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Chelators as Antineuroblastomas Agents

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Summary

Neuroblastoma represents 8-10 % of all malignant tumors in childhood and is responsible for 15 % of cancer deaths in the pediatric population. Aggressive neuroblastomas are often resistant to chemotherapy. Canonically, neuroblastomas can be classified according to the MYCN (N-myc proto-oncogene protein) gene amplification, a common marker of tumor aggressiveness and poor prognosis. It has been found that certain compounds with chelating properties may show anticancer activity, but there is little evidence for the effect of chelators on neuroblastoma. The effect of new chelators characterized by the same functional group, designated as HLZ (1-hydrazino phthalazine), on proliferation (WST-1 and methylene blue assay), cell cycle (flow cytometry), apoptosis (proliferation assay after use of specific pharmacological inhibitors and western blot analysis) and ROS production (fluorometric assay based on dichlorofluorescein diacetate metabolism) was studied in three neuroblastoma cell lines with different levels of MYCN amplification. The molecules were effective only on MYCN-non-amplified cells in which they arrested the cell cycle in the G0/G1 phase. We investigated the mechanism of action and identified the activation of cell signaling that involves protein kinase C.

Key words

Neuroblastoma • Chelators • SHSY5Y cells • MYCN • Cell cycle

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Introduction

Neuroblastoma is a tumor derived from pluripotent stem cells of the sympathetic nervous system called neuroblasts. It is the third most common cancer in children under the age of 10 years where it precedes leukemia and tumors of the central nervous system. In infants less than 12 months of age, it is the most common cancer. Neuroblastomas represent 8-10 % of all malignant tumors in childhood and are responsible for 15 % of cancer-related deaths in the pediatric population [1,2]. However, it is relatively rare in adolescents and young adults. It usually develops in some of the adrenal glands and can spread to other parts of the body such as the chest, abdomen, neck, lymph nodes, pelvis, and bone marrow. It often metastasizes in these organs [2]. Its variability is a clinical hallmark of neuroblastoma. Approximately 75 % of cases with disseminated metastases occur in children older than 1 year. These aggressive neuroblastomas are resistant to chemotherapy and are generally incurable. Conversely, children under one year show a lower degree of disease and well respond to the treatment with chemotherapeutic agents [3].

The International Neuroblastoma Risk Group proposed a classification system from data collected from 8800 patients, dividing neuroblastoma into 4 stages: L1, L2, M, and MS. Tumors in the L1 stage are limited to one part of the body: neck, abdomen, chest, or pelvis. Stage L2 occurs at multiple sites, in stage M tumors are distantly metastasized. Last stage of MS is used to

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categorize patients younger than 18 months with the occurrence of metastasis in the skin, liver, and bone marrow [1,4]. Canonically neuroblastomas are also classified according to the MYCN (N-myc proto-oncogene) gene amplification, the gene encoding for the MYCN protein, a common marker of tumor aggressiveness and poor prognosis [5].

MYCN gene is amplified in some neuroblastomas [6]. It is historically the first genetic marker for the therapeutic stratification of patients in pediatric oncology [7]. MYCN is a member of the MYC family of oncogenes, which encode nuclear proteins serving as transcription factors. The activation of the MYC family genes occurs in human and animal tumors. It is usually caused by genetic changes, resulting in increased expression of wild-type MYCN protein. In neuroblastoma especially in localized tumors, the MYCN gene amplification is an indicator of poor prognosis [8].

There is still no cure for neuroblastoma and often the researchers have to distinguish between compounds active on MYCN amplified and non-amplified tumors and cells [9]. Certain compounds with chelating properties may show anticancer activity [10,11], but there is little evidence for the effect of chelators on neuroblastoma [12]. The role of metal ions in cancer is complex, although many studies identified deviations in accordance with the increased requirement for iron in rapidly proliferating tumor cells. Expression of transferrin receptor (TfR) on the surface of many types of cancer cells, namely prostate, lung, liver, cervical, breast cancer, and choriocarcinoma, is increased [13-15]. These determine the amount of iron that is transported into the cell [16]. Elevated serum ferritin levels have also been observed in several tumors. Serum ferritin was significantly increased in the III and IV stages of neuroblastoma [17]. This observation is used as a prognostic indicator, where a high level of ferritin is associated with a poor prognosis, while a low level indicates a good prognosis [18]. The levels of TfR are inversely proportional to the MYCN expression levels in neuroblastoma [19]. Moreover, it has been recently demonstrated a direct link between the MYCN and ferritin levels associated with prognosis and disease outcome.

Desferrioxamine (DFO) is a well-known studied chelator [18,20,21]. Its cytotoxic activity against neuroblastoma cells *in vitro* is thought to be due to iron chelation. The effects of DFO on neuroblastoma are relatively specific because many non-neuroblastoma cell lines derived from either malignant or normal tissues were not sensitive to DFO [20]. Toxicological experiments showed that treatment with this compound had no side effects [21]. Another study evaluated the effect of treatment with DFO in combination with numerous other anticancer drugs on 57 patients. Complete response was obtained in 24 patients [18]. The reason for the high sensitivity of neuroblastoma cells to DFO is unknown. It seems that these cancer cells contain high levels of ferritin, but the pathophysiological significance of the increase in ferritin expression remains unclear [21].

Materials and Methods

Reagents, cell culture, and plasmids

Synthesis and physicochemical properties of the tested compounds, chelators 3MeOSA, 5tBuSA, Px, Q, PyCopy were published previously [22]. The compounds were dissolved in DMSO in a 10 mM stock solution. The used concentration of chelators in all the experiments was 1 μM. The plasmid for the expression of MYCN, the EMPTY control vector, and human neuroblastoma cells SHSY5Y, LAN1, and LAN5 were kindly provided by prof. Arturo Sala (Brunel University, London, UK). The cells were maintained at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) (Lonza, Basel, Switzerland) containing 10 % fetal bovine serum in a humidified atmosphere of 5 % CO_2. Stable transfections were performed using FUGENE reagent (Promega, Madison, USA) according to the protocol provided by the manufacturer. The clones were kept in Geneticin G418 (Sigma-Aldrich, Munich, Germany). The morphology of the cells was evaluated by bright field image capture using the microscope Olympus IX 81 and processed *via* the Olympus Xcellence rt software.

Proliferation assays

For the WST-1 proliferation assay, the cells were seeded into 96-well plates (1500 cells/well). After 24 h, the cells were exposed to the chelators for the time shown in the figures. After the WST1 reagent (Roche, Basel, Switzerland) was added, the cells were incubated for additional three hours. The optical density (OD) value was determined spectrophotometrically.

Methylene blue was used to evaluate the number of living cells after the application of inhibitors. The cells

were seeded into 12-well plates and one day after the treatment the cells were fixed with 3.7 % paraformaldehyde for 1 h. The fixed cells were stained with methylene blue (1 % solution in borate buffer) for 10 min and washed six times with phosphate buffer (PBS). After removing all the excess of methylene blue, the cells were lysed with HCl solution (0.1 M) and the amount of incorporated methylene blue was measured spectrophotometrically at 595 nm.

The pharmacological inhibitors used U0126 (MEK inhibitor), SP600125 (JNK inhibitor) SB203580 (p38 inhibitor) PKI (5-24) (PKA inhibitor), SH5 (PKB inhibitor), PKC inhibitor and ZDEVD.fmk (caspase inhibitor) were purchased from Santa Cruz Biotechnology (Texas, USA).

Cell cycle analyses

The effects of chelators at a concentration of 1 µM on the neuroblastoma SHSY5Y cell cycle were tested by using commercially available MUSE© Cell Cycle Assay KIT and MUSE© Cell Analyzer (EMD Millipore Corporation, USA) according to the manufacturer´s instructions.

ROS production assay

ROS assays were performed using the fluorometric assay based on the metabolism of dichlorofluorescein diacetate using the kit (OxiSelect™ Intracellular ROS Assay Kit; Cell Biolabs, Inc., San Diego, CA, USA). The assays were performed according to the manufacturer´s instructions and H_2O_2 was used as a positive control of ROS inducing agents.

Western blot analysis

Whole cell extracts, obtained with the use of home-made RIPA lysis and extraction buffer (50 mM Tris, 0.01 % NP40, 12 mM natrium deoxycholate, 3.5 mM SDS, 0.15 M NaCl, 2 mM EDTA, 50 mM NaF), were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking the non-specific interactions with 5 % nonfat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5 % Tween 20) for 60 min, the membrane was washed once with TBST and incubated with the PARP- (1:1000) and β-actin- (1:5000) antibodies at 4 °C for 12 h. The membranes were washed and incubated with the horseradish peroxidase-conjugated with anti-mouse or anti-goat antibodies (1:3000) for 2 h. Then the membranes were washed with TBST and developed with the ECL system (Amersham Biosciences). All the antibodies were purchased from Santa Cruz Biotechnology (Texas, USA).

Statistical analysis

Experiments were always performed in triplicate in three independent trials. The results are expressed as mean ± sampling standard deviation. The data obtained were evaluated using GraphPad Prism 5 software (GraphPad Software, USA) and one-way ANOVA or two-way ANOVA analysis was followed by a *post hoc* analysis by Bonferroni for comparison of values with control values. The significance level for all assays used was $p=0.05$ (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Results

Preliminary studies indicated an anticancer potential of five chelators (3MeOSA, 5tBuSA, Px, Q and PyCopy) belonging to the 1-hydrazino phthalazine (HLZ) group [22].

Effect of chelators on morphology, viability and cell cycle of human neuroblastoma SHSY5Y cells in vitro

The chelators were tested on the SHSY5Y cell line at the final concentration of 1 µM (IC_{50} identified on other cell lines [23]). Microscopic examination of the treated cells after 72 h showed some variation in the response to individual compounds belonging to the same group. Specifically, when exposed to compound PyCopy, the cells initially flattened and then turned rounded and detached from the well surface (Fig. 1a). The proliferation of the neuroblastoma cells affected by the chelators in time course from 24 to 72 h was evaluated by the WST-1 assay (Fig. 1b). Figure 1c shows that only 3MeOSA (1 µM) affected significantly the cell cycle, namely arrested it in the G0/G1 phase.

Mechanism of action of the chelators

Based on proliferation, morphology, and cell cycle data, the mechanisms of action activated by three the most potent compounds (3MeOSA, Px and PyCopy) appeared to be different. To confirm the apoptotic pathway, the cells were pre-incubated with the caspase 3 inhibitor ZDEVD.fmk at the final concentration of 50 µM and then exposed to chelators. The caspase inhibitor did not restore the cell growth and did not compromise the chelators activity, which precludes chelators-induced

activation of the apoptotic machinery (data not shown).

Subsequently, the effect of 3MeOSA, Px and PyCopy chelators has been investigated in the presence of specific kinase inhibitors (PKA, PKB/akt and PKC). The results showed relevant involvement of the PKA pathway during the treatment with Px, and significant participation of PKC pathway on the effect of PyCopy (Fig. 2).

To better understand the cell signaling affected by the chelators, extended analysis was performed by using specific inhibitors of the principal MAPK kinases, as MEK, JNK and p38. No significant changes in the effects of chelators were observed when these kinases were inhibited (data not shown). Similarly, the levels of ROS were not altered by the action of the chelators during 24 h exposure. A non-significant increase in ROS production was observed only for PyCopy (data not shown).

Chelating activity of the tested compounds

To prove that the observed biological effects are due to the chelating activity, the cells were exposed to the chelators in the presence of $CoCl_2$, which, in addition to its toxicity, is a recognized model of hypoxia. $CoCl_2$ at 200 μM concentration killed 75 % of the cells within 24 h. The effect of $CoCl_2$ has been partially reverted by all the chelators at 1 μM concentration. Interestingly, the usual change in cell shape activated by 3MeOSA, Px and PyCopy was partially or totally abrogated (data not shown) and even their antiproliferative activity was reduced (Fig. 3a). Since the chelators are thought to mostly disrupt the iron balance, the same $CoCl_2$ toxicity experiment was performed using the compounds 3MeOSA, Px and PyCopy in combination with a non-toxic concentration of $FeCl_2$ (45 μM). A methylene blue assay performed 72 h after the treatment showed a significant reduction in the antiproliferative effect (Fig. 3b), associated with no change in cell shape (data not shown).

Study of the neuroblastoma resistance mechanism to chelators in vitro

The amplification of MYCN gene that results in the MYCN overexpression, makes the neuroblastoma cells resistant to several chemotherapies. We investigated whether the MYCN-amplified cell lines (LAN1 and LAN5) are resistant to chelators. The results shown in Figure 4 indicated that the chelators were less effective in the MYCN amplifying cells. This applies especially to the compound Px which induced cell proliferation.

In support of the results shown above, we created SHSY5Y clone with an amplified MYCN gene. The plasmid has been used previously to generate MYCN-overexpressing SHSY5Y clones [24]. Figure 5a shows the effect of five chelators used on the morphology or viability of SHSY5Y cells overexpressing MYCN compared to the clone transfected with the empty vector. In the case of 3MeOSA, Px, and PyCopy the MYCN overexpression significantly enhanced the resistance of the SHSY5Y cells (Fig. 5b). Figure 5c shows that the chelators did not affect the cell cycle of the SHSY5Y-MYCN clone.

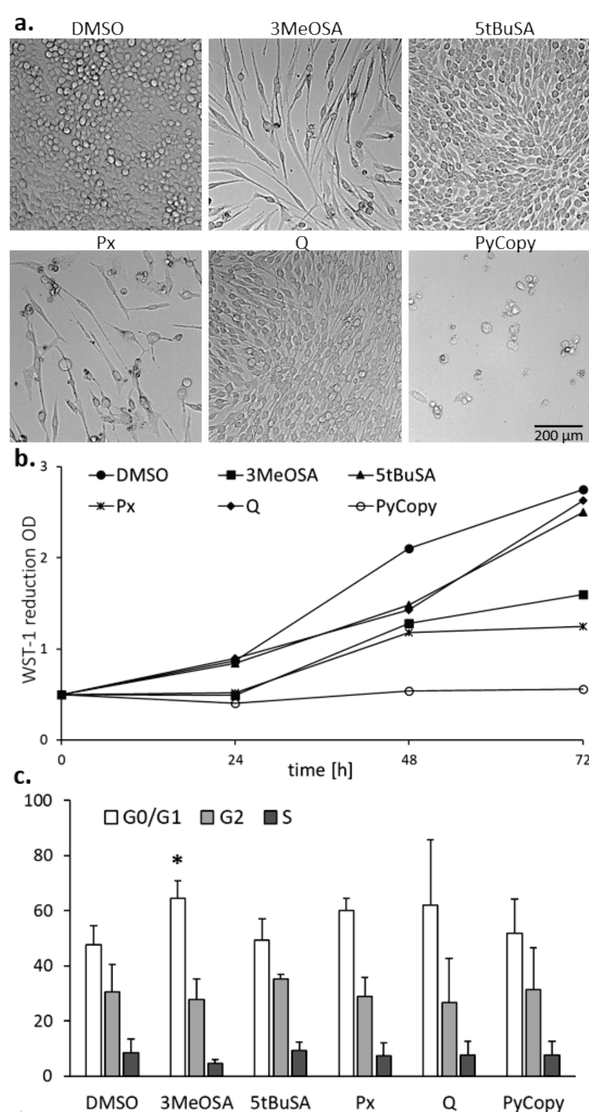

Fig. 1. Effect of chelators on morphology (**a**), viability (**b**) and cell cycle (**c**) of human neuroblastoma SHSY5Y cells *in vitro.* (a – 1 μM, 72 h, b – 1 μM, 24, 48, 72 h, WST-1 assay, c – 1 μM, 24 h, MUSE© cell cycle KIT).

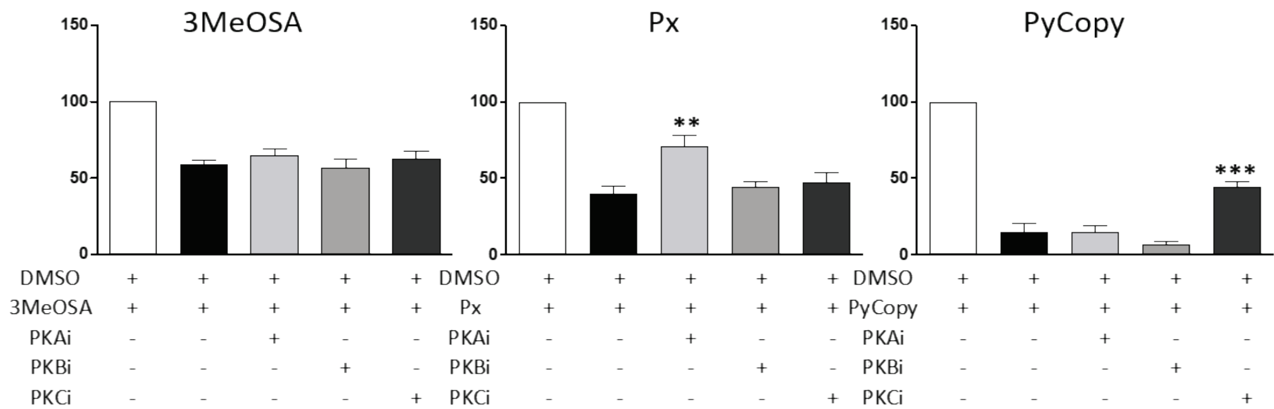

Fig. 2. Effect of chelators on PKA, PKB, and PKC signaling pathway (Human neuroblastoma SHSY5Y cells 3 h pretreatment with individual kinase inhibitors, 48 h exposure to chelators (3MeOSA, Px, PyCopy in concentration of 1 μM, methylene blue assay)).

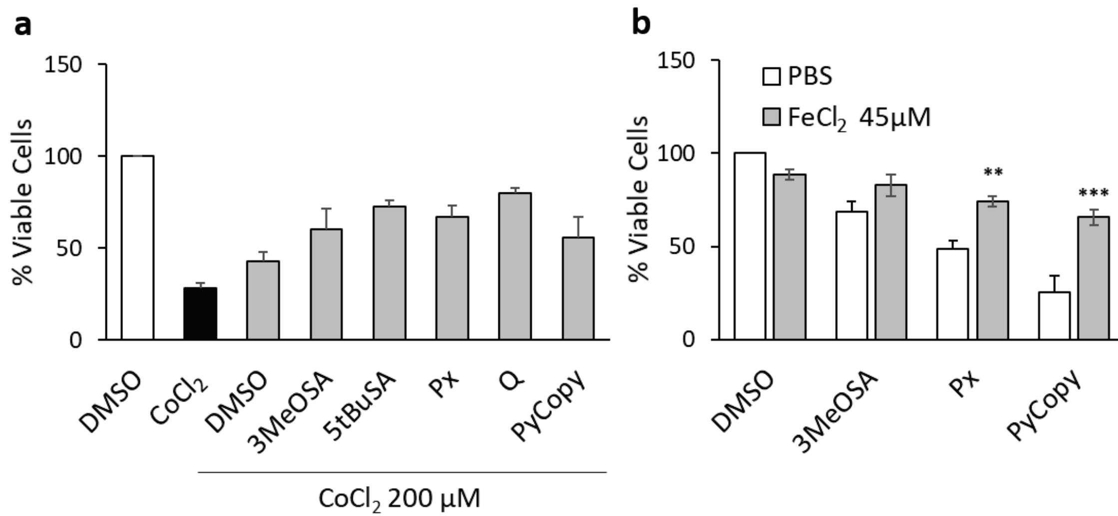

Fig. 3. The ability of chelators to revert the effect of $CoCl_2$ (**a**) and $FeCl_2$ (**b**) on human neuroblastoma SHSY5Y cells (Methylene blue assay, (**a**) $CoCl_2$ – 200 μM, 1 μM chelators, 24 h; (**b**) $FeCl_2$ – 45 μM, 1 μM chelators, 72 h).

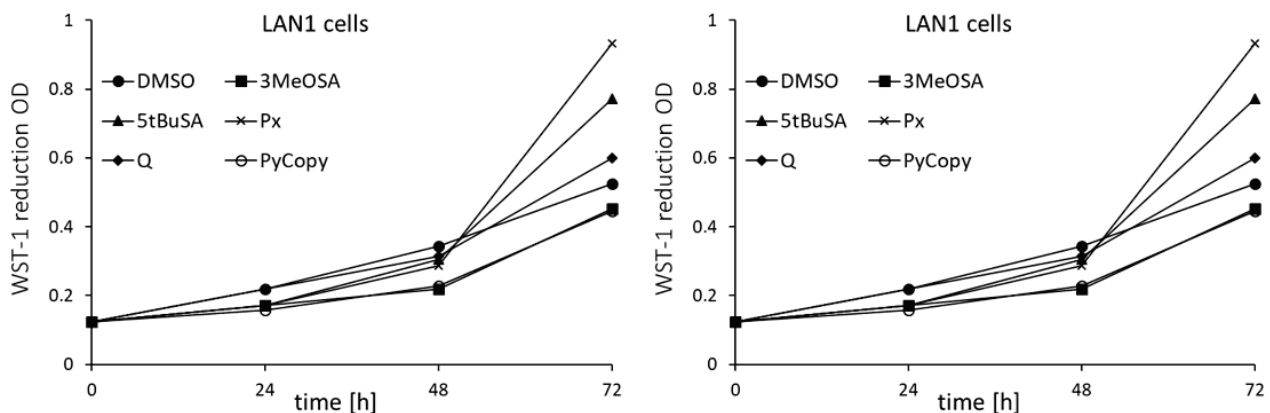

Fig. 4. Effect of chelators on the proliferation of the MYCN-amplified cell lines (Human neuroblastoma cell lines LAN1, LAN2, 1 μM, 24, 48, 72 h, WST-1 assay).

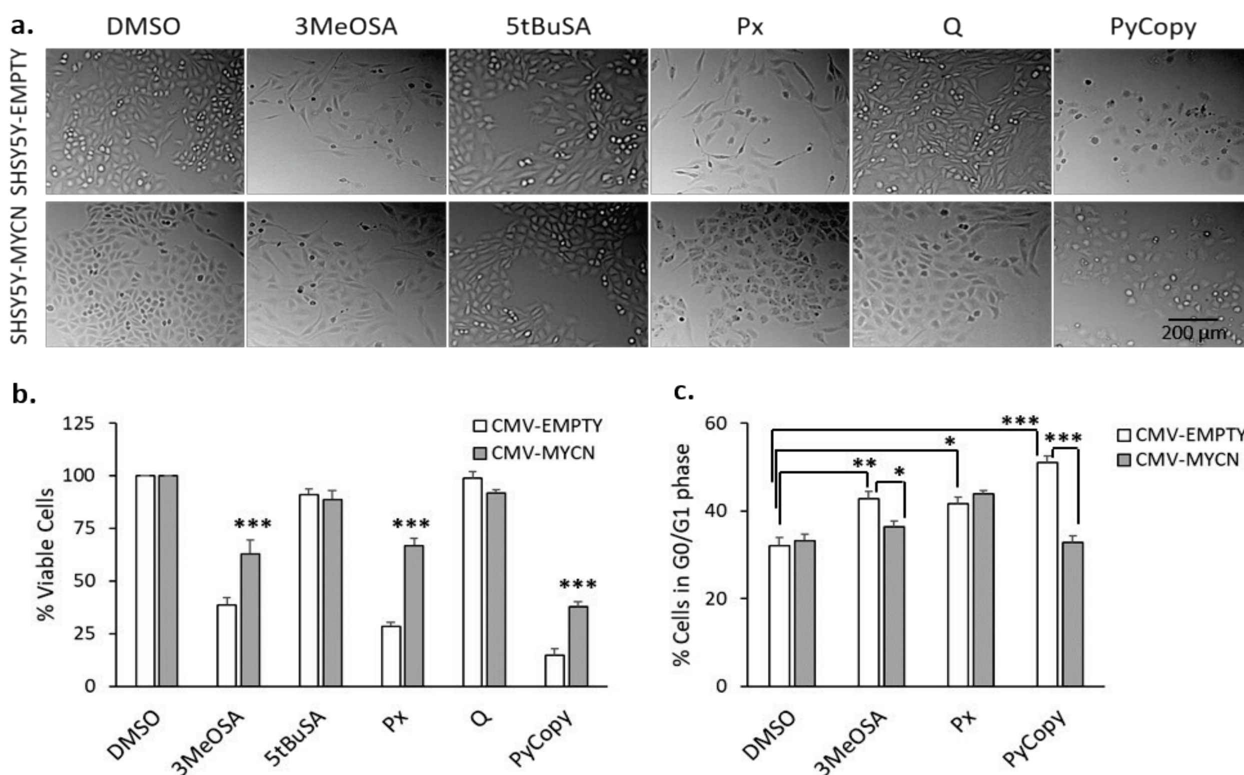

Fig. 5. The effects of chelators on morphology (**a**), proliferation (**b**) and cell cycle (**c**) of the human neuroblastoma MYCN-amplifying SHSY5Y cells. (1 μM, 48 h, **a** – microscopic examination, **b** – methylene blue assay, **c** – MUSE© cell cycle KIT).

Discussion

During preliminary experiments, a library of newly synthesized chelators was tested on several cell lines and the IC_{50} was calculated. The results of the WST-1 test provided information about the significant effect of three chelating agents belonging to one of the tested groups, named HLZ (1-hydrazino phthalazine), namely 3MeOSA, Px and PyCopy, on cell proliferation and paved the way for a more detailed study focused on understanding the mechanism of their action. Their hydrophobic character could probably be responsible for their higher inhibitory effect. Hydrophilic substances penetrate through cell membranes less efficiently, which leads to attempts to synthesize compounds with hydrophobic properties [12]. Shen *et al.* [12] tested the effects of the chelators EDTA, DTPA, DFO, and 2LL on the SHSY5Y cell line, where their most effective chelating agent DTPA reduced cell viability to 30 % at a concentration of 150 μM. Our most potent compound reached this value at 1 μM concentration, so it appears to be much more effective.

Interestingly, the tested chelators induced different morphological changes in the SHSY5Y cells. To understand the reason for the arrest of cell proliferation, the cell cycle was analyzed. A number of studies have shown that some normal and cancer cell lines, especially those sensitive to iron depletion, exhibit cell cycle arrest in G1 (or G1/S phase) [19,25]. Similarly, clinical trials were performed to assess its DFO-mediated antiproliferative activity by blocking G1/S phases for the treatment of neuroblastoma [26-28]. In our study, 3MeOSA, Px, and PyCopy compounds arrested the SHSY5Y cells in the G0/G1 phase. This is consistent with reports showing that some iron chelators induced apoptosis in a large number of cell lines including neuroblastomas [29,30]. ROS-driven caspase-dependent apoptosis was the major mechanism of cell death [29,30]. In contrast to studies demonstrating increased apoptosis in neuroblastoma cell lines compared to non-neuroblastoma solid tumor lines when treated with iron chelators [19], in our experiments, the SHSY5Y cells treated with chelators did not show apoptotic morphology. To exclude the chelators-triggered activation of apoptosis, the cells were preincubated with a known caspase inhibitor (ZDEVD.fmk 50 μM), which should reverse the effect of chelators effect if they induce apoptosis. However, the caspase inhibitor used did not change the effect of the tested chelators. A smaller statistically insignificant effect was only observed when

using PyCopy. As further confirmation that cell death did not occur by the mechanism of apoptosis, a western blot was performed to detect the expression levels and cleavage of PARP, the final substrate of caspase 3. The western blot experiment shows no PARP cleavage (data not shown).

Iron chelation-mediated apoptosis was reported to be induced through ER stress [31,32]. Non-ROS-inducing iron chelator DFO can stimulate JNK signaling [32,33]. In an attempt to identify the mechanism of action of the three effective chelators in terms of cell signaling, SHSY5Y cells were preincubated with selective pharmacological inhibitors of three crucial kinases as PKA, PKB/akt, and PKC. PyCopy affected PKC, the action of Px was significantly reduced by a PKA inhibitor. Based on these results, the same approach was used to evaluate the involvement of the three most common MAPKs (ERK1/2, JNK and p38). The assay showed that specific pharmacological inhibitors of MAPKs do not affect the activity of the chelators (data not shown). The involvement of PKC in iron chelator activity has been already shown in human intestinal epithelial cells [34]. Both PKC and PKA have been described to take a role in the cell death associated with ROS production [35], leading to actin-cytoskeletal alterations. According to the literature, chelators can alter the redox status of cancer cells [36]. However, our experiment did not show any variation in oxidative stress. The results are not so surprising considering that oxidative stress is often caused by free metal ions, like iron or cobalt [37], which can be neutralized by the chelators [37].

To prove that the effect on the cell proliferation and morphology changes was caused by the chelating properties of the tested compounds, we attempted to neutralize chelators effect by adding an excess of free metal ions into the culture media. $CoCl_2$ is known to mimic hypoxia stress [38]. In our experiments, all the chelators significantly reversed the toxicity of $CoCl_2$ at 200 μM concentration. As the effect of chelators is usually associated with altering the iron pathway [39,40], we also exposed the cells to the chelators in combination with a non-toxic concentration of $FeCl_2$ (45 μM). The result clearly shows that the effect of the chelators is neutralized by the $FeCl_2$, even during the most drastic shape change induced by the PyCopy compound. The number of cells surviving to the chelators´ activity is significantly increased during the combined exposure to $FeCl_2$.

Two cell lines, LAN1 and LAN5, carrying the MYCN amplification were tested in parallel with the control MYCN-non-amplified neuroblastoma cells SHSY5Y. The compound Px, able to reduce the cell growth of SHSY5Y cells, increased LAN1 and LAN5 cells proliferation. Both 3MeOSA and PyCopy did not significantly affect LAN1 and LAN5 cells growth. In previously published paper, LAN1 and LAN5 cells were less sensitive to all the chelators [9].

Here we demonstrate the importance of MYCN for resistance. The exact mechanism how MYCN exerts its protective effect will be the focus of future studies. A preliminary experiment suggests that the G0/G1 phase arrest of the cell cycle induced by the 3MeOSA, Px and PyCopy chelators in SHSY5Y cells is partially or totally abrogated in MYCN-overexpressing clone.

Conclusions

New chelators may be promising drugs for the side effects-free treatment of neuroblastoma. We show here that whereas the chelation therapy may not be effective against the MYCN-amplified tumors, the MYCN-non-amplified tumors may be well responsive to the chelators. The HLZ (1-hydrazino phthalazine) is reported here to be a promising functional component of the molecules. The reason for the difference in the effects of the tested chelators is not yet clear. It may be associated with their different ability to enter the cells and localize in specific compartments. The tested compounds do not significantly differ in their lipophilic properties and are not selective for specific metal ions. Further modifications of the structures will be necessary to improve their action and to explain the relation between their structure and activity.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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