametric Kruskal-Wallis test). Me – mean; SD – standard deviation; Med – median; Q1 – lower quartile; Q3 – upper quartile; Min – minimal value; Max – maximal value, *p* – significance.

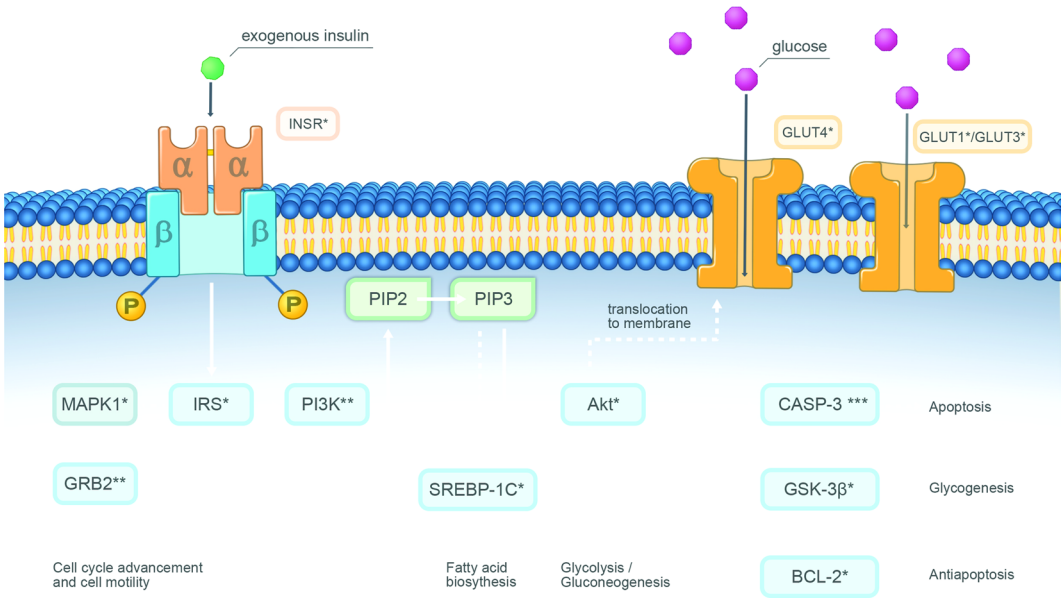

Figure 6. Effects of the therapy on the pathways related to the signaling downstream of insulin receptors. A proposed graphical mechanism illustrates the possible anti-tumor effect of insulin and chemotherapeutic agents in colon cancer cells. Insulin binds to its receptor and initiates a cascade of intracellular processes. Insulin may enhance the therapeutic effect of anticancer drugs through the downregulation of GRB2, a crucial substrate for cell cycle advancement and cell motility, as well as PIK3CA, which plays a pivotal role in growth, proliferation, survival, and migration of cancer cells. *Downregulated after the treatment with cytotoxic agent alone and in combination with insulin. **Downregulated only in insulin pretreated cells.

Study Highlights

New anticancer drugs that marginally improve survival of patients continue to be developed at an unsustainably high cost. The study aimed to elucidate the effects of insulin, an inexpensive drug with a convincing safety profile, on the susceptibility of colon cancer to commonly used chemotherapeutic agents. The *in vitro* and *in vivo* study showed that insulin enhanced the antitumor effect of chemotherapeutics via downregulation of PIK3CA and GRB2, that play a critical role in cell signaling. Insulin might be potentially applied to clinical use to enhance the therapeutic effectiveness of chemotherapeutic drugs. The findings may become a platform for future development of new and inexpensive strategies for the clinical chemotherapy of tumors.

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References

1. Gustavsson, B. *et al*. A review of the evolution of systemic chemotherapy in the management of colorectal cancer. *Clin Colorectal Cancer* **14.1**, 1–10 (2015).
2. Hu, T., Li, Z., Gao, C. & Cho, C. Mechanisms of drug resistance in colon cancer and its therapeutic strategies. *World Journal of Gastroenterology* **22**, 6876 (2016).
3. Hammond, W. A., Swaika, A. & Mody, K. Pharmacologic resistance in colorectal cancer: a review. *Therapeutic Advances in Medical Oncology* **8**, 57–84 (2015).
4. Mailankody, S. & Prasad, V. Comparative Effectiveness Questions in Oncology. *New England Journal of Medicine* **370**, 1478–1481 (2014).
5. Shackney, S. E., McCormack, G. W. & Cuchural, G. J. Jr. Growth Rate Patterns of Solid Tumors and Their Relation to Responsiveness to Therapy. *Annals of Internal Medicine* **89**, 107 (1978).
6. Gross, G. E., Boldt, D. H. & Osborne, C. K. Perturbation by insulin of human breast cancer cell cycle kinetics. *Cancer Res.* **44**, 3570 (1984).
7. Pollak, M. Insulin and insulin-like growth factor signalling in neoplasia. *Nature Reviews Cancer* **8**, 915–928 (2008).
8. De Meyts, P. Insulin and its receptor: structure, function and evolution. *Bioessays* **26**, 1351–1362 (2004).
9. Agrawal, S. *et al*. Insulin-induced enhancement of MCF-7 breast cancer cell response to 5-fluorouracil and cyclophosphamide. *Tumor Biology* **39**, 101042831770290 (2017).
10. Agrawal, S. *et al*. Insulin and novel thioglycosides exert suppressive effect on human breast and colon carcinoma cells. *Oncotarget* **8** (2017).
11. Yang, Y., Wen, F. & Dang, L. Insulin enhances apoptosis induced by cisplatin in human esophageal squamous cell carcinoma EC9706 cells related to inhibition of autophagy. *Chin Med J* **127**, 353–358 (2014).
12. Zou, K., Ju, J. H. & Xie, H. Pretreatment with insulin enhances anticancer functions of 5-fluorouracil in human esophageal and colonic cancer cells. *Acta Pharmacol Sin* **28**, 721–730 (2007).
13. Miglietta, A., Panno, M. L. & Bozzo, F. Insulin can modulate MCF 7 cell response to paclitaxel. *Cancer Lett* **209**, 139–145 (2004).
14. Alabaster, O., Vonderhaar, B. & Shafie, S. Metabolic modification by insulin enhances methotrexate cytotoxicity in MCF-7 human breast cancer cells. *Eur J Cancer Clin Oncol* **17**, 1223–1228 (1981).
15. Lasalvia-Prisco, E. *et al*. Insulin induced enhancement of antitumoral response to methotrexate in breast cancer patients. *Cancer Chemother Pharmacol* **53**, 220–224 (2004).
16. Damyanov, C., Gerasimova, D., Maslev, I. & Gavrilov, V. Low-dose chemotherapy with insulin (insulin potentiation therapy) in combination with hormone therapy for treatment of castration-resistant prostate cancer. *ISRN Urol* **2012**, 140182 (2012).
17. De Both, N. J., Vermey, M., Dinjens, W. N. & Bosman, F. T. A comparative evaluation of various invasion assays testing colon carcinoma cell lines. *Br J Cancer* **81**(6), 934 (1999).
18. Belfiore, A., Frasca, F., Pandini, G., Sciacca, L. & Vigneri, R. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. *Endocr Rev* **30**, 586–623 (2009).
19. Pollak, M. The insulin and insulin-like growth factor receptor family in neoplasia: an update. *Nat Rev Cancer* **12**, 159–169 (2012).
20. Frasca, F. *et al*. The role of insulin receptors and IGF-I receptors in cancer and other diseases. *Arch Physiol Biochem* **114**, 23–37 (2008).
21. Samuels, Y. & Ericson, K. Oncogenic PI3K and its role in cancer. *Curr Opin Oncol* **18**, 77–82 (2006).
22. Song, M. S., Salmena, L. & Pandolfi, P. P. The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol* **13**, 283–296 (2012).
23. Fruman, D. A. & Rommel, C. PI3K and cancer: lessons, challenges and opportunities. *Nat Rev Drug Discov.* **13**, 140–56 (2014).
24. Yap, T. A., Bjerke, L., Clarke, P. A. & Workman, P. Drugging PI3K in cancer: refining targets and therapeutic strategies. *Curr Opin Pharmacol.* **23**, 98–107 (2015).
25. Boucher, J., Kleinridders, A. & Kahn, C. R. Insulin receptor signaling in normal and insulin-resistant states. *Cold Spring Harb Perspect Biol.* **6**, a009191 (2014).
26. Sachdev, D. & Yee, D. Disrupting insulin-like growth factor signaling as a potential cancer therapy. *Mol Cancer Ther.* **6**, 1e12 (2007).
27. Eccleston, A. & Dhand, R. Signalling in cancer, 05/25/print. *Nature* **441**, 423 (2006).
28. Skolnik, E. Y. *et al*. The function of GRB2 in linking the insulin receptor to ras signaling pathways. *Science.* **260**, 1953–1955 (1993).
29. Zhang, Y. *et al*. miR-411-5p inhibits proliferation and metastasis of breast cancer cell via targeting GRB2. *Biochem Biophys Res Commun* **476**, 607–613 (2016).
30. Huebner, K. *et al*. Chromosome locations of genes encoding human signal transduction adapter proteins, Nck (NCK), Shc (SHC1), and Grb2 (GRB2). *Genomics.* **22**(2), 281–7 (1994).
31. Yu, G. Z. *et al*. New insight into the key proteins and pathways involved in the metastasis of colorectal carcinoma. *Oncol Rep.* **19**, 1191–1204 (2008).
32. Hu, C. T., Wu, J. R. & Wu, W. S. The role of endosomal signaling triggered by metastatic growth factors in tumor progression. *Cell Signal* **25**, 1539–45 (2013).
33. Fath, I. *et al*. Cloning of a Grb2 isoform with apoptotic properties. *Science* **264**, 971–4 (1994).
34. Tie, J. *et al*. Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. *Annals of Oncology* **26.8**, 1715–1722 (2015).
35. Pachmann, K. *et al*. Monitoring the Response of Circulating Epithelial Tumor Cells to Adjuvant Chemotherapy in Breast Cancer Allows Detection of Patients at Risk of Early Relapse. *J Clin Oncol* **26**, 1208–1215 (2008).
36. Fujii, T. *et al*. Androgen receptor expression on circulating tumor cells in metastatic breast cancer. *Plos One* **12**, e0185231 (2017).
37. Kitz, J., Lowes, L. E., Goodale, D. & Allan, A. L. Circulating Tumor Cell Analysis in Preclinical Mouse Models of Metastasis. *Diagnostics (Basel)* **8**, 2 (2018).
38. Pachmann, K. *et al*. Standardized quantification of circulating peripheral tumor cells from lung and breast cancer. *Clin Chem Lab Med* **43**(6), 617–627 (2005).

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Author contributions

S.A., M.W. and M.L. had full access to all of the data and are responsible for the integrity of the data and the accuracy of the analysis. M.W., M.L., S.M. and E.P. conducted the *in vitro* studies. S.A., A.K.A. and M.W. contributed to the study concept and design. A.K.A., P.Z. and A.G. supervised the study. M.W. and M.L. performed the flow cytometry and genetic analysis. S.M. and E.P. performed the Western blot. J.W. and J.B. conducted the *in vivo* study. M.P. conducted the CTC analysis. S.A., S.M. and M.W. drafted the manuscript, and all of the authors made critical revisions for important intellectual content and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Research Paper

Insulin and novel thioglycosides exert suppressive effect on human breast and colon carcinoma cells

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ABSTRACT

The rationale for the implementation of novel therapies should be based on hallmarks of cancer. Two novel compounds labelled as thioglycoside A and B were designed and evaluated on breast and colon cancer cell lines. We assessed their cytotoxic effect after sensitizing cancer cells with insulin. In order to explore the underlying mechanisms, we performed tests to assess cell migration and motility, apoptosis, expression of glucose transporter 1 and proapoptotic proteins. Both compounds proved to have an antitumor effect which was significantly enhanced in combination with insulin. Linking glucose and anticancer agent presents an approach that exploits the Warburg effect. Targeting dysfunctional glycometabolism and increased glucose absorption is emerging as a promising anticancer strategy.

INTRODUCTION

Malignancies of diverse origins deviate from healthy tissues in their high consumption of glucose. This phenomenon, recognized as one of the hallmarks of cancer, has attracted a great deal of interest in anticancer therapies. Conjugation of glucose with metabolic agents to selectively target cancer cells was inspired by the widespread use of radiolabeled glucose analog to visualize tumors and their metastases. The field of synthesis and evaluation of sugar-conjugated anticancer agents has grown significantly in recent years, with certain compounds in advanced clinical trials [1].

Thioglycosides have received considerable attention because they are widely employed as biological inhibitors [2–6], inducers [7–9]. Moreover, they are promising candidates in synthetic carbohydrate chemistry as convenient and versatile glycosyl donors. Among these, glycosyl donors are the thioglycosyl heterocycles that are sufficiently stable under a variety of reaction conditions and have the ability to be readily converted into a variety of other functionalities [10–12].

S-Glycosides are very attractive substitutes for O-glycosides, as it is well-known that they are much less susceptible to enzymatic cleavage as well as chemical degradation [13]. Also, they often exhibit a similar conformational solution and similar or even more potent bioactivities compared to the corresponding O-glycosides. (5-Nitro-2-pyridyl) 1-thioglycosides and obtained by their oxidation sulfoxides were assayed for cytotoxicity and *in vitro* antiviral properties against classical swine fever virus (CSFV). The best antiviral activity exhibited sulfoxide derivative of (5-nitro-2-pyridyl) 1-tiolactoside [14]. Also, glycoconjugates formed by the combination of (5-nitro-2-pyridyl) 1-thioglycosides and uridine derivatives showed significant activity against the Flaviviridae family [15]. Pyridine thioglycosides were reported as a new class of antimetabolites which exert inhibitory effects on both DNA and RNA containing viruses [16]. Thioglycosides have been proved to have good cytotoxic effects against

Ehrlich ascites carcinoma cells (EAC cells) and four human cancer cell lines, namely liver Hepg2, breast MCF7, brain U251, lung H460. The postulated mechanism of action of pyridine thioglycosides is a cell cycle arrest in the S phase similar to the antimetabolites and cell cycle arrest in the G2/M phase (M phase) resembling microtubules inhibitors [17]. It was found that antitumour effectiveness of thioglycosides strongly depends on the structure of substituents in the pyridine ring [17]. On the other hand, result presented by Romero-Ramires et al confirmed the higher resistance to enzymatic hydrolysis of thioglycosides as compared to O-glycosyl derivatives. *in vivo* experiments in nude mice bearing an implanted C6 glioma showed that the thioglycoside reduced tumor volume, while the O-glycosyl derivative was inactive, highlighting the importance of using enzyme resistant glycosides [18]. Taking this into account in the planned study, negatively substituted 3-nitro and 5-nitro pirydyl thioglycosides resistance to hydrolysis were selected.

It is well established that insulin exhibits potent metabolic properties and is implicated in many malignancies [19]. Its impact on cellular uptake of many compounds including glucose by facilitated diffusion has been documented [20]. The use of insulin for cancer-specific treatment has been tested in several studies [21–25].

In this research, we have analyzed the antitumor effect of novel compounds: (5-nitro-2-pyridyl) 1-thio-β-D-glucopyranoside labelled as thioglycoside A, and (3-nitro-2-pyridyl) 1-thio-β-D-glucopyranoside labelled as

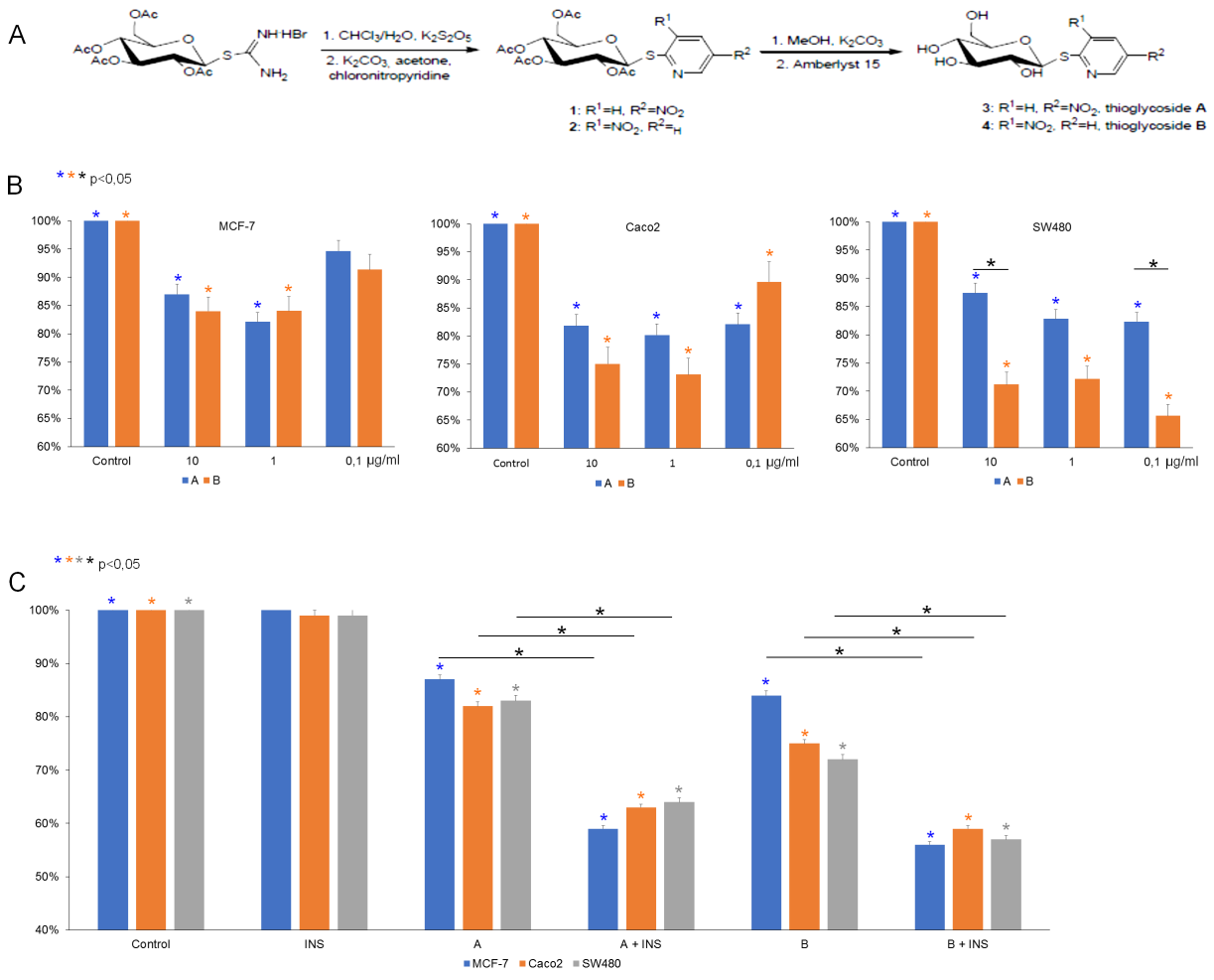

Figure 1: (A) Synthesis of compounds (5-nitro-2-pyridyl) 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside (1, thioglycoside A) and (3-nitro-2-pyridyl) 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside (2, thioglycoside B). **(B)** Activity of thioglycosides A and B on MCF-7, Caco-2, SW480 cancer cell lines. All three cell lines were treated with 10 µg/ml, 1 µg/ml, 0.1 µg/ml of thioglycoside A and B respectively for 24 hours. Cytotoxic effect was measured by MTT assay. Data are shown as mean ±SD from three separate experiments. **(C)** After 8-hour insulin pretreatment (40 µg/ml for MCF-7 and 100 µg/ml for Caco-2 and SW-480) all three cell lines were exposed to 10 µg/ml of thioglycosides A and B respectively for 24 hours. Inhibitory effect was measured by MTT assay. The results are shown as mean ±SD from three individual experiments. Statistically significant variables were marked with * (p<0,05).

thioglycoside B, on three cancer cell lines: MCF-7 human breast cancer cell line and human colon cancer cell lines: Caco-2, SW480.

We further assessed whether insulin can enhance the antitumor effect of these compounds. To investigate and establish the possible mechanisms of this phenomenon, we assessed cell proliferation, cell migration and motility, expression of glucose transporter 1 (GLUT-1) and proapoptotic proteins (caspase-3, BAX).

RESULTS

Thioglycoside A and B exhibit antitumor effect

To identify the optimal concentration of the compounds, various doses were tested. The thioglycosides in concentrations 10 μg/ml and 1 μg/ml exhibited significant inhibition in viability of breast and colon cancer cells (Figure 1B). The effect of both thioglycosides

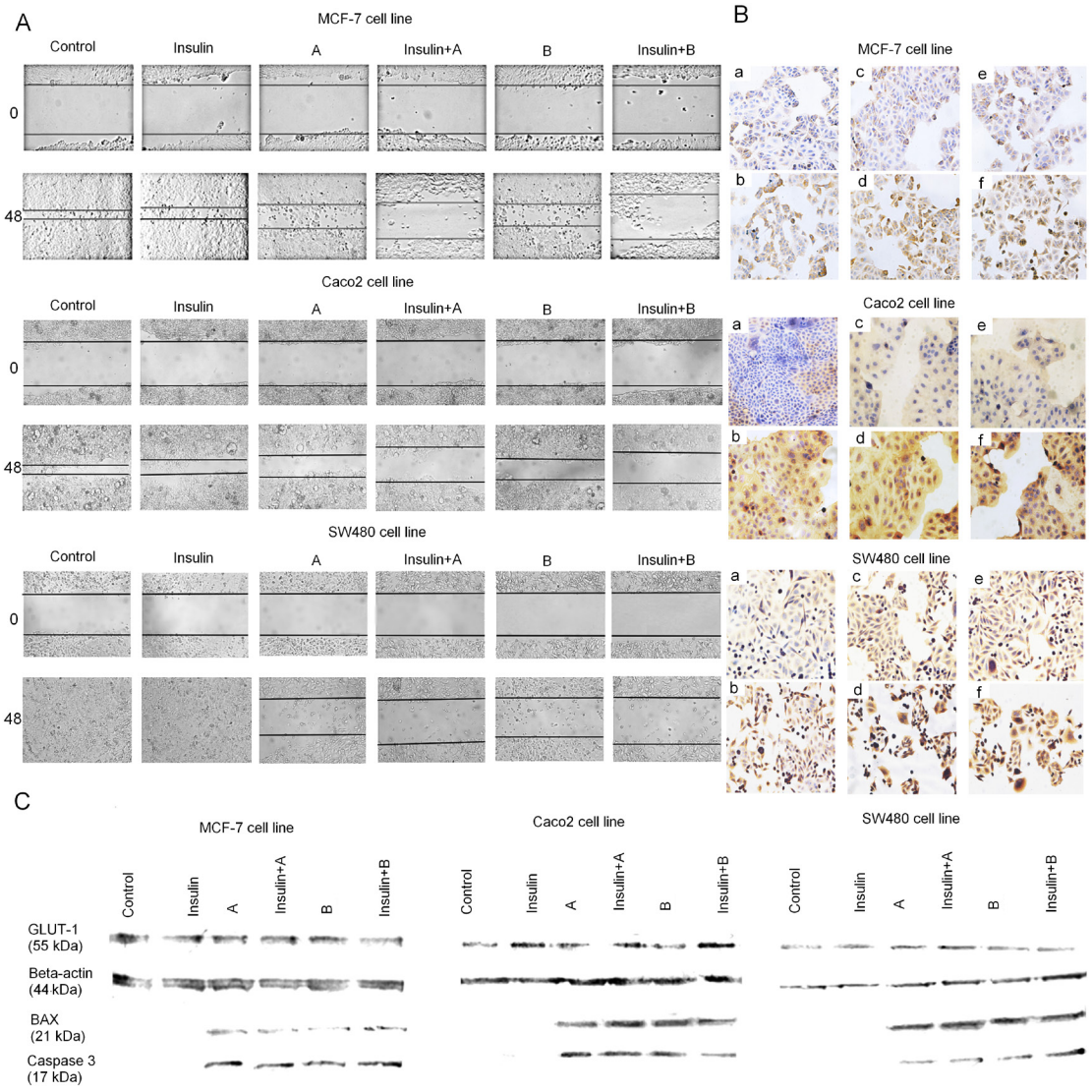

Figure 2: (A) Effect of thioglycosides A and B and thioglycosides A and B with additional insulin pretreatment on migration of MCF-7, Caco-2 and SW-480 cancer cells. Cell migration was evaluated through wound healing assay. Photomicrographs were taken at specific time points with an inverted microscope and digital camera. Control: MCF-7/Caco-2/SW480 cancer cells; Insulin: MCF-7/Caco-2/SW480 exposed to insulin (40 μg/ml for MCF-7 and 100 μg/ml for Caco-2 and SW-480); A: MCF-7/Caco-2/SW480 cancer cells treated with 10 μg/ml of thioglycoside A; A+Insulin: MCF-7 cancer cells pretreated with 40 μg/ml of insulin and Caco-2/SW480 with 100 μg/ml of insulin and treated with 10 μg/ml of thioglycoside A; B: MCF-7/Caco-2/SW480 cancer cells treated with 1 μg/ml of thioglycoside B; B+Insulin: MCF-7 cancer cells pretreated with 40 μg/ml of insulin and Caco-2/SW480 with 100 μg/ml of insulin and treated with 1 μg/ml of thioglycoside B. **(B)** Immunohistochemistry staining for GLUT-1 protein in MCF-7, Caco-2 and SW480 cancer cell lines. LSAB+ method, hematoxylin-counterstained at magnification 100x and 200x (a- control, b- INS, c- compound A, d- compound A+INS, e- compound B, f- compound B+INS). **(C)** Western blotting analysis of expression of apoptosis-related proteins and GLUT-1 receptor in MCF-7, Caco-2 and SW480 cancer cell lines. Cancer cells were incubated with insulin for 8 hours (40 μg/ml for MCF-7 and 100 μg/ml for Caco-2 and SW-480) and treated with 10 μg/ml of compound A and 1 μg/ml of compound B.

on MCF-7 and Caco-2 cell viability was similar. However, by statistical analysis we found that compound B is more cytotoxic to SW480 than compound A. The impact of non-conjugated glucose and other sugars on cell viability of breast cancer cells were assessed during preliminary studies. We found no significant changes in viability of the cells (Supplementary Material 1).

Insulin enhances the inhibitory effect of thioglycosides

MCF-7 cancer cells were pretreated with 40 μg/ml insulin (INS), while colon cancer cells with 100 μg/ml. After incubation with insulin for 8 hours, cells were treated with thioglycosides A and B at concentration 10 μg/ml. Insulin alone had no significant effect on cell growth (Figure 1C). We found that the combination of INS and thioglycosides produced a significant inhibition in growth of both breast and colon cancer cells.

Combination of insulin and thioglycosides inhibits cell motility

Wound-healing assay was performed to assess the combined effect of insulin and thioglycosides on cell proliferation and cell motility (Figure 2A). The results indicate that control and INS-treated cells almost completely filled the "wound" in MCF-7 and Caco-2 cells by 24h. The wound was filled completely in SW480 cells. In case of cells treated only with thioglycosides, a slightly hindered cell was observed. In sharp contrast, an addition of INS with thioglycosides significantly inhibited wound healing. This effect was observed in all cancer cell lines.

Impact on GLUT-1 expression and apoptosis

The effect of insulin and compounds A and B on the expression of glucose transporter was analysed by immunocytochemistry. We found that treatment with insulin caused an elevated cytoplasmic expression of GLUT-1 protein when compared with the control. Comparing to the insulin alone, the combinations of thioglycosides with insulin, produced similar effects (Figure 2B). The findings were confirmed in Western blotting analysis (Figure 2C).

The expression of proapoptotic proteins – caspase 3 and BAX was analysed by Western blotting. We detected high levels of proapoptotic proteins in cells treated with combination of insulin and thioglycosides as well as thioglycosides only (Figure 2C). The flow cytometry analysis showed that insulin had no significant impact on the ratio of apoptosis in all tested cancer cell lines (Figure 3). Over 20% of the MCF-7 breast cancer cells underwent apoptosis when treated with compound A or B. The addition of insulin did not result in significant changes in the ratio of apoptotic cells. The level of apoptosis in SW480 cells treated with thioglycoside A was slightly over 10%. The addition of insulin to compound A resulted in an increased ratio of apoptotic cells, which was slightly over 26%. Compound B with and without insulin produced a similar effect (over 20% of cells undergoing apoptosis). The apoptotic action of compound A on colon

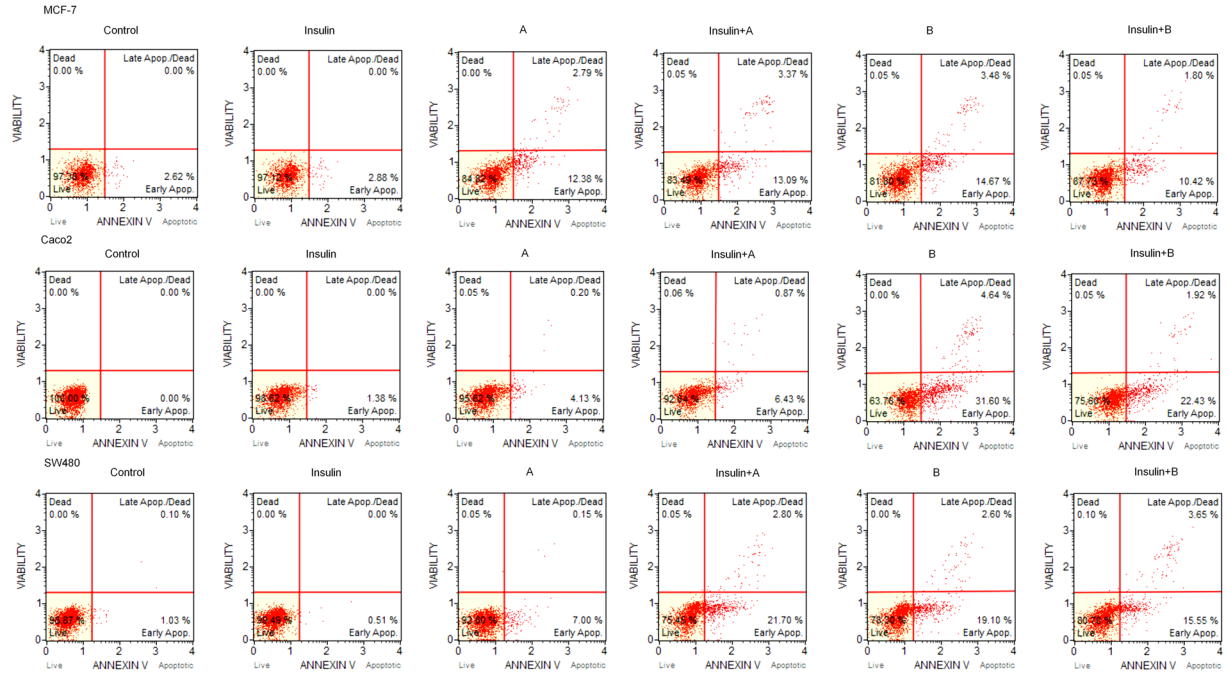

Figure 3: Original histogram plots include a percentage of live, early apoptotic, late apoptotic, total apoptotic, and dead cells differentiated using Muse® Annexin V and Dead Cell Assay Kit.

cancer cell line Caco-2 was similar to that of SW480 (nearly 10%). The addition of insulin to compound A caused an enhancement of apoptosis by slightly over 6%. The highest level of apoptosis was detected in Caco-2 cells treated with compound B (nearly 34%). Interestingly, the combination of insulin and compound B produced 23,33% of apoptosis.

DISCUSSION

Cancer cells, unlike the majority of somatic cells, consume large amounts of glucose and rely on aerobic glycolysis to generate ATP. This observation was described in the 1920s by Otto Warburg and is known as the Warburg effect [26, 27]. The phenomenon is a significant survival advantage as it allows the cancer cells to survive and multiply both in normoxic and hypoxic environment as well to evade immune killing [28, 29].

Glucose as a main fuel for ATP production needs to be transported from extracellular space via cell membrane. Diffusion of extracellular glucose is facilitated by membrane proteins called glucose transporters (GLUTs). Most of the cells which underwent malignant transformation present overexpression of GLUT family members, especially GLUT-1 [30]. Targeting dysfunctional glycometabolism and marked glucose absorption is emerging as a promising anticancer strategy [28]. Linking glucose and anticancer agent presents an approach that exploits the Warburg effect.

Insulin is known to be implicated in many malignancies [19]. It affects the cell metabolism by increasing transcription, stimulating DNA synthesis as well as increasing the turnover of cellular carbohydrates and lipids [20]. The cellular uptake of glucose and numerous ions is markedly enhanced in the presence of insulin. Due to its complex influence on malignant cell metabolism, insulin has been exploited as a potential sensitizing agent in cancer therapy. Several *in vitro* and clinical studies have found that the inhibitory effect of various cytotoxic agents can be enhanced in the presence of insulin [21–25].

Herein we report the discovery of novel compounds in which sugar is linked to aglycone in an efficient manner through S-glycosidic bond. Moreover, this is the first study that investigated the combined use of insulin and glucose linked to anticancer agent. Our research conducted on breast and colon cancer cell lines demonstrated cytotoxic activity of thioglycoside A and thioglycoside B. Interestingly, this effect was significantly enhanced in the presence of insulin. We found that insulin can increase the cytotoxic action of glucose-conjugates up to two-fold. These potential candidates for future anticancer therapy displayed antiproliferative as well as proapoptotic action *in vitro*. These results can be explained by: 1/ inhibition of metabolic pathways that lead to formation of purines and pyrimidines as well as 2/ inhibition of tubulin polymerization [17].

In present study, we assessed the impact of insulin on GLUT-1 expression. The results showed an elevated expression of GLUT-1 in insulin treated cells. These findings are consistent with previous studies [25]. We suggest that an overexpression of GLUTs is responsible for a higher uptake of the novel compounds, thus leading to enhanced cytotoxicity. It is hypothesized that the preferential uptake of glucose into malignant versus normal tissues, which is further enhanced by insulin, is responsible for the observed anticancer effect.

In conclusion, our study demonstrates the pioneer use of novel thioglycosides with supplementary insulin to selectively target cancer cells *in vitro*. Further *in vivo* studies are required to assess its application. This approach has a great deal of potential and a considerable opportunity for growth.

MATERIALS AND METHODS

Chemistry

The synthesis and assessment of antitumor activity of number of thioglycoside derivatives of substituted dihydropyridine have been performed. From the results of studies on the antitumor activity and structure-activity relationship, it can be concluded that the effect of the substituent in the aryl group placed at position 4 as well as the glycopyranosylthio moiety in the pyridine ring was obvious [31]. Many methods are presented for the efficient preparation of thioglycosides [32]. The conventional synthesis of dihydropyridine thioglycosides is achieved through the reaction of piperidinium salts of dihydropyridine thiolates with per-*O*-acetyl glycopyranosyl halides. Treatment of the glycosides with saturated solution of ammonia in methanol at room temperature afforded the free glycosides. A simple approach for the stereoselective synthesis of 1,2-trans 1-thioglycosides is based on the utilization of glycosyl isothiourea derivatives as precursors. Conversion of glycosyl isothiourea into per-*O*-acetyl-1-thio-β-D-hexopyranoses followed by treatment with substituted aryl chlorides under basic conditions provides an efficient method for the synthesis of aryl 1-thio-β-glycosides [33]. We have found that this method is an effective procedure of the synthesis of hetaryl thioglycosides (Figure 1A).

General information

The ^{1}H NMR and ^{13}C NMR spectra were recorded with an Agilent spectrometer at a frequency of 400 MHz using TMS as an internal standard and $CDCl_3$ or CD_3OD as solvents. NMR solvents were purchased from ACROS Organics (Geel, Belgium). Chemical shifts

(δ) are expressed in ppm and coupling constants (*J*) in Hz. The NMR spectra are shown in the Supplementary Material 2. Optical rotations were measured with a JASCO P-2000 polarimeter using a sodium lamp (589.3 nm) at room temperature. Melting point measurements were performed on OptiMelt (MPA 100) Stanford Research Systems. Mass spectra were recorded with a WATERS LCT Premier XE system (high resolution mass spectrometer with TOF analyzer) or with a 4000 QTRAP ABSciex mass spectrometer using electrospray-ionization (ESI) technique. Reactions were monitored by TLC on precoated plates of silica gel 60 F254 (Merck Millipore). The TLC plates were inspected under UV light (λ = 254 nm) or charring after spraying with 10% sulfuric acid in ethanol. Crude products were purified using column chromatography performed on Silica Gel 60 (70-230 mesh, Fluka) developed with toluene/EtOAc or $CHCl_3$/MeOH solvent systems. All evaporations were performed on a rotary evaporator under diminished pressure at 40 °C.

Isothiouronium salt of tetra-*O*-acetyl-β-D-glucose was prepared as described in the literature [34]. All chemicals used in experiments were of analytical grade and purchased from Sigma-Aldrich, Fluka and ACROS Organics.

General procedure for synthesis of per-*O*-acetylated nitropyridyl 1-thioglycosides

Synthesis of (5-nitro-2-pyridyl) and (3-nitro-2-pyridyl) per-*O*-acetyl-1-thio-β-D-glucosides (**1**) [35] and (**2**) [36], respectively, was performed from the isothiouronium salt of tetra-*O*-acetyl-β-D-glucose according to the following procedure.

A solution of $K_2S_2O_5$ (6.84 g, 82 mmol) in water (60 mL) was heated up to 80°C for 2 minutes. After cooling down to 50°C, the isothiouronium salt of tetra-*O*-acetyl-β-D-glucose (15 g, 30.8 mmol) in $CHCl_3$ (200 mL) was added to this solution. The resulting mixture was heated at the reflux for 0.5 h. Then the layers were separated, the organic layer was washed with brine (3×100 mL), dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was dissolved in dry acetone (500 mL). To this solution were added sequentially 2-chloro-5-nitropyridine or 2-chloro-3-nitropyridine (4.87 g, 31 mmol) and K_2CO_3 (8.28 g, 60 mmol). The resulting mixture was stirred at room temperature for 0.5 or 2 h, then filtered and evaporated. The crude products were purified by crystallization from ethyl alcohol. Residue after crystallization was purified by column chromatography (toluen: ethyl acetate; gradient: 10:1 to 4:1).

(5-nitro-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (1, thioglycoside A)

Reaction time: 30 minutes. Product **1** (12.3 g, 82%) was obtained as a white solid after purification by a crystallization from ethyl alcohol. $[\alpha]_n^{20}$ = 14 ($CHCl_3$, c = 5); m.p. 181-183°C; ^{1}H NMR (400 MHz, CD_3OD): δ 2.02, 2.03, 2.04, 2.05 (4s, 12H, CH_3CO), 3.92 (ddd, 1H, *J* = 2.4 Hz, *J* = 4.7 Hz, *J* = 10.2 Hz, H-5), 4.11 (dd, 1H, *J* = 2.4 Hz, *J* = 12.5 Hz, H-6a), 4.27 (dd, 1H, *J* = 4.7 Hz, *J* = 12.5 Hz, H-6b), 5.17 (dd, 1H, *J* = 9.0 Hz, *J* = 10.2 Hz, H-4), 5.25 (dd, 1H, *J* = 9.8 Hz, *J* = 10.6 Hz, H-2), 5.38 (dd, 1H, *J* = 9.0 Hz, *J* = 9.8 Hz, H-3), 6.11 (d, 1H, *J* = 10.6 Hz, H-1), 7.34 (d, 1H, *J* = 8.8 Hz, H-3_{pyr}), 8.31 (dd, 1H, *J* = 2.6 Hz, *J* = 8.8 Hz, H-4_{pyr}), 9.27 (d, 1H, *J* = 2.6 Hz, H-6_{pyr}).^{13}C NMR (100 MHz, CD_3OD): δ 20.08, 20.10, 20.19 ($\underline{C}H_3CO$), 61.29 (C-6), 67.56, 68.62, 73.42, 75.72 (C-2, C-3, C-4, C-5), 80.55 (C-1), 121.82, 130.68, 141.56, 144.52, 163.09 (C_{pyr}), 168.89, 168.94, 169.57, 170.02 (CO).

(3-nitro-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (2, thioglycoside B)

Reaction time: 2 hours. Product **2** (12.7 g, 85%) was obtained as a white solid after purification by a crystallization from ethyl alcohol. $[\alpha]_n^{20}$ = 64 ($CHCl_3$, c = 1); m.p. 125-126°C; ^{1}H NMR (400 MHz, CD_3OD): δ 2.02, 2.03, 2.04, 2.05 (4s, 12H, CH_3CO), 3.92 (ddd, 1H, *J* = 2.4 Hz, *J* = 4.7 Hz, *J* = 10.2 Hz, H-5), 4.23 (dd, 1H, *J* = 2.4 Hz, *J* = 12.5 Hz, H-6a), 4.23 (dd, 1H, *J* = 4.7 Hz, *J* = 12.5 Hz, H-6b), 5.18 (dd, 1H, *J* = 9.0 Hz, *J* = 10.2 Hz, H-4), 5.32 (dd, 1H, *J* = 9.4 Hz, *J* = 10.2 Hz, H-2), 5.38 (dd, 1H, *J* = 9.0 Hz, *J* = 9.4 Hz, H-3), 6.07 (d, 1H, *J* = 10.2 Hz, H-1), 7.32 (dd, 1H, *J* = 4.7 Hz, *J* = 8.2 Hz, H-5_{pyr}), 8.53 (dd, 1H, *J* = 1.6 Hz, *J* = 8.2 Hz, H-6_{pyr}), 8.73 (dd, 1H, *J* = 1.6 Hz, *J* = 4.7 Hz, H-4_{pyr}).^{13}C NMR (100 MHz, CD_3OD):δ 20.26, 20.59, 20.66 ($\underline{C}H_3CO$), 61.85 (C-6), 68.20, 68.90, 74.38, 75.96 (C-2, C-3, C-4, C-5), 79.85 (C-1), 120.09, 133.95, 142.32, 152.98, 154.33 (C_{pyr}), 169.23, 169.34, 170.22, 170.56 (CO).

Deprotection of (5-nitro-2-pyridyl) or (3-nitro-2-pyridyl) per-*O*-acetylated 1-thioglycosides

Compounds **1** or **2** (5 g, 10.2 mmol) were suspended in MeOH (300 mL). To the resulting mixture K_2CO_3 (5.5 g, 40 mmol) was added. The whole mixture was stirred at room temperature. The reaction was monitored by TLC on silica gel plates using $CHCl_3$: MeOH (5:1, v/v) solvent system. After the completion of the reaction, the solid was filtered off, washed with MeOH and filtrate was neutralized by adding ion exchange resin Amberlyst 15. The resin was filtered off and the organic layers were concentrated. For purification of compound **4** the residue was redissolved in a MeOH (10 mL) and concentrated with a small amount of silica-gel in order to prepare sample for purification by column chromatography.

(5-nitro-2-pyridyl) 1-thio-β-D-glucopyranoside (3)

Reaction time: 30 minutes. Product **3** (4.79 g, 95%) was obtained as a white solid after purification by a crystallization from anhydrous ethanol. $[\alpha]_n^{20}$ = 110 (MeOH, c = 0.8); m.p. 60-64°C; ^{1}H NMR (400 MHz, CD_3OD): δ 3.43-3.51 (m, 2H, H-2, H-4), 3.54 (ddd, 1H, *J* = 2.2 Hz, *J* = 5.8 Hz, *J* = 9.3 Hz, H-5), 3.55 (dd, 1H, *J* = 8.6 Hz, *J* = 9.4 Hz, H-3), 3.73 (dd, 1H, *J* = 5.8 Hz, *J* =

12.1 Hz, H-6a), 3.92 (dd, 1H, J = 2.2 Hz, J = 12.1 Hz, H-6b), 5.51 (d, 1H, J = 9.8 Hz, H-1), 7.63 (dd, 1H, J = 0.8 Hz, J = 9.0 Hz, H-3_{pyr}), 8.46 (dd, 1H, J = 2.7 Hz, J = 9.0 Hz, H-4_{pyr}), 9.26 (d, 1H, J = 2.7 Hz, H-6_{pyr}).^{13}C NMR (100 MHz, CD_3OD):δ 62.87. (C-6), 71.43, 73.77, 79.97, 82.49 (C-2, C-3, C-4, C-5), 85.41 (C-1), 123.31, 132.74, 143.36, 145.89, 167.90 (C_{pyr}).

(3-nitro-2-pyridyl) 1-thio-β-D-glucopyranoside (4)

Reaction time: 30 minutes. Product **4** (4.94 g, 98%) was obtained as a solidifying oil after purification by a column chromatography (chloroform: methanol; gradient: 100:1 to 10:1). $[\alpha]_n^{20}$ = 59 (MeOH, c = 0.8); ^{1}H NMR (400 MHz, CD_3OD): δ 3.36-3.45 (m, 2H, H-4, H-5), 3.45-3.52 (m, 2H, H-2, H-3), 3.63 (dd, 1H, J = 5.1 Hz, J = 12.1 Hz, H-6a), 3.86 (dd, 1H, J = 1.9 Hz, J = 12.1 Hz, H-6b), 5.81 (m, 1H, H-1), 7.37 (dd, 1H, J = 4.7 Hz, J = 8.2 Hz, H-5_{pyr}), 8.55 (dd, 1H, J = 1.6 Hz, J = 8.2 Hz, H-6_{pyr}), 8.76 (dd, 1H, J = 1.6 Hz, J = 4.7 Hz, H-4_{pyr}).^{13}C NMR (100 MHz, CD_3OD): δ 62.67 (C-6), 71.34, 73.21, 80.14, 82.17 (C-2, C-3, C-4, C-5), 83.77 (C-1), 121.20, 134.97, 144.01, 154.56, 156.30 (C_{pyr}).

In vitro analysis of biological activity

Cell culture and experiment conditions

The human breast cancer cell line MCF-7, the human colon cancer cell lines Caco-2 and SW480 were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. MCF-7 and SW480 cell lines were cultured in DMEM/F12 + heat-inactivated 10% foetal bovine serum + 1% glutamine and Caco-2 was cultured in 80% MEM (with Earle's salts) + 20% heat inactivated foetal bovine serum + 1% glutamine. Cells were incubated at 37°C in a 5% CO_2 and a 95% humidified atmosphere. When cells reached 80% confluence, they were digested with 0.25% trypsin for the following experiments. Cell culture reagents were obtained from Gibco, Invitrogen (Thermo Fisher Scientific Inc., Carlsbad, CA, USA). For all of the experiments, the cells were cultured 24 hours after seeding for adherence and the culture medium was replaced. The next day, the cells were exposed to insulin (Insulin solution human, Sigma Aldrich, Germany) for 8 hours in dose 40 μg/ml for MCF-7 and 100 μg/ml for Caco-2 and SW480 and then treated for a further 24 hours by synthesized compounds in an optimal concentration evaluated after the viability assay. Test solutions of the tested compounds (1 mg/ml) were prepared by dissolving the substances in 100 μl of the dimethyl sulfoxide (DMSO, BioShop Canada Inc., Ontario, Canada) complemented with 900 μl of the tissue culture medium. Afterwards, the tested compounds were diluted in the culture medium to reach the final concentrations. The synthesized compounds 1 and 2 were further used in studies of their biological activities as compounds A and B, respectively.

Cell viability and proliferation assay

The viability of MCF-7, Caco-2, SW480 cells in response to thioglycoside A and B was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. To assess the proper drug concentration, cells were seeded at a density of $7x10^3$/well in 96-well culture plates and treated as described in experimental conditions with different thioglycosides concentrations 10 μg/ml, 1 μg/ml, 0,1 μg/ml for 24 hours. MTT solution (Sigma Aldrich, Germany) was added to the wells on a 96-well plate to a final concentration of 0.5mg/ml and incubated at 37°C for 4h. Following incubation, the formazan crystals were solubilized with 100 μl DMSO (Sigma Aldrich, Germany) for 15 minutes. The optical absorbance (A) was measured at 490nm using a BioTek ELX800 multi-well reader (BioTek, Winooski, VT, USA). The absorbance in the untreated control group was regarded as 100% cell viability. The percentage of viable cells (VC) was calculated according to: VC (%) = (A of experimental group/A of control group)x100. All assays were repeated 3 times. For further experiments a concentration of 10 μg/ml and 1 μg/ml of compound A and B respectively, was used.

Immunocytochemistry

For the immunocytochemistry analysis, cells were seeded at 4 × 10^4 cells per well in 4-well imaging slides (Eppendorf, Hamburg, Germany). After treatment, cells were placed in 4% paraformaldehyde at 4°C for 10 min, washed with PBS and permeabilized in 0.1% Tween 20 in PBS for 10 min. Immunocytochemistry was performed using the LSAB+ method (LSAB+ System HRP from DAKO, Glostrup, Denmark). After permeabilization, the cells were washed with PBS and incubated with the endogenous peroxidase-blocking buffer and then were incubated with the protein-blocking buffer. Next, the primary antibody against GLUT-1 receptor (Atlas Antibodies, Stockholm Sweden, dilution 1:100) was used, and slides were stored overnight at 4°C. The following day, the slides were washed with PBS and incubated for 1 hour with a secondary anti-rabbit-HRP conjugated antibody from DAKO Kit. Then the slides were rinsed twice with PBS and stained with 3,3'-diaminobenzidine in chromogen solution. Finally, the cells were counterstained with Mayer's haematoxylin and then dehydrated in graded alcohols, cleared in xylene, and mounted with xylene-based mounting medium. The negative control was obtained by omitting the first antibody. Images of immunocytochemistry results were taken by a light microscope fitted with a digital camera (Nikon Eclipse 80i with camera DS-Fil-U2, Amsterdam, The Netherlands) at magnification of 100x and 200x.

Western blotting analysis

The cells were plated at a density of $8x10^4$/well in 6-well culture plates. The cells were washed twice with pre-cooled PBS and, subsequently, cell lysates were prepared using RIPA buffer containing protease and phosphatase inhibitors (1% cocktails, all from Sigma Aldrich, Germany). The lysates were incubated with low agitation for 30 min at 4°C and then cleaned by centrifugation at 16 000xg for 15 minutes. The supernatants were collected, and the protein concentration was measured at 280nm using the Qubit Protein Assay for the Qubit Fluorometer (Invitrogen, Thermo Fisher Scientific Inc., Carlsbad, CA, USA).

Total 50 ug protein extracts were separated on 4% to 12% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis, all Western blotting reagents and equipment from Invitrogen, Thermo Fisher Scientific Inc., Carlsbad, CA, USA) and transferred to the nitrocellulose membrane. The membrane was blocked with phosphate-buffered saline containing 0.1% Tween 20 (Sigma Aldrich, Germany) with 10% bovine serum albumin (Sigma Aldrich, Germany) for 1h at room temperature. Subsequently, the membrane was incubated overnight at 4°C with the first antibodies' solution. The primary antibodies used in this study included anti-β-actin for protein normalization (dilution 1:1000, Abcam, Cambridge, UK), anti-caspase 3 (dilution 1:500, Abcam, Cambridge, UK), anti-bax (dilution 1:1000, Abcam, Cambridge, UK), anti-GLUT1 (dilution 1:500, Atlas Antibodies, Stockholm, Sweden). After washing twice with PBS, the membrane was incubated with horseradish peroxidase-labelled secondary anti-rabbit antibody in dilution 1:1000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1h at room temperature and thereafter washed three times with PBS. The final detection was performed with enhanced colorimetric Western blotting visualization reagent using the 1-Step Ultra TMB Blotting Solution (Thermo Fisher Scientific Inc., Carlsbad, CA, USA). The results were documented using appropriate Bio-Rad equipment (Molecular Imager Gel Doc TMXR+, BioRad, Hercules, CA, USA).

Wound-healing assay

The cells were seeded in the 2 Well Culture Insert in u-Dish (Ibidi, Martinsried, Germany) according to manufacturer's instructions. After appropriate cell attachment (24 hours), Culture Inserts were gently removed and the cells were treated either with cell medium or were exposed to 10 μg/ml or 1 μg/ml of compound A and B, with or without 8 h of insulin sensitization (40 μg/ml for MCF-7, and 100 μg/ml for Caco-2, SW480). Images of cell migration in 500 μm gap were taken at time point 0 hours and over a 24-hours time period by phase-contrast microscope fitted with a digital camera (Nikon Eclipse 80i with camera DS-Fil-U2, Amsterdam, The Netherlands) at magnification of 40x.

Flow cytometry analysis

The ratio of apoptosis was measured using Muse® Annexin V and Dead Cell Assay Kit. The cells were detached from their culture vessel (each well of 24-well plate), using Gibco™ Trypsin-EDTA as a dissociation reagent. The amount of 100 μl of Gibco™ Trypsin-EDTA was added to each well. After 5 minutes of incubation in 37°C, the cells were gently scraped off and the amount of 300 μl of medium (appropriate for each cell line) was added. The dissociated cells were transferred to Eppendorf® tubes of 1.5 ml volume and gently mixed on vortex. Then the amount of 100 μL of each cell sample was added to 100 μl of Muse™ Annexin V & Dead Cell reagent and gently mixed. After 20 minutes of incubation at room temperature in darkness, samples were gently mixed and then loaded onto the Muse™ Cell Analyzer, where the test of degree of apoptosis was performed following the Muse™ Annexin V & Dead Cell Kit User's Guide. The obtained results include a percentage of live, early apoptotic, late apoptotic, total apoptotic, and dead cells.

Statistical analysis

The conformity of distribution of analyzed parameters with the normal distribution was checked. The conformity was assessed by the Shapiro–Wilk test. The homogeneity of variance was tested with Bartlett's test. The significance of differences in mean values (M) in more than two populations for parameters of normal distribution and homogeneous variances was assessed with analysis of variance (ANOVA). In case of rejection of the null hypothesis of homogeneity of variance, to verify the differences between the mean values in pairs, Scheffe's test was performed. The critical significance level was set at $p=0.05$. The data were expressed as mean ± standard deviation (M ± SD) and analyzed with the statistical program STATISTICA v.12 (StatSoft, Inc., Tulsa, OK, USA).

Author contributions

SA, MW, ML had full access to all of the data and are responsible for the integrity of the data and the accuracy of the analysis. SA, MW, PZ, WS, AG contributed to the study concept and design. AG, PZ supervised the study. GG and WS performed the synthesis of the compounds. SA, EP, MW, KW contributed to the analysis and interpretation of the data. SA conducted the statistical analyses. SA, EP, AG, WS drafted the manuscript, and all of the authors made critical revisions for important intellectual content and approved the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

1. Calvaresi EC, Hergenrother PJ. Glucose conjugation for the specific targeting and treatment of cancer. Chem Sci. 2013; 4: 2319-2333. https://doi.org/10.1039/C3SC22205E.
2. Brajeswar P, Walter K. S-, N-, and O-glycosyl derivatives of 2-acetamido-2-deoxyglucose with hydrophobic aglycons as potential chemotherapeutic agents and N-acetyl-β-glucosaminidase inhibitors. Carbohydr Res. 1984; 126: 27-43. https://doi.org/10.1016/0008-6215(84)85124-1.
3. Kuhn CS, Lehmann J, Steck J. Syntheses and properties of some photolabile β-thioglycosides. Potential photoaffinity reagents for β-glycoside hydrolases. Tetrahedron. 1990; 46: 3129-3134. https://doi.org/10.1016/S0040-4020(01)85452-1.
4. Blanc-Muesser M, Vigne L, Driguez H, Lehmann J, Steck J, Urbahns K. Spacer-modified disaccharide and pseudo-trisaccharide methyl glycosides that mimic maltotriose, as competitive inhibitors for pancreatic alpha-amylase: a demonstration of the "clustering effect". Carbohydr Res. 1992; 224: 59-71. https://doi.org/10.1016/0008-6215(92)84093-8.
5. Apparu C, Driguez H, Williamson G, Svensson B. Chemoenzymatic synthesis of 6ω-S-α-d-glucopyranosyl-6ω-thiomaltooligosaccharides: their binding to Aspergillus niger glucoamylase G1 and its starch-binding domain. Carbohydr Res. 1995; 277: 313-320.
6. Marino C, Mariño K, Miletti L, Manso Alves MJ, Colli W, de Lederkremer RM. 1-Thio-β-D-galactofuranosides: synthesis and evaluation as β-D-galactofuranosidase inhibitors. Glycobiology. 1998; 8: 901-904. https://doi.org/10.1093/glycob/8.9.901.
7. Rho D, Desrochers M, Jurasek L, Driguez H, Defaye J. Induction of cellulose in Schizophyllum commune: thiocellobiose as a new inducer. J Bacteriol. 1982; 149: 47-53.
8. Defaye J, Guillot JM, Biely P, Vršanská M. Positional isomers of thioxylobiose, their synthesis and inducing ability for D-xylan-degrading enzymes in the yeast Cryptococcus albidus. Carbohydr Res. 1992; 228: 47-64. https://doi.org/10.1016/S0008-6215(00)90548-2.
9. Birk R, Ikan A, Bravdo B, Braun S, Shoseyov O. Synthesis of lsopropyl-1-thio-B-D-glucopyranoside (IPTGlc), an inducer of aspergillus niger b1 B-glucosidase production. Appl Biochem Biotechnol. 1997; 66: 25-30.
10. Chen Q, Kong F. Stereoselective glycosylation using fully benzylated pyrimidin-2-yl 1-thio-β-d-glycopyranosides. Carbohydr Res. 1995; 272: 149-157. https://doi.org/10.1016/0008-6215(95)00031-N.
11. Hanessian S, Bacquet C, Lehong N. Chemistry of the glycosidic linkage. Exceptionally fast and efficient formation of glycosides by remote activation. Carbohydr Res. 1980; 80: C17-C22.
12. Ding X, Yang G, Kong F. Synthesis and glycosylation of pyrimidin-2-yl 1-thio-α-d-manno-and-α-l-rhamnopyranoside. Carbohydr Res. 1998; 310: 135-139.
13. Driguez H. Thiooligosaccharides as tools for structural biology. Chembiochem. 2001; 2: 311-318.
14. Krol E, Pastuch-Gawolek G, Nidzworski D, Rychlowski M, Szeja W, Grynkiewicz G, Szewczyk B. Synthesis and antiviral activity of a novel glycosyl sulfoxide against classical swine fever virus. Bioorg Med Chem. 2014; 22: 2662-70. https://doi.org/10.1016/j.bmc.2014.03.027.
15. Pastuch-Gawolek G, Chaubey B, Szewczyk B, Krol E. Novel thioglycosyl analogs of glycosyltransferase substrates as antiviral compounds against classical swine fever virus and hepatitis C virus. Eur J Med Chem. 2017; 137: 247-262. https://doi.org/10.1016/j.ejmech.2017.05.051.
16. Scala S, Akhmed N, Rao US, Paull K, Lan LB, Dickstein B, Lee JS, Elgemeie GH, Stein WD, Bates SE. 1997. P-glycoprotein substrates and antagonists cluster into two distinct groups. Mol Pharmacol. 1997; 51: 1024-1033. https://doi.org/10.1124/mol.51.6.1024.
17. Elgemeie GH, Mahdy EM, Elgawish MA, Ahmed MM, Shousha WG, Eldin ME. A new class of antimetabolites: pyridine thioglycosides as potential anticancer agents. Z Naturforsch C. 2010; 65: 577-587.
18. García-Álvarez I, Groult H, Casas J, Barreda-Manso MA, Yanguas-Casás N, Nieto-Sampedro M, Romero-Ramírez L, Fernández-Mayoralas A. 2011. Synthesis of antimitotic thioglycosides: *in vitro* and *in vivo* evaluation of their anticancer activity. J Med Chem. 2011; 54: 6949-55. https://doi.org/10.1021/jm200961q.
19. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer. 2008; 8: 915. https://doi.org/10.1038/nrc2536.
20. De Meyts P. Insulin and its receptor: structure, function and evolution. Bioessays. 2004; 26: 1351-1362. https://doi.org/10.1002/bies.20151.
21. Lasalvia-Prisco E, Cucchi S, Vazquez J, Lasalvia-Galante E, Golomar W, Gordon W. Insulin-induced enhancement of antitumoral response to methotrexate in breast cancer patients. Cancer Chemother Pharmacol. 2004; 53: 220-224. https://doi.org/10.1007/s00280-003-0716-7.
22. Yang Y, Wen F, Dang L, Fan Y, Liu D, Wu K, Zhao S. Insulin enhances apoptosis induced by cisplatin in human esophageal squamous cell carcinoma EC9706 cells related to inhibition of autophagy. Chin Med J (Engl). 2014; 127: 353-358.

23. Zou K, Xie H. Pretreatment with insulin enhances anticancer functions of 5-fluorou-racil in human esophageal and colonic cancer cells. Acta Pharmacol Sin. 2007; 28: 721-730. https://doi.org/10.1111/j.1745-7254.2007.00554.x.

24. Damyanov C, Gerasimova D, Maslev I, Gavrilov V. Low-dose chemotherapy with insulin (insulin potentiation therapy) in combination with hormone therapy for treatment of castration-resistant prostate cancer. ISRN Urol. 2012; 2012: 140182. https://doi.org/10.5402/2012/140182.

25. Agrawal S, Łuc M, Ziółkowski P, Agrawal AK, Pielka E, Walaszek K, Zduniak K, Woźniak M. Insulin-induced enhancement of MCF-7 breast cancer cell response to 5-fluorouracil and cyclophosphamide. Tumor Biol. 2017; 39: 1010428317702901. https://doi.org/10.1177/1010428317702901.

26. Warburg O. The metabolism of carcinoma cells. J Cancer Res. 1925; 9: 148-163. https://doi.org/10.1158/jcr.1925.148.

27. Warburg O. On the origin of cancer. Science. 1956; 123: 309-314. https://doi.org/10.1126/science.123.3191.309.

28. Vander Heiden MG. Targeting cancer metabolism: a therapeutic window opens. Nat Rev Drug Discov. 2011; 10: 671-684. https://doi.org/10.1038/nrd3504.

29. Jones NP, Schulze A. Targeting cancer metabolism - aiming at a tumour's sweet-spot. Drug Discov Today. 2012; 17: 232-241. https://doi.org/10.1016/j.drudis.2011.12.017.

30. Medina RA, Owen GI. Glucose transporters: expression, regulation and cancer. Biol Res. 2002; 35; 9-26. https://doi.org/10.4067/S0716-97602002000100004.

31. Abbas HA, El Sayed WA, Fathy NM. Synthesis and antitumor activity of new dihydropyridine thioglycosides and their corresponding dehydrogenated forms. Eur J Med Chem. 2010; 45: 973-982. https://doi.org/10.1016/j.ejmech.2009.11.039.

32. Zhong W, Boons GJ, Crich D, Bowers AA. 2008. Glycoside Synthesis from 1-Sulfur/Selenium-Substituted Derivatives: Thioglycosides in Oligosaccharide Synthesis. In Handbook of Chemical Glycosylation: Advances in Stereoselectivity and Therapeutic Relevance. Demchenko AV, editor. John Wiley and Sons, Weinheim. 261-303. https://doi.org/10.1002/9783527621644.ch4a.

33. Driguez H, Szeja W. Facile synthesis of 1, 2-trans-nitrophenyl-1-thioglycopyranosides. Synthesis. 1994; 1994: 1413-1414. https://doi.org/10.1055/s-1994-25704.

34. Whistler RL, Wolfrom ML, BeMiller JN. 1963. Methods in Carbohydrate Chemistry. In Reactions of Carbohydrates, Volume 2. Academic Press, New York and London. 434. https://doi.org/10.1021/ed041p352.2.

35. Pastuch G, Szeja W. A facile and efficient synthesis of S-glycosylated derivatives of 5-nitropyridine. Carbohydr Lett. 1997; 2: 281-286.

36. Niemiec-Cyganek A. Heteroaryl thioglycosides, a new class of substrates for glycosidases. Pol J Chem. 2003; 77: 969-973.

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Insulin-induced enhancement of MCF-7 breast cancer cell response to 5-fluorouracil and cyclophosphamide

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Abstract

The study was designed to evaluate the potential use of insulin for cancer-specific treatment. Insulin-induced sensitivity of MCF-7 breast cancer cells to chemotherapeutic agents 5-fluorouracil and cyclophosphamide was evaluated. To investigate and establish the possible mechanisms of this phenomenon, we assessed cell proliferation, induction of apoptosis, activation of apoptotic and autophagic pathways, expression of glucose transporters 1 and 3, formation of reactive oxygen species, and wound-healing assay. Additionally, we reviewed the literature regarding theuse of insulin in cancer-specific treatment. We found that insulin increases the cytotoxic effect of 5-fluorouracil and cyclophosphamide in vitro up to two-fold. The effect was linked to enhancement of apoptosis, activation of apoptotic and autophagic pathways, and overexpression of glucose transporters 1 and 3 as well as inhibition of cell proliferation and motility. We propose a model for insulin-induced sensitization process. Insulin acts as a sensitizer of cancer cells to cytotoxic therapy through various mechanisms opening a possibility for metronomic insulin-based treatments.

Keywords

5-Fluorouracil, breast cancer, chemotherapy, cyclophosphamide, insulin

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Background

It is well established that malignancies exhibiting rapid growth and high-growth fraction are more susceptible to chemotherapeutic regimens than slow-growing, low-growth fraction malignancies.[1,2] The potency of clinically known chemotherapeutic drugs depends upon disruption of metabolic pathways. The activity of one biochemical pathway determines the effectiveness of a particular cytotoxic agent. Therefore, it is desirable to alter the metabolic profile of a resistant tumor in such a way that it develops specific drug sensitivity.[3]

Currently, almost 60% of all patients with early breast cancer receive chemotherapy but only a minority will benefit from it.[4] While different cytotoxic therapies are employed in the management of breast carcinoma, response rates are low, and acquired resistance is common.[5] The chemotherapeutic agents 5-fluorouracil (5FU) and cyclophosphamide (CPA) have been widely used in the clinic and incorporated in the treatment of several malignancies, that is, colorectal, gastric, pancreatic, breast, or head and neck cancer. However, drug resistance of malignant cells and systemic toxicity in the course of treatment are alarming causes of failure of chemotherapy.[4–6] It is therefore desirable to explore novel therapeutic

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approaches, including adjuvant drugs that enhance cancer cell death resulting from standard therapies.

Insulin exhibits potent anabolic properties and has been implicated in many malignancies including breast cancer.[7] It is known to regulate carbohydrate and lipid metabolism, stimulate DNA synthesis, and modulate transcription.[8] Insulin has an impact on cellular uptake of various nutritional substances by facilitated diffusion. The uptake of materials such as glucose, amino acids, potassium, magnesium, and phosphate ions is vastly enhanced in its presence.[9–11] Hence, insulin is known to be a modifier of the metabolism of cancer cells.

The study was designed to examine the effect of insulin on the sensitivity of breast cancer cell line MCF-7 to chemotherapeutic agents 5FU and CPA. To investigate and establish the possible mechanisms of this phenomenon, we assessed cell proliferation, induction of apoptosis, activation of apoptotic and autophagic pathways, expression of glucose transporters (GLUTs) 1 and 3, and formation of reactive oxygen species (ROS). Additionally, we reviewed the literature regarding the use of insulin in cancer-specific treatment.

Methods

Cell culture and experiment conditions

The human breast cancer cell line MCF-7, obtained from Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures, was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with heat-inactivated 10% fetal bovine serum and 1% glutamine. Cells were incubated at 37°C in a 5% CO_2 and 95% humidified atmosphere. When cells reached 80% confluence, they were digested with 0.25% trypsin for the following experiments. All cell culture reagents were purchased from Gibco, Invitrogen (Thermo Fisher Scientific Inc., Carlsbad, CA, USA).

Unless otherwise specified, MCF-7 cells were seeded at a density of 7×10^3 per well in 96-well culture plates or 8×10^4 per well in 6-well culture plates, and after 24 h of incubation for adherence, the cells were exposed to insulin (Insulin solution human; Sigma Aldrich, Schnelldorf, Germany) 40 µg/mL for 8 h and then treated for a further 72 h by 5FU or CPA (Sigma Aldrich) of different concentrations 1000, 500, and 100 µM and 4000, 2000, and 400 µg/mL, respectively. The drugs were freshly diluted to the final concentration in culture medium before experiment.

Cell viability and proliferation assay

The viability of MCF-7 cells following treatment was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. MTT solution (Sigma Aldrich) was added to the wells on a 96-well plate to a final concentration of 0.5 mg/mL and incubated at 37°C for 4 h. Following incubation, the formazan crystals were solubilized with 100 µL dimethyl sulfoxide (DMSO; Sigma Aldrich) for 15 min. The optical absorbance (A) was measured at 490 nm using a BioTek ELX800 multi-well reader (BioTek, Winooski, VT, USA). The absorbance in the untreated control group was regarded as 100% cell viability. The percentage of viable cells (VC) was calculated according to the following formula: VC (%) = (A of experimental group/A of control group) × 100. All assays were carried out three times.

Table 1. The immunoreactivity score (IRS) calculation method.

Intensity of staining	Number of stained cells
0 = negative	0 = ≤10%
1 = weak	1 = 11%–30%
2 = moderate	2 = 31%–60%
3 = strong	3 = ≥61%

The final score was calculated by multiplying the staining intensity value by the percentage of staining.

Immunocytochemistry

For the immunocytochemistry analysis, MCF-7 cells were seeded at 4×10^4 cells per well in three-well chamber slides (Thermo Fisher Scientific Inc.). After treatment, cells were placed in 4% paraformaldehyde at 4°C for 10 min and then washed with phosphate-buffered saline (PBS; Sigma Aldrich). Immunocytochemistry was performed using the labeled streptavidin biotin (LSAB)+ method (LSAB+ System HRP from Dako, Glostrup, Denmark). Glass slides with cells were incubated with the endogenous peroxidase-blocking buffer and then were incubated with the protein-blocking buffer. Next, primary antibodies against examined proteins (anti-GLUT-1 and anti-GLUT-3; Santa Cruz Biotechnology, Inc., Heidelberg, Germany, dilution 1:25) were used, and slides were stored overnight at 4°C. The following day, the slides were washed with PBS and incubated for 1 h with a secondary anti-mouse-HRP antibody (dilution 1:100; Santa Cruz Biotechnology, Inc.). Then, the slides were rinsed twice with PBS and stained with 3,3′-diaminobenzidine in chromogen solution. Finally, cells were counterstained with Mayer's hematoxylin and then dehydrated in graded alcohols, cleared in xylene, and mounted with xylene-based mounting medium. The negative control was obtained by omitting the first antibody. Photographs were taken by light microscope fitted with a digital camera (Nikon Eclipse 80i with camera DS-Fil-U2, Amsterdam, The Netherlands) at magnifications of 100× and 200×.

The immunostaining for GLUT-1 and GLUT-3 was reported according to the following standards (Table 1). For each glass slide, the immunoreactivity score (IRS) was calculated by multiplying the staining intensity value by percentage of stained cells. The final score of immunoreactivity

ranged from 0 to 9. Three independent pathologists evaluated the glass slides in a blind manner using the light microscope at 100× magnification. Their validation was in agreement in the case of 65% of the slides. Any disagreements were discussed, and then, slides were classified to the most adequate category of IRS.

Terminal deoxynucleotide transferased dUTP nick end labeling apoptosis assay

The ApopTag Peroxidase in Situ Apoptosis Detection Kit (Merck Millipore, Darmstadt, Germany) detects apoptotic cells in situ by labeling fragmented DNA by the Terminal Deoxynucleotide Transferase enzyme in terminal deoxynucleotide transferased dUTP nick end labeling (TUNEL) method. MCF-7 cells were seeded at 4×10^4 cells per well in three-well chamber slides (Thermo Fisher Scientific Inc.). After treatment, the cells were assayed according to the manufacturer's instructions. Briefly, cells were fixed in 1% formaldehyde in PBS 7.4 pH. After washed twice with PBS, cells were incubated with TdT enzyme at 37°C for 1 h. The slides were washed three times in PBS and incubated with anti-digoxigenin peroxidase conjugate in a humidified chamber for 30 min at room temperature (RT), followed by three rinses with PBS at RT. Then, slides were incubated with peroxidase substrate and after that counterstained with hematoxylin, dehydrated, and mounted in medium. To detect peroxidase-stained apoptotic bodies, bright-field microscopy was used. The number of TUNEL-positive cells was determined. The rate of TUNEL-positive cells (peroxidase-positive) was determined by dividing the number of TUNEL-positive cells by the total number of cells in the slides.

Flow cytometry analysis

Staining was performed using Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Kit Plus (BioVision, Milpitas, CA, USA). Up to 5×10^5 cells were resuspended in 500 µL of binding buffer and stained using 5 µL of FITC-conjugated Annexin V and 1 µL of SYTOX Green Dye. After 10 min of incubation in RT, cells were analyzed in cytometer.

Analysis was performed using BD FACS Calibur flow cytometer (FC) and BD CellQuest Pro Software (BD Biosciences, San Jose, CA, USA). With one exception, at least 12,000 total events were recorded from each tube. After gating cells on Forward Scatter (FSC)/Side Scatter (SSC) dot plot to remove debris from analysis, fluorescence detected in FL1 channel was analyzed on histograms. Populations showing weak to high (lower than dead cells peak) fluorescence were considered to represent apoptotic cells.

Western blot analysis

MCF-7 cells were plated at a density of 8×10^4 per well in six-well culture plates. After treatment, cell supernatants were collected, and they were washed with PBS and centrifuged at 125*g* for 10 min. Cell pellets were lysed. Cells on plates were washed twice with pre-cooled PBS and, subsequently, were treated with lysis buffer (4% sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol (DTT), in 0.1 M Tris–HCl buffer pH 7.6, 300 µL/well) containing protease and phosphatase inhibitors (1% cocktails; Sigma Aldrich). The both lysates were cleaned by centrifugation at 16,000*g* for 15 min. The supernatant was collected, and the protein concentration was measured at 280 nm using a spectrophotometer, PicoDrop 2000 (Thermo Fischer Scientific, Inc., Waltham, MA, USA). Total protein extracts were separated on 4%–12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE; equipment from Invitrogen, Carlsbad, CA, USA), and transferred to the nitrocellulose (Amersham Hybond; Healthcare Bio-sciences AB, Uppsala, Sweden). The membrane was blocked with PBS containing 0.1% Tween 20 (Sigma Aldrich) with 10% goat serum (Sigma Aldrich) for 1 h at RT. Subsequently, the membrane was incubated overnight at 4°C with the first antibodies' solution. The primary antibodies used in this study included anti-β-actin (dilution 1:500; Abcam, Cambridge, UK), anti-caspase 3 and anti-caspase 8 (dilution 1:500; Merck Millipore), anti-bax (dilution 1:100; Santa Cruz Biotechnology, Inc.), anti-atg 7 (dilution 1:100; Atlas Antibodies, Stockholm, Sweden), and anti-GLUT-1 and anti-GLUT-3 (dilution 1:25; Santa Cruz Biotechnology, Inc.). After washing twice with PBS, the membrane was incubated with horseradish peroxidase–labelled secondary anti-rabbit antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h at RT and thereafter washed three times with PBS. The final detection was performed with enhanced colorimetric western blotting visualization reagents using the DAB Enhanced Liquid Substrate System for Immunochemistry (Sigma Aldrich). The results were documented using appropriate Bio-Rad equipment (Molecular Imager Gel Doc TMXR+; Bio-Rad, Hercules, CA, USA). Loading differences were normalized by the usage of a monoclonal β-actin antibody against the housekeeping control β-actin.

ROS determination

ROS generation was measured using manufacturer's instruction of 2′,7′-dichlorofluorescein diacetate (DCFDA) Cellular ROS Detection Assay Kit (Abcam). MCF-7 cells were seeded into 96-well plates and treated as described in *Cell experiment conditions*. After treatment, the cells were washed and stained with 25 µM DCFDA in a buffer for 45 min at 37°C. After washing once with a buffer, ROS generation was analyzed by fluorescent plate reader Experion using excitation/emission wavelengths of 485/535 nm, respectively (Bio-Rad).

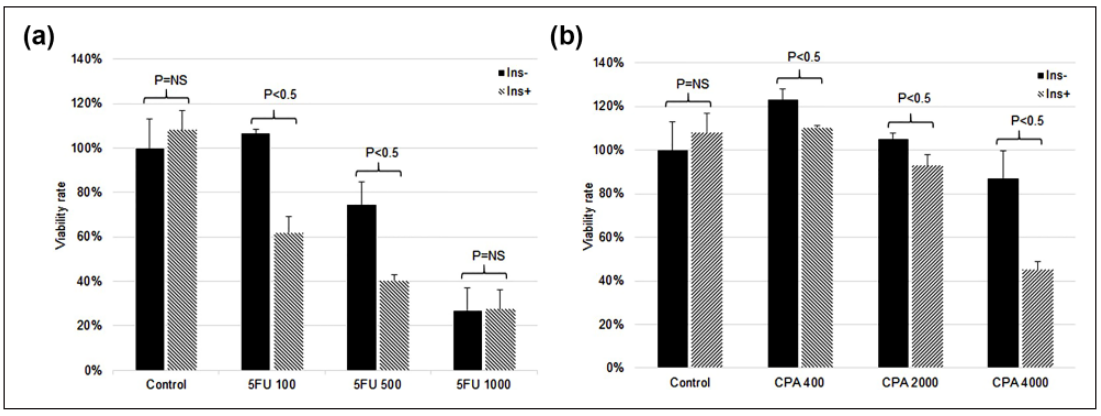

Figure 1. The inhibitory effect of insulin/5FU and insulin/CPA on the growth of MCF-7 cells. After insulin exposure (40 µg/mL) for 8 h, MCF-7 cells were treated with (a) 100, 500, and 1000 µM 5FU and (b) 400, 2000, and 4000 µg/mL CPA for 72 h. The inhibition rate was measured by MTT assay. The results are expressed as mean ± SD from triplicate experiments.

Wound-healing assay

MCF-7 cells were grown in six-well plates until about 80%–90% confluency was reached at which point a 10 µL pipette tip was used to create a scratch/wound with clear edges across the width of a well. Wells were treated either with cell medium or were exposed to 200 µM 5FU or 4000 µg CPA, with or without 8 h of insulin sensitization (40 µg/mL). Photomicrographs were taken over a 48-h time period. A Nikon TS100 inverted microscope was used to measure and photograph the cell migration from the wound/scratch edge.

Statistical analysis

For all quantitative parameters, the conformity of their distribution with the normal distribution was checked. The conformity assessment was carried out by the Shapiro–Wilk test. The homogeneity of variance was tested with Bartlett's test. The significance of differences in mean values (M) in more than two populations for parameters of normal distribution and homogeneous variances was assessed with analysis of variance (ANOVA). In case of rejection of the null hypothesis of homogeneity of variance, to verify the differences between the mean values in pairs, post hoc tests were performed (Scheffe's test). The level $p = 0.05$ was assumed as the critical significance level. Data were expressed as mean ± standard deviation (M ± SD) and analyzed with the statistical program STATISTICA v.12 (StatSoft, Inc., Tulsa, OK, USA).

Results and discussion

Insulin potentiates the cytotoxic effect of 5FU and CPA

To identify the optimal concentration of insulin (INS) and the incubation hours for sensitizing effect, various doses of insulin and time of exposure were tested in the preliminary study [Supplementary Figure]. Our findings were consistent with the previous literature.[12,13]

In our study, 40 µg/mL of insulin alone for 8 h of incubation had no significant effect on cell growth (Figure 1(a) and (b)). A combination of 500 µM 5FU with previous insulin sensitization led to significant decrease of viability of MCF-7 compared with 500 µM 5FU alone. Evidently, 100 µM 5FU had an inhibitory effect only in the presence of insulin. However, 5FU alone at high concentration resulted in an effect similar to that in the presence of additional insulin. Combination of 4000 µg/mL CPA with INS produced a significant inhibition in viability when compared with CPA alone. Similarly, 2000 and 400 µg/mL CPA had inhibitory effect on MCF-7 cells only in the prior presence of INS.

Insulin enhances apoptosis

We determined the apoptosis by FC analysis and TUNEL assay. In most tubes (with exception of 5FU-treated cells), dead cells could be differentiated from viable and apoptotic cells on the basis of FSC and SSC properties (Figure 2). Dead cells showed significantly lower FSC and slightly lower SSC values. In all samples treated with INS, including INS-treated control, a significantly higher apoptotic cell percentages and higher apoptosis/necrosis ratios were detected in comparison to samples treated without INS (Table 2). A high difference in apoptosis percentage was observed in CPA ± INS-treated samples, while the difference in samples treated with 5FU ± INS was comparable to control samples. Interestingly, in CPA-only treated sample, cell suspension was strikingly hypocellular and showed high percentage of necrosis, while in CPA + INS-treated sample, cell suspension cellularity was comparable to other samples and showed significantly higher apoptosis/necrosis ratio.

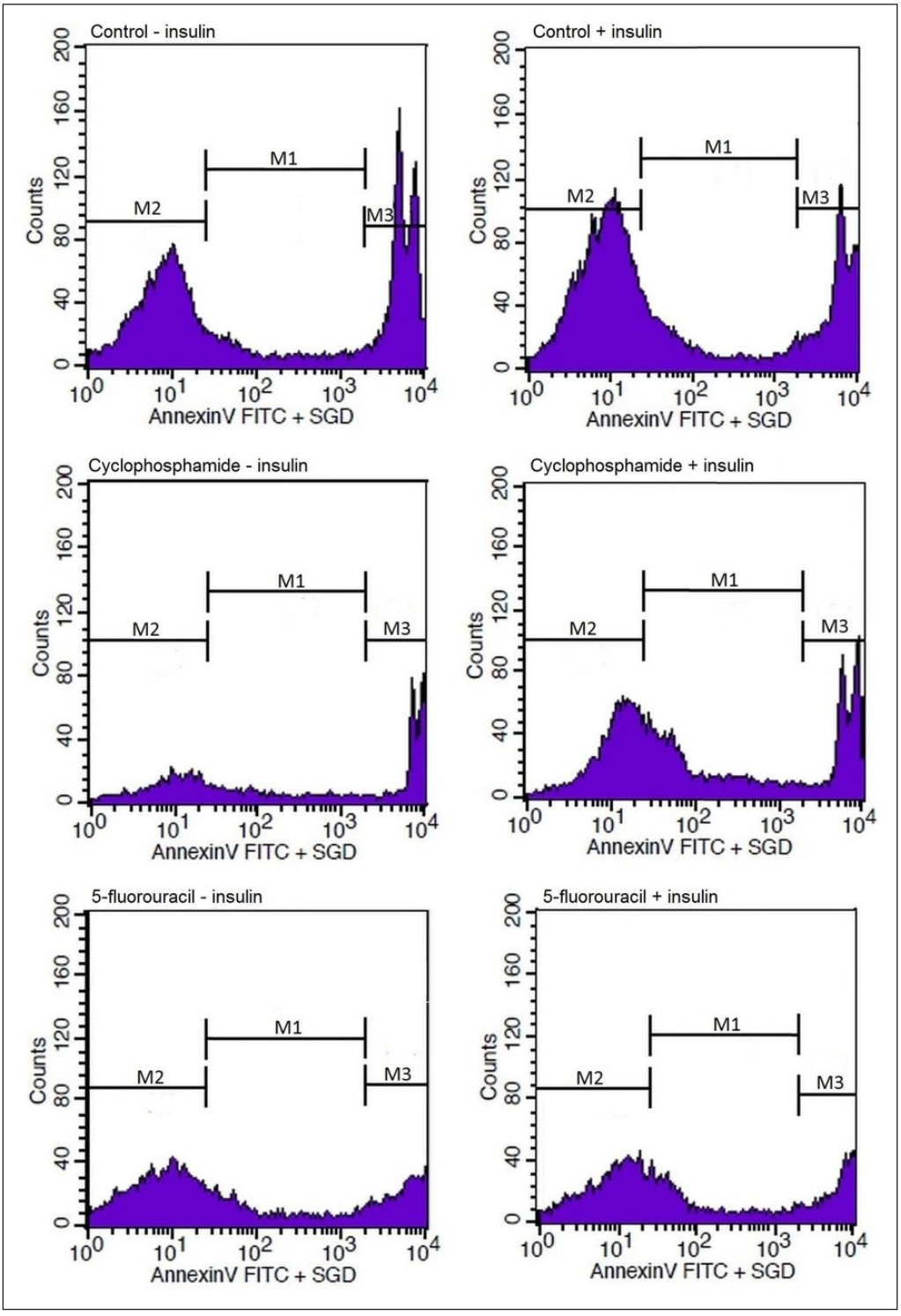

Figure 2. Original histogram plots presenting living, apoptotic, and dead cells, differentiated on the basis of their fluorescence after staining with Annexin V-FITC and SYTOX Green Dye. M1 marked events represent apoptotic cells (M2—living cells and M3—necrotic cells).

The number of TUNEL-positive cells was counted and presented as a percentage of apoptotic cells in relation to cells in the slide (Figure 3(a) and (b)). When tumor cells were treated with INS for 8 h followed by 5FU treatment, the apoptotic population increased significantly compared with 5FU treatment alone. Similarly, the ratio of apoptotic cells in insulin-pretreated cells in CPA group was significantly higher than in CPA alone.

Table 2. Apoptosis (%) and necrosis (%) of MCF-7 human breast cancer cells.

	Apoptotic M1	Viable M2	Necrotic M3
Control	11.82	48.80	39.50
Control + INS	17.26	59.45	23.44
CPA	20.08	36.11	43.99
CPA + INS	33.61	40.95	25.64
5FU	21.26	55.86	23.10
5FU + INS	27.04	51.14	22.13

INS: insulin; CPA: cyclophosphamide; 5FU: 5-fluorouracil.

Insulin activates apoptotic and autophagic pathways

To examine possible molecular pathways affected by the treatment, we have assessed the presence of both apoptotic and autophagic proteins (Figure 3(d)). Cells treated with 5FU as well as CPA presented higher expression of proapoptotic Bax protein compared with the control group or cells treated with insulin only. More interestingly, combination of 5FU with insulin resulted in higher protein expression of caspase 8 in comparison with

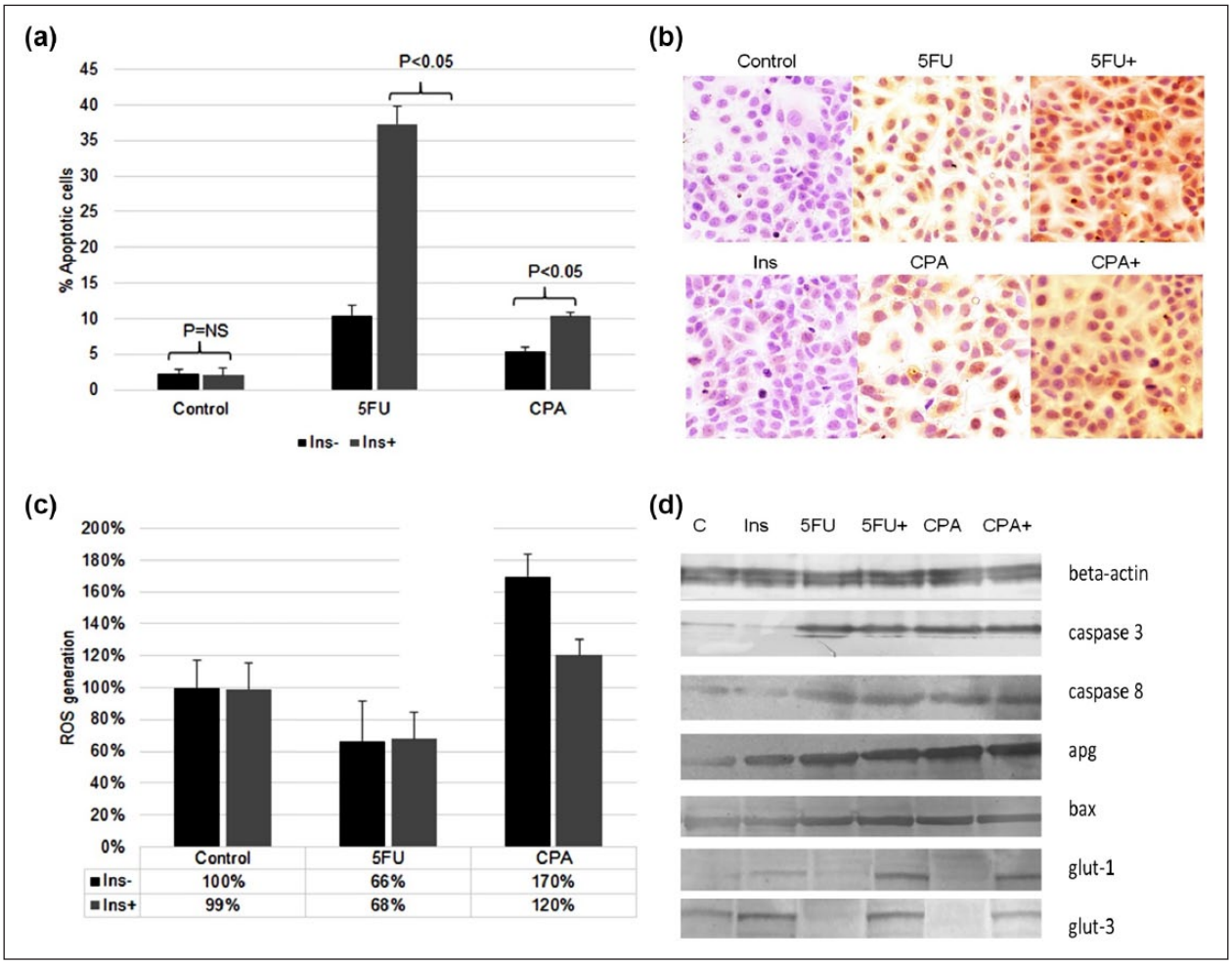

Figure 3. Results of (a and b) TUNEL apoptosis detection assay, (c) ROS detection assay, and (d) western blot analysis in MCF-7 cell line. Cells were incubated with 40 µg/mL insulin for 8 h and then treated with 200 µM 5FU and 2000 µg CPA for 72 h. (a) and (b) Apoptosis was recognized due to an increased number of apoptotic bodies. The rate of TUNEL-positive cells (peroxidase-positive) was determined by dividing the number of TUNEL-positive cells by the total number of cells in the slides. TUNEL method is based on the ability of terminal deoxynucleotidyltransferase (TdT) to label blunt ends of double-stranded DNA breaks independent of a template. A brown color of peroxidase indicates TUNEL-positive apoptotic cell death through condensation of chromatin and cell blebbing. Peroxidase in situ TUNEL method, hematoxylin-counterstained (magnification 100×). (c) ROS generation assay. The results are expressed as mean ± SD from triplicate experiments. (d) Western blot analysis of apoptosis, autophagy-related proteins, and GLUT-1 and GLUT-3 proteins in MCF-7 cell line.
C: control; Ins: insulin; 5FU: 5-fluorouracil; 5FU+: insulin with 5-fluorouracil; CPA: cyclophosphamide; CPA+: insulin with cyclophosphamide.

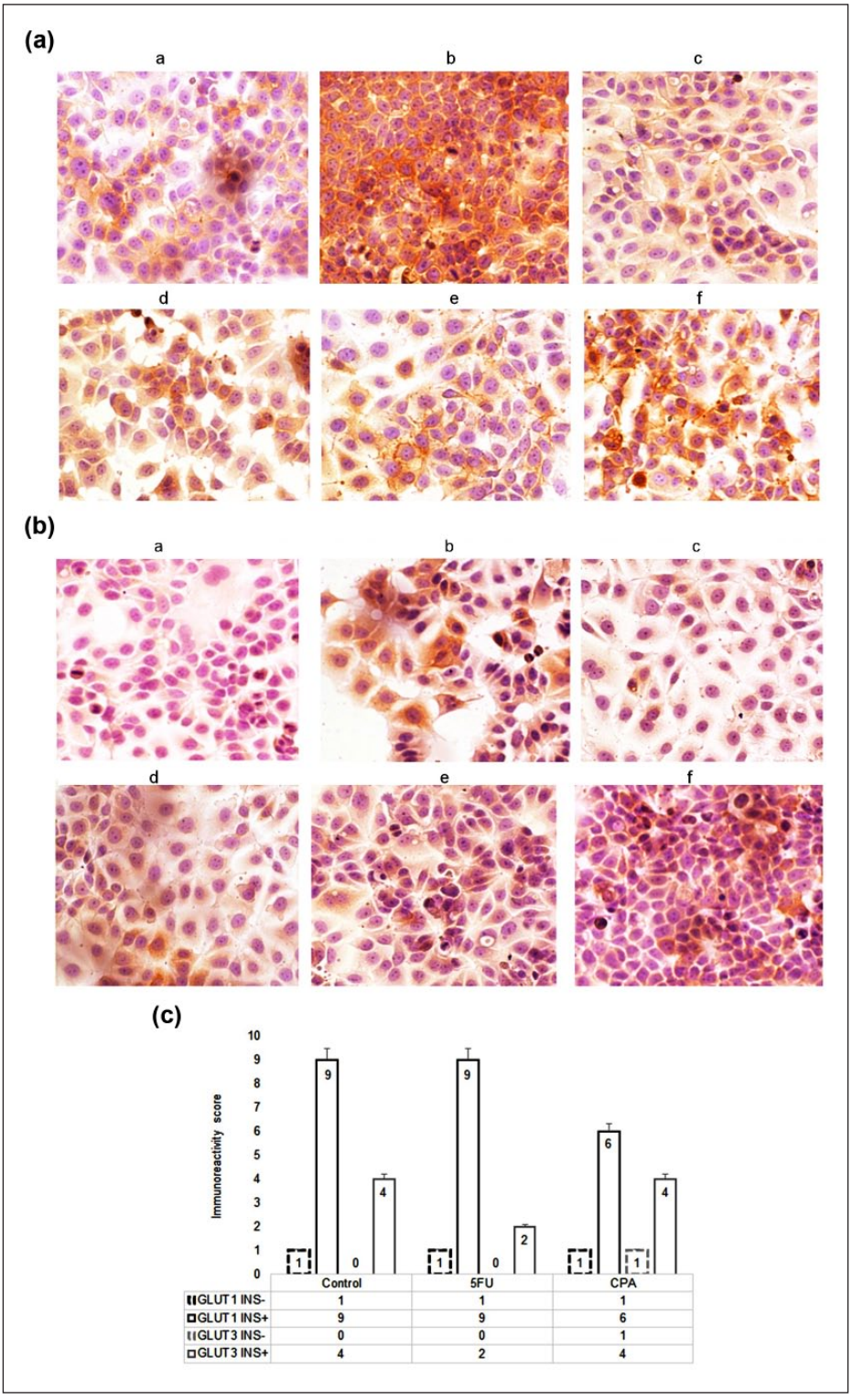

Figure 4. Immunostaining results of (A) GLUT-1 and (B) GLUT-3 protein expression in MCF-7 cell line. DAB method, hematoxylin-counterstained; magnification 100×. Cells were exposed to 200 µM fluorouracil or 2000 µg cyclophosphamide for 72 h, with or without 8 h of insulin sensitization (40 µg/mL). (a) Control, (b) insulin, (c) 5FU, (d) INS/5FU, (e) CPA, and (f) INS/CPA. (C) Immunoreactivity score (IRS) of the anti-GLUT-1 and anti-GLUT-3 immunostaining. The IRS calculation method is presented in Table 1.

5FU-only group. Comparable effect was observed for the cells treated with both CPA and insulin, which presented higher expression of caspase 3, placed downstream of the apoptotic pathway. Autophagy-related protein 7, Atg-7, was detected in higher levels in cells treated with drug/insulin combination, a result that can be connected to the increase in cells undergoing apoptosis.

Insulin-induced expression of GLUT-1 and GLUT-3

The impact of insulin on GLUT-1 and GLUT-3 protein expression was analyzed by immunocytochemistry. The tumor cells were exposed to insulin at 40 μg/mL for 8 h and then treated with 5FU at 200 μM or CPA at 2000 μg/mL for 48 h. The results showed that treatment with insulin caused an elevated expression of GLUT-1 and GLUT-3 proteins when compared with the control. Comparing to the insulin alone, the combinations of 5FU with insulin, or CPA with insulin, produced similar effects (Figure 4). The findings were confirmed in western blot analysis.

Insulin pretreatment inhibits cell proliferation and cell motility

To further examine how insulin pretreatment affected cell proliferation along with cell motility, "wound-healing" assays were performed. The results indicated that control- and INS-treated MCF-7 cells nearly completely filled the "wound" by 48 h (Figure 5). In stark contrast, combination of 4000 μg CPA as well as 200 μM 5FU with INS slightly hindered the motility/proliferation of these cells comparing to CPA or 5FU alone. These results are a further indication that combination of CPA and 5FU with INS impairs ability of breast cancer cell proliferation and motility.

ROS formation

To establish the effect of combined insulin/5FU or insulin/CPA on the intracellular redox status, we determined the intracellular ROS formation. We observed that 5FU-treated cells exhibited decreased ROS formation. The addition of insulin to 5FU did not influence ROS generation. Conversely, CPA-treated cells significantly enhanced ROS generation. Insulin-/CPA-treated cells exhibited decreased levels of ROS compared with CPA-treated alone group (Figure 3(c)).

Novel application of insulin

The use of insulin as an adjunct in the management of malignant neoplasia was proposed by Ayre et al.[14] over 30 years ago. It was suggested that insulin could increase membrane permeability of cancer cells, which leads to an increased uptake of cytotoxic agents. It was hypothesized that insulin-induced hypoglycemia causes stress to cancer tissue and allows selective endocytosis of metabolic agents even in low doses.

Although the role of insulin in cancer therapy is not fully understood, there are studies reporting its novel application. In a clinical trial conducted by Lasalvia-Prisco et al.,[12] it was reported that combination of methotrexate and insulin produced a significantly better clinical outcome in patients with multidrug-resistant metastatic breast cancer, compared to patients treated with methotrexate or insulin alone. In vitro studies[12–15] found that activity of various chemotherapeutic agents, such as paclitaxel, 5FU, cisplatin, or methotrexate, can be considerably enhanced in the presence of insulin. It was also reported in a small-scale clinical trial that insulin improves the outcome of hormone therapy in castration-resistant prostate cancer.[16]

Impact on cell proliferation, apoptosis, and autophagy

In this study, we were focused on the effects of insulin on the activity of 5FU and CPA and the underlying mechanisms. Our findings indicate that insulin can significantly raise the susceptibility of MCF-7 human breast cancer cells to 5FU and CPA. It was observed that insulin increases the cytotoxic effect of 5FU and CPA in vitro up to two-fold. These findings are consistent with the results from previous studies.[12–15] It is well established that resistance to apoptosis leads to uncontrolled proliferation, resulting in tumor survival, therapeutic resistance, and recurrence of cancer.[17] Induction of apoptosis plays an important role in enhancing the activity and function of cytotoxic agents. In our study, combination of insulin/5FU and insulin/CPA produced increase in apoptosis rate compared with 5FU or CPA alone. Evidently, when the exposure of cancer cells to insulin is optimal, the ratio of cells sensitive to 5FU and CPA significantly raises. Results of our research indicate that not only does insulin promote apoptosis when followed by anticancer drugs but also the mechanism of this phenomenon may be linked to autophagy, which has been proved to correlate with cell death or survival. Previous research showed that depending on the context, autophagy can serve as a destructive or protective factor in tumor cells.[18] The increase in autophagy observed in our work may promote apoptosis due to degrading various crucial cellular factors.[19] Another suggested mechanism of enhancing cell death via autophagy relies on its energetic effect, which provides adenosine triphosphate (ATP) needed to undergo apoptosis.[20] Experiments using metformin on breast cancer prove that insulin causes an increase in caspase-3 expression, which is a result of cells entering the apoptotic pathway.[21]

Role of GLUT-1 and GLUT-3

Cancer cells depend on glucose metabolism for energy production. Glucose uptake across the plasma membrane is considered the rate-limiting step for glucose metabolism. The facilitative GLUT family mediates a bidirectional and energy-independent process of glucose transport in most tissues and cells. Elevated expression of GLUTs has been reported in the majority of malignancies.[2,22] GLUT-1 is an isoform that exhibits a high affinity for glucose and can also transport galactose, mannose, glucosamine, and docosahexaenoic acid (DHA).[23] It is responsible for basal glucose uptake and is expressed in all tissues under normal conditions. Overexpression of GLUT-1 has been reported in a number of malignancies including lung, brain, breast, bladder, cervical, colorectal, esophageal, hepatocellular, head and neck, gastric, ovarian, renal cell, pancreatic, thyroid,

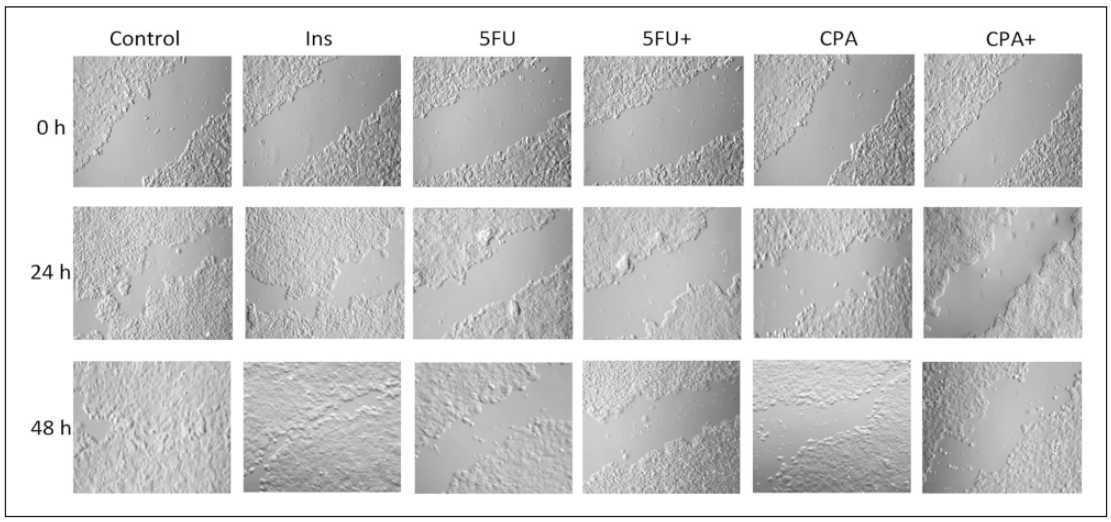

Figure 5. In vitro wound-healing/scratch assay. Photomicrographs were obtained at the indicated time points using a 10× objective on a Nikon eclipse TS100 inverted microscope and recorded using NIS-Elements F 3.2 software.
Control: control MCF-7 cancer cells; Ins: MCF-7 cancer cells treated with 40 µg/mL INS; 5FU: MCF-7 cancer cells treated with 200 µM 5FU; 5FU+: MCF-7 cancer cells treated with INS 40 µg/mL and 200 µM 5FU; CPA: MCF-7 cancer cells treated with 4000 µg CPA; CPA+: MCF-7 cancer cells treated with INS 40 µg/mL and 4000 µg CPA.

penile, and uterine cancers.[24–26] While it is well established that most cancer types overexpress GLUT-1, previous research has documented mixed evidence for the expression of GLUT-1 in breast cancer varying from 40% to 90%.[27–29] Similarly, GLUT-3 is a high-affinity GLUT that can also transport galactose, mannose, maltose, xylose, and DHA.[23] It is detected mainly in the central nervous system; hence, it is considered to be a neuron-specific GLUT. The expression of GLUT-3 in breast cancer is not well understood, with some studies showing higher GLUT-3 expression in poorly versus well-differentiated breast tumors.[30] In our study, we examined the impact of insulin on expression of GLUT-1 and GLUT-3. We found that insulin enhances significantly the expression of these transporters. These findings can be explained through the phosphatidylinositol 3-kinase (PI3K)–Akt pathway. Akt, a serine/threonine kinase downstream of PI3K, is shown to induce the expression of GLUT-1 and GLUT-3 in cancer cells, leading to stimulation of glucose transport and higher metabolic activity.[31] The activity of PI3K pathway is known to induce the metabolic processes regulating growth.[32] We hypothesize that activation of this pathway followed by consecutive cytotoxic treatment can be responsible for higher susceptibility of cancer cells to chemotherapeutic agents.

Formation of ROS

In malignant cells, elevated levels of ROS depend on a variety of processes including the following: increased metabolic activity, mitochondrial dysfunction, peroxisome activity, increased cellular receptor signaling, and oncogene activity.[33,34] The link between ROS and insulin signaling is known but not fully understood.[35,36] It is known that ROS enhances sensitivity to insulin.[37] In a tumor microenvironment, the cells respond to insulin stimulation in a more evident manner. In our study, we hypothesized that insulin may enhance the formation of ROS by cytotoxic agents. We found that combined INS/5FU produced similar effects to 5FU alone, whereas INS/CPA resulted in a decrease in ROS generation comparing with CPA alone, which can partially be explained by its increased cytotoxicity. It is therefore suggested that insulin does not play a pivotal role in the generation of ROS when combined with 5FU or CPA.

Insulin receptors and drug uptake

Membrane receptors for insulin (type 1 insulin-like growth factor receptor (IGF-1R), insulin receptor (IR)-A, IR-B, and their hybrids) play an important role in the regulation of metabolism in both malignant and non-malignant cells. The downstream effects of activation of insulin membrane receptors involve glucose uptake, glycogen synthesis, protein synthesis, cell survival, and transcription. It is well established that IR, IGF-1R, and their hybrids exhibit overexpression in many malignancies including breast cancer.[3,38] Therefore, it is assumed that cancer cells exhibit a more potent response to insulin stimulation. The cellular permeability to chemotherapeutic agents can be enhanced via a process known as *insulin-mediated endocytosis*.[39,40] This specific process enables cells to take up small and large molecular ligands, such as hormones, growth factors, enzymes, and plasma proteins.

It is suggested that through combined therapy of insulin and cytotoxic agents, the latter can accumulate intracellularly in a more significant manner. The findings of Zou et al.[16] confirm this assumption, as the uptake of 5FU was enhanced in the presence of insulin.

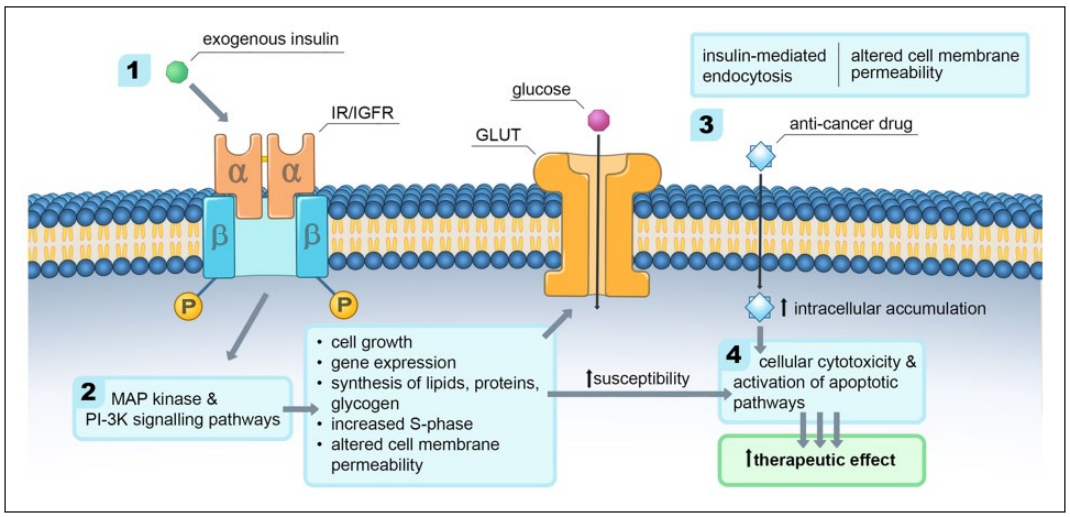

Figure 6. Insulin-induced sensitization of cancer cell to chemotherapeutic agent. Exogenous insulin binds to insulin receptor (IR) or insulin growth factor receptor (IGFR), leading to the activation of the mitogen-activated protein (MAP) kinase and the phosphatidylinositol-3-kinase (PI3K) signaling pathways. This results in the stimulation of various intracellular processes including cell growth; gene expression; synthesis of lipids, proteins, and glycogen; increase in S-phase cells; and altered cell membrane permeability. Consecutive administration of cytotoxic agents can lead to higher intracellular accumulation due to processes such as insulin-mediated endocytosis and insulin-induced alteration of membrane permeability. Activation of various metabolic and mitogenic pathways raises the susceptibility of cancer cell, which in the presence of high intracellular drug concentration results in increased cytotoxicity, apoptosis, and the enhancement of therapeutic effect.

Insulin-induced sensitization of malignancies to chemotherapy

We propose a model for insulin-induced sensitization process (Figure 6). Exogenous insulin binds to an IR or an insulin growth factor receptor (IGFR), leading to activation of the mitogen-activated protein kinase (MAPK) and the PI3K signaling pathways. This results in stimulation of various intracellular processes including cell growth, gene expression; synthesis of lipids, proteins, and glycogen; and increase in S-phase cells. Consecutive administration of cytotoxic agents causes a higher intracellular accumulation due to mechanisms such as insulin-mediated endocytosis and insulin-induced alteration of membrane permeability. Activation of various metabolic and mitogenic pathways raises the susceptibility of a tumor, which in the presence of high intracellular drug concentration results in increased cytotoxicity and apoptosis.

Conclusion

Our findings support the use of insulin as a cell sensitizing agent. We conclude that insulin can enhance the effects of cytotoxic therapy through suggested mechanisms including MAPK and PI3K pathways. The cellular processes involved in this phenomenon need further study. The concept of treating tumors by prior sensitization opens a possibility for metronomic insulin-based treatments.

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Authors' contributions

S.A., M.W., and M.L. participated in study design and coordination, carried out the molecular studies and immunoassay, and prepared draft of the manuscript; E.P., K.W., and K.Z. carried out flow cytometry and western blot analysis; and P.Z. participated in the design of the study and coordination. All authors read and approved the final manuscript

Consent for publication

Not applicable

Ethics approval and consent to participate

Not applicable

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References

1. Shackney SE, McCormack GW and Cocheral GJ. Growth rate patterns of solid tumors and their relation to responsiveness to therapy. *Ann Intern Med* 1970; 89: 107–121.
2. Talmadge JE and Fidler IJ. AACR centennial series: the biology of cancer metastasis: historical perspective. *Cancer Res* 2010; 70(14): 5649–5669.

3. Gatenby RA, Silva AS, Gillies RJ, et al. Adaptive therapy. *Cancer Res* 2009; 69(11): 4894–4903.
4. Colombo PE, Milanezi F, Weigelt B, et al. Microarrays in the 2010s: the contribution of microarray-based gene expression profiling to breast cancer classification, prognostication and prediction. *Breast Cancer Res* 2011; 13(3): 212.
5. Florea A-M and Busselberg D. Breast cancer and possible mechanisms of therapy resistance. *J Local Glob Health Sci* 2013; 2: 4.
6. Higgins CF. Multiple molecular mechanisms for multidrug resistance transporters. *Nature* 2007; 446: 749–757.
7. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer* 2008; 8(12): 915–928.
8. De Meyts P. Insulin and its receptor: structure, function and evolution. *Bioessays* 2004; 26: 1351–1362.
9. Acevedo CG, Marquez JL, Rojas S, et al. Insulin and nitric oxide stimulates glucose transport in human placenta. *Life Sci* 2005; 76: 2643–2653.
10. Gu S, Villegas CJ and Jiang JX. Differential regulation of amino acid transporter SNAT3 by insulin in hepatocytes. *J Biol Chem* 2005; 280: 26055–26062.
11. Rosic NK, Standaert ML and Pollet RJ. The mechanism of insulin stimulation of (Na+,K+)-ATPase transport activity in muscle. *J Biol Chem* 1985; 260: 6206–6212.
12. Lasalvia-Prisco E, Cucchi S, Vazquez J, et al. Insulin-induced enhancement of antitumoral response to methotrexate in breast cancer patients. *Cancer Chemother Pharmacol* 2004; 53: 220–224.
13. Alabaster A, Vonderhaar B and Shafie S. Metabolic modification by insulin enhances methotrexate cytotoxicity in MCF-7 human breast cancer cells. *Eur J Cancer Clin Oncol* 1981; 17: 1223–1228.
14. Ayre SG, Bellon DPGY and Perez GD Jr. Insulin potentiation therapy: a new concept in the management of chronic degenerative disease. *Med Hypotheses* 1986; 20(2): 199–210.
15. Yang Y, Wen F, Dang L, et al. Insulin enhances apoptosis induced by cisplatin in human esophageal squamous cell carcinoma EC9706 cells related to inhibition of autophagy. *Chin Med J* 2014; 127: 353–358.
16. Zou K, Ju JH and Xie H. Pretreatment with insulin enhances anticancer functions of 5-fluorouracil in human esophageal and colonic cancer cells. *Acta Pharmacol Sin* 2007; 28: 721–730.
17. Miglietta A, Panno ML, Bozzo F, et al. Insulin can modulate MCF 7 cell response to paclitaxel. *Cancer Lett* 2004; 209: 139–145.
18. Goldstein BJ, Mahadev K, Wu X, et al. Role of insulin-induced reactive oxygen species in the insulin signalling pathway. *Antioxid Redox Signal* 2005; 7(7–8): 1021–1031.
19. Loh K, Deng H, Fukushima A, et al. Reactive oxygen species enhance insulin sensitivity. *Cell Metab* 2009; 10: 260–272.
20. Jing K and Lim K. Why is autophagy important in human diseases? *Exp Mol Med* 2012; 44: 69–72.
21. Yu L, Wan F, Dutta S, et al. Autophagic programmed cell death by selective catalase degradation. *Proc Natl Acad Sci U S A* 2006; 103: 4952–4957.
22. Damyanov C, Gerasimova D, Maslev I, et al. Low-dose chemotherapy with insulin (insulin potentiation therapy) in combination with hormone therapy for treatment of castration-resistant prostate cancer. *ISRN Urol* 2012; 2012: 140182.
23. Zhao FQ and Keating AF. Functional properties and genomics of glucose transporters. *Curr Genomics* 2007; 8: 113–128.
24. Barron CC, Bilan PJ, Tsakiridis T, et al. Facilitative glucose transporters: implications for cancer detection, prognosis and treatment. Facilitative glucose transporters: implications for cancer detection, prognosis and treatment. *Metabolism* 2016; 65(2): 124–139.
25. Ganapathy V, Thangaraju M and Prasad PD. Nutrient transporters in cancer: relevance to Warburg hypothesis and beyond. *Pharmacol Ther* 2009; 121: 29–40.
26. Macheda ML, Rogers S and Best JD. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. *J Cell Physiol* 2005; 202: 654–662.
27. Godoy A, Ulloa V, Rodriguez F, et al. Differential subcellular distribution of glucose transporters GLUT1–6 and GLUT9 in human cancer: ultrastructural localization of GLUT1 and GLUT5 in breast tumor tissues. *J Cell Physiol* 2006; 207: 614–627.
28. Younes M, Brown RW, Mody DR, et al. GLUT1 expression in human breast carcinoma: correlation with known prognostic markers. *Anticancer Res* 1995; 15: 2895–2898.
29. Kang SS, Chun YK, Hur MH, et al. Clinical significance of glucose transporter 1 (GLUT1) expression in human breast carcinoma. *Jpn J Cancer Res* 2002; 93: 1123–1128.
30. Ravazoula P, Batistatou A, Aletra C, et al. Immunohistochemical expression of glucose transporter Glut1 and cyclin D1 in breast carcinomas with negative lymph nodes. *Eur J Gynaecol Oncol* 2003; 24: 544–546.
31. Krzeslak A, Wojcik-Krowiranda K, Forma E, et al. Expression of GLUT1 and GLUT3 glucose transporters in endometrial and breast cancers. *Pathol Oncol Res* 2012; 18: 721–728.
32. Morcavallo A, Stefanello M, Iozzo RV, et al. Ligand-mediated endocytosis and trafficking of the insulin-like growth factor receptor I and insulin receptor modulate receptor function. *Front Endocrinol* 2014; 5: 220.
33. Xiao G and Gan L-S. Receptor-mediated endocytosis and brain delivery of therapeutic biologics. *Int J Cell Biol* 2013; 2013: 703545.
34. Storz P. Reactive oxygen species in tumor progression. *Front Biosci* 2005; 10: 1881–1896.
35. Szatrowski TP and Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res* 1991; 51(3): 794–798.
36. Besse-Patin A and Estall JL. An intimate relationship between ROS and insulin signalling: implications for antioxidant treatment of fatty liver disease. *Int J Cell Biol* 2014; 2014: 519153.
37. Barthel A, Okino ST, Liao J, et al. Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1. *J Biol Chem* 1999; 274: 20281–20286.
38. Wong KK, Engelman JA and Cantley LC. Targeting the PI3K signaling pathway in cancer. *Curr Opin Genet Dev* 2010; 20: 87–90.
39. Pandini G, Vigneri R, Costantino A, et al. Insulin and insulin-like growth factor-I (IGF-I) receptor overexpression in breast cancers leads to insulin/IGF-I hybrid receptor overexpression: evidence for a second mechanism of IGF-I signaling. *Clin Cancer Res* 1999; 5: 1935–1944.
40. Hunker CM, Kruk I, Hall J, et al. Role of Rab5 in insulin receptor-mediated endocytosis and signaling. *Arch Biochem Biophys* 2006; 449(1–2): 130–142.