Impact, and treatment possibility of chemotherapy resistance in cancer

theses of doctoral dissertation

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Introduction

Human malignancies are among the leading cause of death worldwide, with a death toll of over 18 Million people every year. Besides surgical intervention and radiotherapy, most patients are treated with chemotherapy regimens. While there is a broad selection of durgs available, therapy resistance and formation of distant metastases occur regularly, causing two-thirds of cancer-related deaths.

The efficacy of chemotherapy is depending on drug absorbance and distribution in the body, tumor microenvironment, its ability to enter the tumor and the tumor cells, cellular uptake and metabolism, and excretion both from the cell and the body. This complex system is relying on the properties of the drug, the tumor microenvironment, and the cancer cell itself, their characteristics are determining therapeutic success.

In my theses I investigated three aspects of therapy resistance in detail: targeted therapy inefficacy due to mutation of the target protein; hypoxia-driven increase of cell motility and metastatic potential; drug elimination from the cells by drug efflux transporters. These processes lead directly to therapy failure, and poor prognosis of cancer patients in clinics.

In the first part of my theses I investigated the effects of the activating mutations of the K-Ras oncogene in non-small cell lung cancer. Mutations of the Ras oncogene occur in multiple cancer types, leading to well-known consequences. In non-small cell lung cancer, mutations of the K-Ras isoform (affecting about one-third of the patients) has a deleterious effect on the targeted therapies against epidermal growth factor receptor (EGFR). In the mechanism of action of Zoledronic acid treatment, the inhibition of K-Ras posttranslational prenylation, and subsequent membrane-associated localization is essential. Zoledronate is in clinical use in the treatment of NSCLC patients with bone metastases, however, in some cases, the treatment has no benefit. Based upon its mechanism, it is reasonable to propose that the genetic variations of K-Ras may confer to the abolishment of zoledronic acid response.

In the second part of my theses I investigated the effects of the hypoxic environment of the tumors. In solid tumors, the rapid and uncontrolled cell proliferation is usually not accompanied by the establishment of the proper vasculature, which leads to insufficient oxygen and nutrient supply of the tumor cells. This, however, does not always result in cell death, due to their ability to adapt to the severe environmental changes. The Hif-1α

transcription factor is the main intracellular effector protein of low oxygen tension in cells. Under hypoxic conditions, the rapid proteasomal degradation of Hif-1α is blocked, and Hif-1α protein accumulates and gets into the nucleus, enhancing the transcription of more than 60 target genes as a part of a transcription complex. The upregulated genes contribute to substantial changes in cell survival and proliferation, cellular metabolism, cell-matrix adhesion, and cell motility. This increased motility helps the cancer cell leaving its original position, which is identified as the first step of metastasis formation. Critical importance of Rho GTPase proteins RhoA, Rac1 and Cdc42 is known in the promotion of cell motility, as well as their connection with Hif-1α signaling. Our former experience, however, pointed out that hypoxic condition does not always promote cell motility. As motility and metastasization is a keystone of therapy resistance, understanding how hypoxia impacts cell motility is of great importance.

In the third part of the theses I focused on the multidrug resistance caused by P-glycoprotein (Pgp), the transmembrane efflux transporter encoded by the ABCB1 gene. Pgp is able to recognise and export numerous substrate molecules from the cell. This phenomenon results in insufficient drug accumulation, and therapy failure. It has been shown in multiple cancer types that increased ABCB1 expression acts as negative predictive and prognostic factor, which also highlighted the potential of therapies targeting Pgp. However, the Pgp inhibitors performing effectively *in vitro* mcould not be used in clinics, since the presence and activity of Pgp in healthy tissues is necessary in the self-defense against xenobiotics. While the expression of the transporter confers to increased resistance against numerous clinical antitumor agents (multidrug resistance, MDR), some molecules have been discovered that were surprisingly more toxic on MDR cells. This MDR-selective toxicity was successfully proven for many compounds later. Pgp-mediated efflux is responsible for resistance, however, the mechanism of action of MDR-selective toxicity was yet to be unveiled. Several hypotheses arose, one of which put responsibility on the active transport mechanism. For its activity, Pgp binds ATP intracellularly, and hydrolizes it to gain energy for conformational change and substrate efflux. It was proposed that the continuous stimulation of this ATP activity can lead to intracellular ATP depletion, and cell death. Another hypothesis is based upon our previous findings that the numerous effective MDR-selective agents derived from the 8-hydroxiquinoline all have the ability for the chelation of heavy metal ions. This led to the assumption that these compounds might form complex with intracellular iron. Since a number of vital enzymes require the presence of iron for their proper function, and cancer cells are known for their high iron demand, the removal of iron from the intracellular enzymes might contribute to MDR-selective toxicity.

Objectives:

Key gene mutations in K-Ras, the hypoxia-driven increase of cell motility and the drug efflux by P-glycoprotein can all result in cancer chemotherapy resistance Therefore I set up the following objectives during my work:

1. **Investigating the role of K-Ras mutations in the zoledronate therapy efficacy of NSCLC tumors.**
2. **Characterizing the effects of hypoxia and Hif-1α expressionon cancer cell motility and metastatic activity in a variety of cencer cell models.**
3. **Investigating the impact of MDR-selective drugs on the intracellular ATP pool depletion in Pgp-expressing cancer cells.**
4. **Discovering the suspected connections between iron chelation of the MDR-selective compounds, iron depletion in Pgp-expressing cells, and Pgp activity.**

Materials & Methods:

Chemicals

NSC297366 was obtained from the compound library of the Developmental therapeutic programme (NCI, NIH). A zoledronate (Zometa) and cisplatin were purchased from Novartist, doxorubicine from TEVA. The P-gp inhibitor tariquidar (XR9576) was a kind gift from Dr. Susan Bates (NCI). Cobalt chloride, iron chloride (·6 H_2O), deferasirox (DFS), deferiprone (DFPR), 2-hidroxyurea, verapamil, human holotransferrin, chetomin, colchicine, 2-deoxi-glucose, vinblastine, MTT and sulphorhodamine B (SRB) were bought from Sigma-Aldrich.

Iron chloride, cobalt chloride, transferrin, zoedronate, MTT, A vas-klorid, kobalt-klorid, transzferrin, zoledronsav MTT, SRB and cisplatin were dissolwed in sterile water, while the other compounds were dissolved in sterile DMSO.

Cells

For the K-Ras mutation experiments we used human NSCLC cell line H358 harboring K-Ras G12C mutation, and K-Ras G12S mutant A549 cells. We also used K-Ras wild-type cell lines H1650, H1975 and LCLC-103H. For the hypoxia measurements we used the *in vivo* liver metastasis selected HT-168-M1 cells derived from the malignant melanoma A2058, human fibrosarcoma HT-1080, human colon carcinomas HT-25 and HT-29, and squamous cell carcinoma of the head and neck region PE/CA PJ15 cell models.For the investigation of the MDR-selective toxicity, we used ABCB1-negative MES-SA human uterine sarcoma, doxorubicine-selected ABCB1-positive MES-SA/DX5 cell line, and A431 epidermoid carcnioma cell lines. We produced ABCB1 expressing derivatives MES-SA/B1, A431/B1 of the cells by lentiviral transduction (made by Katalin Német and Nóra Kucsma). For drug interaction measurements We repeated our measurements on human cervical adenocarcinoma cell lines selected for different levels of ABCB1 expression (KB-3-1, KB-8, KB-8-5 and KB-8-5-11). Hif-1α silencing was performed using shRNS system (Sigma). A431, MES-SA and KB cells were cultured in high-glucose DMEM (Gibco), PE/CA PJ15 cells in IMDM (Lonza), all other cells in RPMI-1640 (Sigma) medium, supplemented by 10% FBS (Gibco) and 50 unit/ml penicillin/streptomycin (Gibco).

Hypoxia treatment

We established low-oxygen millieu using a gas control chamber (Billups-Rothenberg) attached to air supply containing low levels (5%, 1%) of oxygen. The chamber temperature was kept at 37°C during the experiments. After opening the chamber, we put the samples to ice and processed (Western Blot, RT-PCR) all of them within five minutes in order to avoid the effects of reoxygenation.

Cell proliferation assays

Cells were seeded in 96-well plates and treated with different drugs in a range of concentrations. After treatment (48 or 72 hours), viable cell quantity was measured using Sulphorodamine B (SRB) or MTT assays. For drug interaction experiments (made by Szilárd Tóth) we treated cells seeded on 384-well plates with varying concentrations of the two drugs. The fluorescence representing viable cell number was read after 96 hours.

ATP pool measurements

Intracellular ATP pools were measured using the ATPLite luminescence based kit (PerkinElmer). 96-well white OptiPlate plates with transparent bottom (PerkinElmer) were used to seed cells. Following 4-hour treatments we lysed the cells, added substrate (luciferin), and measured the hydrolysis of cellular ATP by the detection of oxyluciferin formation accompanied by luminescent signal. ATP content was interpreted as relative percentage compared to untreated controls.

Western blot

We prepared whole protein lysates from cell cultures after treatment. We applied a differential centrifugation on manually homogenized NSCLC cell cultures in order to separate intracellular and membrane-associated protein fractions. Depending on protein size, we performed SDS-PAGE electrophoresis using 7,5-10% gels. The separated proteins were then blotted to a PVDF membrane using a wet blot system (BIO-RAD). Primary antibodies: anti-K-Ras (ab55391, Abcam) anti-Na/K-ATPase (BML-SA247-0100, Enzo Life Sciences), anti-Rho (-A, -B, -C) (#17-294, Millipore), anti-Rac1 (23A8 klón, Millipore), anti-cdc42 (#17- 441, Millipore), anti-HIF-1α (ab51608, Abcam), cell cycle and cell cycle regulation sampler kit antibodies (#9932 & #9870, Cell Signaling Technologies), anti-p53 (#2524, Cell Signaling), anti–β-aktin (AC-15, Sigma). Horseradis-peroxidase (HRP) conjugated goat anti-mouse and goat anti-rabbit IgG secondary antibodies were used, and the signals were detected using enhanced WesternBright chemiluminescent system.

mRNA expression measurements

For mRNA expression quantification, we prepared the cell culture treatment samples in 6-well plates, up to 0,5-1 * 10^6 cell number per well. The cells were lysed in Trizol (Life Technologies) reagent, whole RNA content was purified, and cDNA was produced by reverse transcription. We used Taqman probes (HIF-1α, RHOA, RAC1, CDC42, β-actin, TFR1, RPLP0) for RNA amplification (Thermo Scientific) by real-time qPCR (Step One Plus, Life Technologies). RPLP0 and β-aktin was used as endogenous control, relative gene expression levels were calculated using the $2^{-\Delta\Delta C}{}_t$ method.

Flow cytometry

For cell cycle analysis, we used Propidium Iodide and 5-Bromo-2'-deoxyuridine labeling. For apoptosis measurements we used Annexin V – Propidium Iodide Apoptosis kit (Dojindo), or quantified Sub-G1 fraction after Propidium Iodide labeling. We performed Calcein-AM assay to measure ABCB1 transporter function. All cells were measured using Attune or Attune NxT flow cytometer (Life Technologies).

ATPase activity measurements

Az ABCB1 transporter activity exports substrates via active transport, fueled by ATP hydrolysis. The resulting anorganic phosphate can be measured by colorimetric detection. Spodoptera frugiperda (Sf9) insect cell line-derived membranes expressing human ABCB1 were used to measure the hydrolysis of added ATP in cell-free experiments. Verapamil was used as positive control for ATPase activity stimulation. NSC297366, or 8-hydroxiquinoline complexes were prepared using 1:2 metal ion:ligand ratio.The measurements were performed by Ágnes Telbisz and Melinda Gera.

dNTP szintek meghatározása

After 24-hour treatment (DMSO control; NSC297366; 2-hydroxyurea), cells were trypsinized and cell extractions were produced using 60% methanol. The centrifuged supernatant was dried by vacuum centrifuge, and the pellet containing dTNPs was dissolved in nuclease free water. Cellular dNTP levels were quantified in a polymerase-based fluorescent assay by Judit Eszter Szabó.

Videomicroscopy

Cells were plated on 24-well plates, and placed into a custom-built inverse microscope with a joint cell culture incubator (World precision Instruments). Cell culture photos were taken every 5 minutes, of three non-adjacent fields of view. In hypoxia treatments, we applied lowered oxygen tensions of 5% and 1%. The evaluating software developed by the research group counted the random movement distance over time for every cells. The measurements were performed by Tamás Márton Garay and István Kenessey.

Total X-ray fluorescence

To fill up intracellular iron pools of the seeded cells in 6-well plates, we applied holotransferrin (25 µM, 37°C, 4h), followed by 8-hour treatment with serum free medium with or withour 5 µM chelátor: iron chloride 2:1 ratio complex. After treatment and trypsinization, the samples underwent acidic extraction, and were dried onto quartz slides. The Total X-Ray fluorescence (TXRF) measurements were performed using an Atomika 8030C (atomika Instruments GmbH) spectrometer. All measurements were done by Anikó Gaál, Veronika Pape and Norbert Szoboszlai. Gallium content was used as endogenous control.

Mass sppectrometry

We treated the seeded cells with 2:1 ratio chelator:iron chloride on 6-well plates (5µM, 37°C, 4h). Trypsinized cells were pelleted and disintegrated by acetonitrile. The samples were measured using Sciex 3200 Qtrap hybrid tandem mass spectrometer (Applied Biosystems) with joint HPLC system (series 200, PerkinElmer) by Pál Szabó.

Cell migration

48-well Boyden-chambers with 8 µm pore size membrane (Whatman) were used to analyze the directional movement of the cells, using 100 µg/ml fibronectin (Sigma) as chemoattractant. After 6-20 hours of incubation, the migrated cells were fixed by methanol, and stained by toluidine blue. Migrated cell number was counted under microscope by József Tóvári and Enikő Tátrai.

***In vivo* xenograft tumor growth and metastasis models**

The animal models were planned and performed under the supervision of József Tóvári, in the experimental animal house of the National Institute of Oncology, using 6-week old male SCID mice. For the *in vivo* investigation of effects of hypoxia on metastasis, we injected HT-168-M1 melanoma cells intrasplenically, followed by liver colonization by the tumor cells. Subcutaneously grown tissue pieces of HT-29 colon carcinoma were implanted orthotopically (to the colon wall) , and liver and lung metastases were recorded. The animals were grouped to control (saline i.p. twice a week), hypoxic (260mg/l $CoCl_2$ for four weeks in the drinking water - Cobalt inhibits the

degradation of Hif-1α, mimicking hypoxia), and Hif-1α inhibition (1mg/body mass kg chetomin i.p. twice a week) arms. Livers and lungs were formaline-fixed and metastatic sites were counted under microscope.

In the *in vivo* NSCLC experiments, we measured the growth of subcutaneously injected human NSCLC (LCLC-103H, A549, H1650) xenograft tumors. With or without cisplatin (0,2mg/ body mass kg, i.p., weekly) administration, we investigated the tumor growth in control (saline i.p. weekly), human dose zoledronic acid (50µg/body mass kg i.p., weekly), and high-dose zoledronic acid (500 µg/ body mass kg i.p., weekly) groups. 41 days after inoculation, we measured tumor weight, and fixed the samples in formaline.

Immunhistochemistry

Formaline-fixed, paraffin-embedded tumor samples were used to produce 5µm microscopic slices for immunostaining. The samples were stained using anti-human smooth muscle actin (SMA-1) antibody (Agilent) labeling the blood vessel walls containing smooth muscle cells.The samples were made by Katalin Derecskei, evaluated by István Kenessey.

Statistical analyses

The cell viability data were analysed using the GraphPad Prism software's in-built dose-respponse model, the fitted normalized curves provided IC_{50} values, the concentration of the drug causing the death of 50% of the cells. Using IC_{50} values, we characterized the combination index (CI) in the co-treatment experiments, profiling synergistic, additive or subadditive drug-drug interactions. Group mean comparisons were analysed by Student's t-tests, using Statistica 12.0 (Statsoft) software.

Results

The effects of KRAS mutations on the zoledronic acid therapy efficacy

We investigated the impact of zoledronic acid treatment on NSCLC cell line K-Ras prenylation and translocation. Based on literature, prenylation inhibition results in inhibition of membrane-association of K-Ras. We treated the cells with 25µM zoledronate for 24 hours, and detected K-Ras protein expression by western blot on intracellular and membrane-associated protein lysates. Among wild-type cells, we observed full inhibition of K-Ras translocation in LCLC-103H cells, a partial inhibition in H1975 cells, however, in the H1650 cells we did not measure any changes. Importantly, the latter two cell lines harbor mutation, and deletion of the K-Ras activating EGF receptor, respectively, which might contribute to the weaker effect of zoledronic acid treatment. Similar measurements in K-Ras G12C mutant H358 and K-Ras G12S mutant A549 cells showed that zoledronic acid was ineffective in the inhibition of the membrane-association of the mutant K-Ras protein. Emphasizing the role of K-Ras mutation in zoledronate resistance.

In vitro toxicity tests showed that K-Ras wild type cells show decreased proliferation at lower (10 µM) zoledronic acid concentration, while the cells harboring K-Ras mutation are resistant until higher (50-100 µM) dose treatment. Migration tests performed in Boyden-chamber showed a general suppressive effect of zoledronic acid treatment on cell motility, differences between cell sensitiviy did not show any connections with K-Ras status. Zoledronic acid monotherapy showed significantly increased apoptotic activity in the K-Ras wild-type LCLC-103H cells only. Combination cytotoxicity tests showed that in cells with wild-type K-Ras, the zoledronate could effectively potentiate the cytotoxic effect of cisplatin, while only moderate effect was observed on H358, and no synergism on A549 cells. These findings are all in line with the Western blot results depicting K-Ras localization inhibition.

We performed *in vivo* xenograft experiments to characterize the antitumor effect of zoledronic acid in NSCLC models. LCLC-103H and H1650 cells with wild-type K-Ras, and A549 cells with K-Ras G12S mutation were used. In the animal models, only LCLC-103H cells showed sensitivity to zoledronate monotherapy. Similarly, combined therapy with cisplatin showed lower tumor sizes in higher zoledronic acid groups, but this trend

was not statistically significant. Immunohistochemical staining of the tumor samples showed that zoledronic acid treatment induced intensive blood vessel formation, which suggests the importance of the angiogenic environment on zoledronic acid therapy efficacy.

Our results showed increased zoledronate resistance of NSCLC cells harboring mutations of the K-Ras oncogene, however, the heterogeneity of response among the K-Ras wild type cells indicate that other factors might contribute to zoledronic acid treatment failure.

Effects of tumor hypoxia and the activation of Hif-1α signaling on tumor cell proliferation, motility, and metastasis *in vitro* and *in vivo*

We used five human tumor cell line to assess the effects of hypoxic environment: HT1080 fibrosarcoma, HT-168-M1 malignant melanoma, HT-25 and HT-29 colon adenocarcinoma, PE/CA PJ15 head and neck squamous cell carcinoma models were examined. Cells were investigated in hypoxic conditions by 5% and 1% oxygen tension, or by chemical induction of Hif-1α accumulation by cobalt chloride. The cell proliferation experiments did not show drastic changes in cell viability and proliferation in hypoxia, compared to normoxic controls. The non-directional motility was measured by videomicroscopy, while directed migration was investigated in Boyden-chamber. The colon adenocarcinoma models HT-25 and HT-29 showed very low motility, while HT168-M1, HT1080 and PE/CA PJ15 cells were moving intensively without any stimulation. The movement speed of the more motile cells increased even further in hypoxia, while it was unable to stimulate the movement of the more sessile cell lines. This pattern was generally seen in both assays, with an interesting exception of PJ15 cells in the Boyden chamber, where 5% oxygen stimulated the migration activity, while 1% oxygen did not, suggesting some possible feedback mechanisms.

The intracellular effector of hypoxia is Hif-1α, a major transcription factor, which has known linkage to the Rho GTPase (RhoA, Rac1, Cdc42) protein expressions, therefore we examined the mRNA expression changes of the four genes (includung HIF-1α). In HT-168-M1 cells, all the four genes had increased expression in hypoxia. In case of HT1080 and PE/CA PJ15 cells, 1% oxygen tension resulted in the feedback of the changes, similarly to migration activity of PE/CA PJ15. However, HT-25 did not show overexpression of the four targets, and HT-29 responded with downregulation of all four genes in hypoxia.

Based on these data, we chose two models with distinct hypoxic response, the highly motile, hypoxia-responsive HT-168-M1 and HT-29, more sessile, and not mobilizable by hypoxia. Examination of Hif-1α and Rho GTPase protein levels showed, that while hypoxia results in Hif-1α accumulation in both cells, the Rho GTPase levels remained unaffected in the HT-168-M1 cells, and showed strong downregulation in HT-29 cells, in accordance with the lack of mobilization in hypoxia.

Besides *in vitro* testing, we used *in vivo* xenograft colonization and metastasis models to investigate the effects of hypoxia on metastatic activity. *In vivo* hypoxia could be mimicked by Hif-1α stabilization and accumulation after cobalt chloride administration, while inhibition of Hif-1α activity was assessed by chetomin treatment. The number of hepatic metastases of HT-168-M1 xenografts increased in the hypoxic setup, while significantly decreased upon Hif-1α inhibition. However, no such changes were observed in the HT-29 model, nor in lung metastasis, nor in liver metastasis.

To further clear whether the motility and metastasis were dependent of hypoxia and Hif-1α expression, we used shRNA to silence HIF-1α expression in both HT-168-M1 and HT-29 cells. The repeated motility tests showed that the hypoxia-dependent increase of cell motility could be reversed by HIF-1α silencing. However, HT-29 did not show any changes among oxygen levels or HIF-1α status. The in vivo examination of Hif-1α induction on metastatic activity showed that the abrogation of HIF-1α expression could rescue the hypoxia-related increase of the metastatic activity.

In summary, we could identify different, cell-line specific hypoxia-related phenotypes in our in vitro and in vivo systems. This could inspire further investigations to characterize the determinants of hypoxia-dependent and hypoxia-independent motility and metastasis, which can help application of more accurate targeted therapies in the future.

The mechanism of action of the MDR-selective compound NSC297366, and the selective killing of ABCB1-expressing cells through the depletion of intracellular iron pools

Of numerous effective MDR-selective molecules, we selected NSC297366 (6-Chloro-3-[(2-fluorophenyl)methyl]-2,4-dihydropyrido [3,2-h][1,3]benzoxazine), an 8-

hydroxyquinline derivative compound with high selectivity towards MDR cells. We used MES-SA and KB-3-1 cell panels to show that NSC297366 toxicity is proportionally increasing with Pgp expression.

Investigation of the ATP hydrolysis and possible intracellular ATP depletion showed that neither Pgp substrate vinblastine nor ATP-ase stimulating Pgp inhibitor verapamil nor MDR-selective NSC297366 were able to decrease the cellular ATP levels. Glycolysis inhibitor 2-deoxy-glucose was efficient in decreasing cellular ATP pools similarly in Pgp-negative A431 and Pgp-overexpressing A431/B1 cells. Our combination treatment experiments showed that not even in case of inhibited ATP production (2-deoxy-glucose) was possible to further deplete ATP levels by Pgp transport stimulation.

The structure-activity relationship and binding property results showed that the chemical group of 8-hydroxyquinolines with numerous MDR-selective members (including NSC297366) is a group of potent metal ion chelators. Iron levels are strictly regulated in cells, essentially needed for crucial enzymatic processes. Using Pgp-negative MES-SA and Pgp-expressing MES-SA/B1 cells, NSC297366 shows MDR-selective toxicity, which could be reversed by the addition of exogenous iron. While iron increased NSC297366 tolerance in both cells, the effect was stronger in MES-SA/B1 cells, abolishing the Pgp-dependent selectivity. Additionally, co-treatment experiments with NSC297366 and non-selective chelator deferiprone showed synergistic effect in MES-SA/B1 cells and additive effect in MES/SA cells.

Examining the cellular responses of the suggested iron depletion we proved that NSC297366 treatment selectively increases the expression of transferrin receptor (the cell surface protein responsible for cellular iron uptake) mRNA in MES-SA/B1 cells, an effect reversible by transporter inhibition (by tariquidar) or exogenous iron addition. Prolyl hydroxylases responsible for Hif-1α degradation have iron dependent functionality, too. Lack of iron thus leads to Hif-1α accumulation, an effect which was shown in case of NSC297366 treatment. Importantly, lower drug concentrations were effective in MES-SA/B1 cells. This effect was also found Pgp-function and iron dependent. Hif-1α activity was also selectively increased upon NSC297366 treatment of MDR cells, represented by increased VEGFA mRNA levels.

Further experiments were performed to clarify the possible connection between iron depletion and MDR-selective toxicity. Measurement of iron-dependent enzyme activity Ribonucleotide reductase, the enzyme responsible for production of dNTPs necessary for DNA synthesis showed that universal inhibitor 2-hydroxyurea inhibited dNTP synthesis in both cells, while NSC297366 selectively blocked it in MES-SA/B1 cells. Also, flow cytometry experiments showed MDR-selective G1/S phase cell cycle arrest, resulting in the sharp decrease of G2 phase cells. All these effects were reversible by Pgp inhibition. Detailed analysis of cell cycle regulation expression pattern identified multiple key protein expressions (Cyclin B1, Cyclin D3, $^{p21Cip1/Waf1}$, $p27^{Kip1}$, CDK4, p53) changing selectively in MES-SA/B1 cells after 24 hours of NSC297366 treatment. Selective induction of p53 was suggesting apoptotic activity changes. Indeed, NSC297366 caused MDR-selective, Pgp-function dependent apoptosis.

All these evidences were in line with the iron depletion theory, however, we wanted to perform direct measurements involving Pgp function. Intracellular iron levels were quantified using total X-ray fluorescence, showing that NSC297366 could selectively deplete iron from MES-SA/B1 cells, in contrary to non-selective chelator 8-hydroxyquinoline, or by inhibited Pgp. Treatment with NSC297366:$FeCl_3$ complex leads to both iron and chelator accumulation in the cells, an effect largely abrogated in the presence of functional Pgp. Furthermore, cell-free membranes containing Pgp were used to measure ATPase activity of the transporter. NSC297366:$FeCl_3$ complex stimulated the ATPase activity of Pgp, pointing out that the complex is a substrate of P-glycoprotein and can be exported from the cells through Pgp.

Clinically used iron chelator deferasirox, and other potent iron chelators were also examined, proving that efficient iron complexing property per se is not enough to express MDR-selective toxicity. Our experiments with Hif-1α silenced cells and A431 and A431/B1 models lacking functional p53 proved that, while these proteins can be accumulated due to iron depletion, are not crucial during the process of MDR-selective toxicity of NSC297366.

Based on our results, we built a model of the mechanism of action behind MDR-selective toxicity. This includes drug entry to the cells, complex formation with available free iron, thus pushing the metabolically iron-dependent cancer cells into iron hunger. The chelator-iron complex then can be recognised and exported by Pgp in multidrug-resistant cells, causing more severe, and eventually fatal iron depletion, and MDR-selective toxicity. This approach can serve as a new strategy designing drugs targeting MDR cancer.

Conclusions

The effects of KRAS mutations on the zoledronic acid therapy efficacy in NSCLC

We showed that the *in vitro* antiproliferative and anti-migratory effects of zoledronic acid treatment are absent in the examined NSCLC tumors harboring K-Ras mutations.

We proved that membrane-associated localization of K-Ras, thus its activity can be inhibited – though to different extent – by zoledronate administration in K-Ras wild type NSCLC cells, while no such effects were recorded in K-Ras mutant NSCLC cells.

We reported the cell-line dependent increase apoptotic activity and potentiation of cisplatin cytotoxicity by zoledronic acid in K-Ras wild type NSCLC models.

Our *in vivo* experiments approved the antitumor and angiogenic switch effect of zoledronic acid treatment in K-Ras wild type LCLC-103H xenograft tumors.

Effects of tumor hypoxia and the activation of Hif-1α signaling on tumor cell proliferation, motility, and metastasis *in vitro* and *in vivo*

We showed that no dramatic changes are occurring in cell proliferation under hypoxic circumstances.

We measured that *in vitro* motility and migration – and their inducibility in hypoxia – are profoundly different among the investigated cancer cell lines.

We reported that Hif-1a Rho GTP-ase protein expression change patterns due to hypoxia are different among cell lines both on mRNA and protein level, which is well reflected in cell motility changes

Our *in vivo* xenograft metastasis models showed different Hif-1α dependency of metastasis formation: while HT-168-M1, a highly motile malignant melanoma model increased metastatic activity on Hif-1α stimulation, and decreased metastasis number while Hif-1α activity was inhibited, no such changes were observed in the HT-29 colon carcinoma model.

We successfully approved the differences in Hif-1α dependency of cell motility and metastatic activity changes in hypoxia by using Hif-1α silenced cells *in vitro* and *in vivo*.

Pgp-expression in cancer is a double-edged sword: it contributes to multidrug resitance and therapy failure, while it is also a potential target to eliminate MDR cancer cells via selective depletion of intracellular iron through Pgp.

We reported MDR-selective toxicity of numerous 8-hydroxiquinoline derived compounds in our cellular model system.

We showed that the selective toxicity of NSC297366 can be rescued by the addition of excess iron, and that NSC297366 shows synergism with iron chelator deferiprone.

We identified the early signals of iron depletion in Pgp-expressing cells upon NSC297366 treatment: overexpression of transferrin receptor, accumulation of Hif-1α protein, and upregulation of its transcriptional targets.

We proved that the iron deficiency caused by NSC297366 results in the inhibition of ribonucleotide reductase activity, cell cycle arrest in the G1/S phase, and enhanced apoptotic activity in Pgp-expressing MES-SA/B1 cells.

Direct measurement of intracellular iron and NSC297366 showed that the accumulation of both molecules are obstacled by Pgp activity. We proved that intracellular iron pools are decreased upon NSC297366 treatment of cancer cells with functional Pgp. Our ATP-ase assays confirmed that NSC297366:iron complex is a substrate of Pgp, stimulating ATP hydrolysis and Pgp-mediated transport from the cells.

Additionally, we proved that iron chelation property of compounds is not sufficient to achieve MDR-selective toxicity. We also showed that induction of Hif-1α and p53 proteins by NSC297366 treatment are signals, but not necessary for MDR-selective toxicity.

Ultimately, based on the results with NSC297366, we established the model of cellular iron depletion through Pgp-mediated export of iron from the cells, which is a starting point of a promising new strategy in the treatment of multidrug-resistant cancers.

Publications related to the theses

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