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Mdm2 inhibitors as a platform for the design of P-glycoprotein inhibitors

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Abstract:

Chemoresistance is thought to be the cause of low treatment efficacy and mortality in more than 90% of patients with advanced cancer. The activation of drug efflux by P-glycoprotein is the key mechanism of resistance. All known P-gp inhibitors are used only in the combination therapy. We propose a new approach based on the multitarget rational design of drugs, which possess both the antitumor and efflux pump inhibitory activity. In this work, the principle possibility of combining the ability to inhibit P-gp and p53-Mdm2 protein-protein interaction in one structure is considered. The biological activity of a number of known and newly synthesized compounds was evaluated using cell lines with different p53 status. The possibility of using computer modeling for the search for P-glycoprotein inhibitors among Mdm2 inhibitors was analyzed; P-gp interaction site and binding modes of substrates and inhibitors were identified. The results obtained in cells that have the native balance of drug resistance and sensitivity showed the ability of the cells to both actively throw out xenobiotics and to lose this ability using P-gp inhibitors. The data obtained indicate that Mdm2 inhibitors are a promising platform for the development of multitarget drugs that can overcome tumor resistance by inhibiting the P-glycoprotein activity.

Keywords: multitarget drug design; P-glycoprotein; MDR1; p53-Mdm2; drug resistance; efflux inhibition; cell-based assay; computer modeling.

The problem of multiple drug resistance (MDR) development is becoming increasingly acute with the improvement of anticancer therapy methods and effectiveness of first-line therapy. Proliferation of a small number of survived cancer cells leads to the development of a secondary tumor that is insusceptible to the initial set of drugs. This is manifested as tumor progression after stabilization or significant regression. Thus, chemotherapy, which was successful at the first stage, becomes ineffective. Chemoresistance is thought to be the cause of low treatment efficacy and mortality in more than 90% of patients with advanced cancer. [1] The phenomenon of chemoresistance is associated not only with the development of relapses, but also with metastasis. [2]

Drug resistance is associated with impaired functioning of various cellular mechanisms associated with the transport of substances, DNA repair, apoptosis and others. Overexpression of transporters that prevent a cytostatic from entering the cell is considered the most important mechanism of drug resistance. [2-4]

P-glycoprotein (P-gp, MDR1) is a transmembrane efflux transporter of the ATP binding cassette (ABC) family, which pumps out various xenobiotics from the cell. It captures molecules from the intracellular side of the membrane and throws them out, which prevents the accumulation of the drug in the tumor and reduces the therapy effectiveness. P-gp overexpression was established in leukemia, ovarian and breast cancers, neuroblastomas and a number of other hematological and solid tumors. It was found that high level of P-gp expression can both be originally specific for cancer cells and appear during chemotherapy. [2] Overexpression of P-gp in cancers reduces the concentration of drugs in the cell and allows it to develop resistance to a number of topical cytostatics such as taxanes (paclitaxel), vinca alkaloids (vinblastine), and anthracyclines (daunorubicin). [5] The use of P-gp pump blockers allows one to restore the ability of the drug to enter the tumor cell and suppress its vital activity.

The third generation P-gp inhibitors are under clinical trials. They are developed using modern methods of drug design and showed good efficiency against P-gp with high specificity and minimum own toxicity. [6] Zosuquidar (NCT00046930) and valspodar (NCT00003190) could not improve the treatment of acute myeloid leukemia and epithelial ovarian cancer in phase II and III trials. [7, 8] And although tariquidar successfully passed phase II trials with docetaxel, [9] the phase III with vinorelbine and paclitaxel/carboplatin were terminated (NCT00042315, NCT00042302). Nevertheless, trials of P-gp inhibitors is ongoing as a part of the study on the CNS functioning (NCT03809234, NCT03809234).

The feature of the use of modern P-glycoprotein inhibitors is their ancillary role; they serve to increase the effectiveness of the main chemotherapeutic agent (taxanes, vinorelbine, etc.) [7-9]. At the same time, even if the side effects of the ancillary agent are very low, they are combined with the side effects of the main drug in any case, and it not only increases the toxic load on the organism, but also complicates the scheme of drug combination to increase the therapy effectiveness.

In this paper, we propose to look at P-glycoprotein inhibitors from a fundamentally different point of view: to make them an independent combat unit capable of alone exhibiting targeted biological activity. As a platform for the rational design of P-glycoprotein inhibitors, we consider p53-Mdm2 protein-protein interaction (PPI) inhibitors. The choice of the platform is determined by several reasons:

- these compounds are relevant for a wide range of solid tumors, since the p53-Mdm2 pathway is compromised in more than 50% of all human cancers, [10]

- pharmacophore hypothesis of p53-Mdm2 PPI inhibitors was thoroughly developed (see Supplementary), there are a lot of data on the structure–activity relationship obtained in recent years that can be used for the rational design of small molecule compounds, [11-13]

- the ability to inhibit P-glycoprotein was shown for these agents, [14, 15]

- their structure is very variable and allows various modifications, while the structure of the known p53-Mdm2 PPI inhibitors is not antagonistic to the structure of the known glycoprotein inhibitors (fig. 1). [16-20]

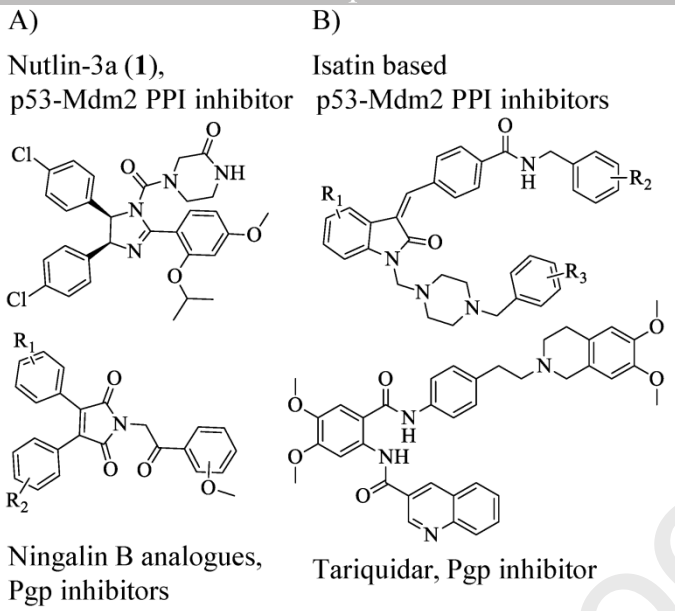

Fig. 1 – The structure similarity of p53-Mdm2 PPI inhibitors and P-glycoprotein inhibitors: A) Nutlin-3a, MDM2 inhibitor, [16] and Ningalin B analogues, P-glycoprotein inhibitor series, [17] B) Isatin based p53-Mdm2 PPI inhibitors, [19] and tariquidar, P-glycoprotein inhibitor [20].

In particular, the structural features suggest the presence of anti-Pgp activity in the developments of our group – Isatin based p53-Mdm2 PPI inhibitors (fig. 2, compound **3** [19, 21]), and our optimized compounds of Prof. Hardcastle (fig. 2, compound **4** [12, 22], for synthesis see Supplementary). Tariquidar was considered as the reference P-gp inhibitor. Nutlin-3a (compound **1**) is a p53 reactivator of the cis-imidazoline class, which was shown to competitively inhibit P-gp. [15] AMG232 (compound **2**), a p53-Mdm2 PPI inhibitor, is currently undergoing phase II clinical trials against Merkel Cell Carcinoma (ClinicalTrials.gov Identifier: NCT03787602), Acute Myeloid Leukemia (NCT03787602), while its trials against myeloid leukemia are initiated (NCT04190550). [23-25]

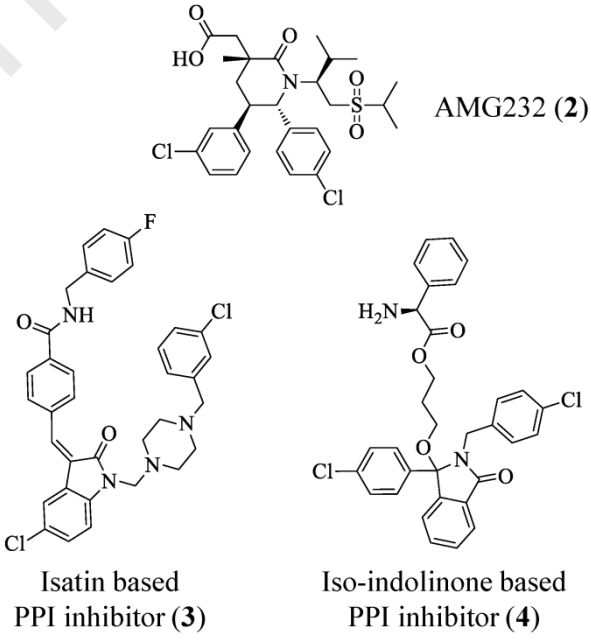

Fig. 2 – Structures of p53-Mdm2 PPI inhibitors **2**, **3**, **4**.

To evaluate the biological activity of the compounds, the following cell lines were used: HCT116 (p53+/+), H1299 (p53-/-), and MCF7, which is most often used for P-glycoprotein studies. All lines were routinely cultivated on Petri dishes (63.5 cm^2) at 37°C in a CO_2 incubator according to ATCC recommendations. For experiments, cells were plated in wells of 96-well plates with an optical bottom in DMEM growth medium (Gibco, United States) and after 48 h cultivation, the required treatment was performed. As controls, similarly cultured cells were used without the treatment with the test compounds. All experiments were performed in triplicate; average values (means ± SEM) are presented.

To assess the effect of the compounds on tumor cell growth, the solutions of the studied substances in the medium obtained by 200X dilution of stock DMSO solutions were added to the wells. Evaluation of the results was performed after 48 h according to the confluence of the monolayer after washing with PBS using the Operetta CLS™ multi-parametric cell imaging system and Harmony 3.1 software (PerkinElmer, United States) in the brightfield mode. [9] The results are presented in fig. 3.

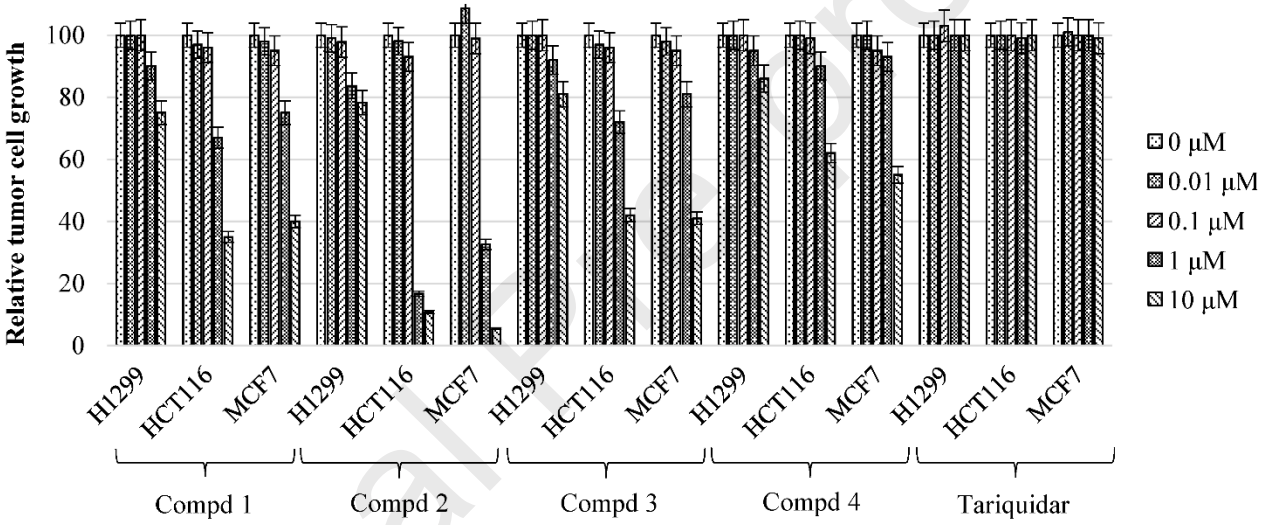

Fig. 3 – The effect of compounds on tumor cell growth.
Growth of corresponding cell line in the absence of substances was taken as 100%.

As expected, PPI inhibitors suppressed growth of p53+ cells, AMG232 showed the best results in terms of both the target effect and selectivity: the difference between the effects in p53+ and p53- cells was the largest. Tariquidar did not affect the cells of all the studied lines in the entire range of the considered concentrations up to 10 μM, which confirms the absence of its own therapeutic effect.

Along with the antitumor activity, we were interested in the ability of compounds to inhibit the transport activity in real time. To this end, we studied the effect of the compounds on the accumulation of Rhodamine 123, a classical P-glycoprotein substrate, in the cells. [17, 26] Tariquidar was used as a positive control. The cells plated in the wells of a 96-well plate were cultured to the confluency of 30%, then solutions of the substances and Rhodamine 123 were added (the concentration of Rhodamine in the solution was 1 μM). The uniform distribution of the cells in the wells was controlled using the Operetta CLS™ imager (PerkinElmer). After 40 min, the cells were washed and the fluorescence intensity of Rhodamine 123 (excitation and emission wavelengths of 488 nm and 515-575 nm, respectively) in different wells was compared using a ClarioStar plate reader (BMG Labtech).

It was found that in the case of HCT116 line under reproducible experimental conditions, the effect of tariquidar is already observed at 0.5 μM, and this concentration was further used to conduct comparative studies of the substances on all cell lines. The results are presented in fig. 4.

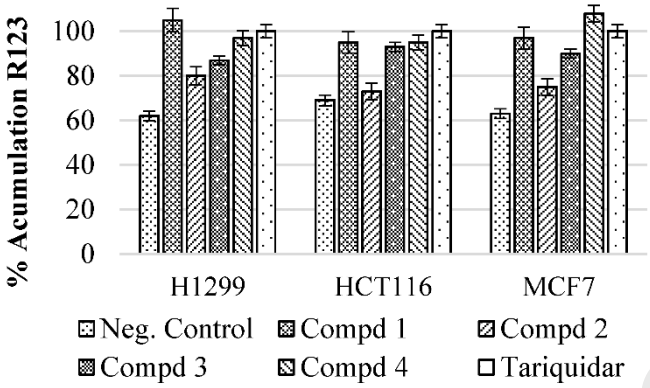

Fig. 4 – The effect of the compounds on Rhodamine 123 accumulation in cells. The fluorescence in corresponding cell lines when treated with 0.5 μM tariquidar was taken as 100%).

Nutlin-3a (compound **1**) has significant P-gp inhibitory activity in cells with negative p53 status; similarly, AMG232 (compound **2**) is the most active against H1299 cells. This fact can be explained only by the absence of specific transcriptional activity of p53. Cells with positive p53 status (HCT116, MCF7) show a significant structure – P-gp inhibitory activity relationship for Mdm2 inhibitors. Compound **2**, although it is an effective Mdm2 inhibitor, does not have a pronounced ability to inhibit P-gp, while Mdm2 inhibitors based on indolinone (compound **3**) and isoindolinone (compound **4**) are significantly more effective. It should be noted that compound **4**, which showed the inhibition efficacy at the level of the tariquidar, was obtained during optimization study of isoindolinone derivatives. [12, 27] We showed that the modification led to an increase in the lipophilicity of the compounds and, consequently, both membrane permeability and target binding. The data obtained indicate that in the case of P-glycoprotein, an increase in the lipophilicity made it an inhibitor of P-glycoprotein comparable with tariquidar.

Since the approach involves optimization of the proposed Mdm2 inhibitors, we were interested in whether computer simulation can be used at this stage. Earlier, we showed that in the case of p53-Mdm2 PPI, computer simulation can be applied quite successfully. [12, 19, 28, 29] To evaluate the adequacy for the computer prediction of the biological activity of compounds in the case of P-gp, we performed docking using CCDC GOLD Suite v5.2.2 software package. [30]

It is known that to release substances from the cell, P-gp changes its conformation from inward-facing to outward-facing. [31, 32] The X-ray structure of inward-facing P-glycoprotein, PDB ID: 4Q9H [33] was chosen for the analysis. A region that includes binding sites of known P-gp inhibitors and substrates was chosen as a site for docking. [33, 34] Bis-Benzimide (Huechst 33258) was analyzed as a substrate, and tariquidar and zosuquidar were used as inhibitors.

Analysis of the substrate binding (bis-Benzimide, fig. 5, red) and the binding of the known inhibitor (tariquidar, fig. 5, blue) with P-gp suggests that their key difference lies in the binding mode of the small molecule compound in the protein cavity. If we represent P-gp in the form of the letter V, then bis-Benzimide is compactly located between the alpha-helices of one branch of the letter V, while the tariquidar acts as a spacer between the branches, preventing conformational rearrangements required for the release of a substance (fig. 5).

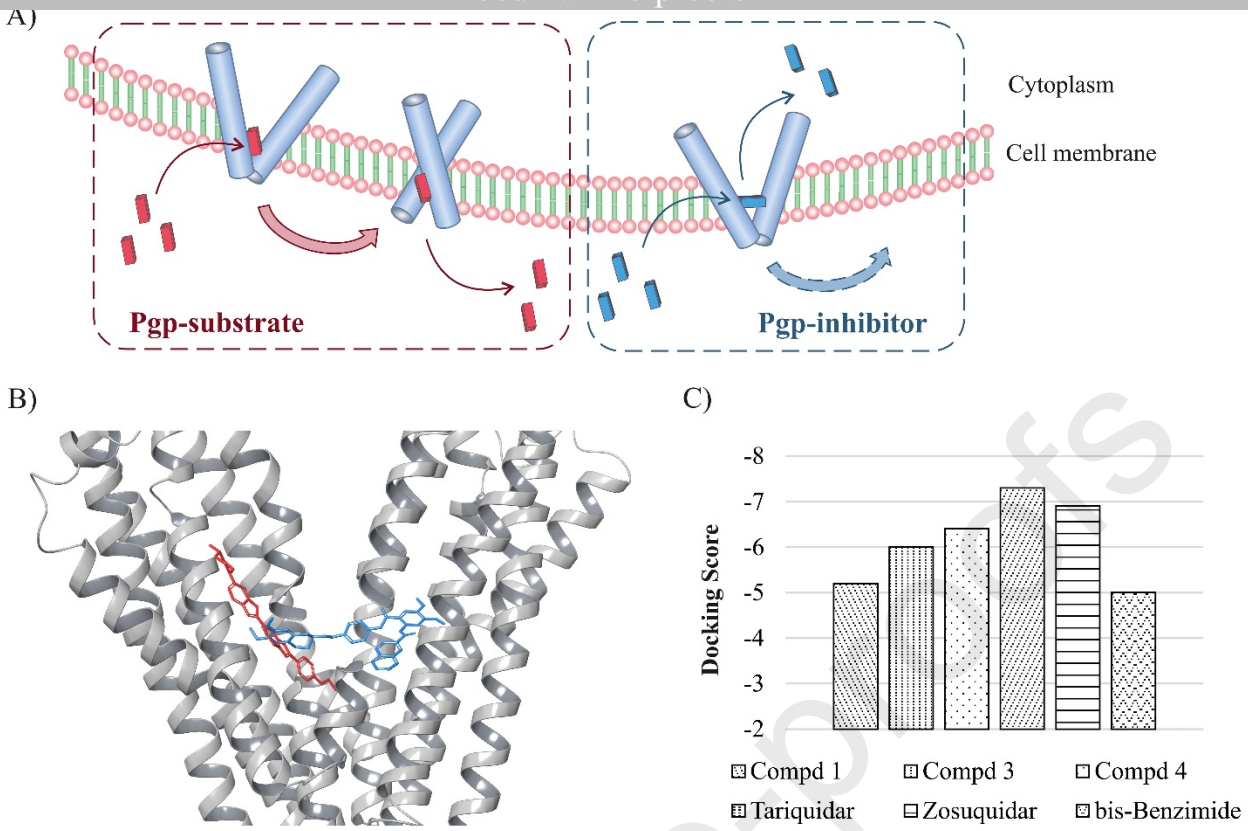

Fig. 5 – Simulation of the interaction of small molecules with P-gp.
Substrate (bis-Benzimide) is in red, inhibitor (tariquidar) is in blue.
A) Binding of the substrate and the inhibitor to P-gp is shown schematically,
B) Binding mode of compounds in the P-gp cavity according to the docking results,
C) Energy parameters of the interaction.

The energy parameters of the interaction indicate a stronger binding of the inhibitor compared with the substrate (fig. 5, C). In accordance with this assumption, Nutlin-3a (compound **1**) should rather act as a substrate. This result is in good agreement with the proposed hypothesis that Nutlin-3a reduces transport of other substrates due to competitive inhibition of P-gp. [13] Binding mode and molecular dynamics confirm that Nutlin-3a is not able to interfere with P-gp conformational transitions to a degree sufficient for inhibition. Compounds **3** and **4**, in turn, not only fit between the V branches, but also bind quit strongly to the target, which confirms their inhibitory ability with respect to P-gp. Binding modes of Nutlin-3a and compound **4** are represented in Supplementary.

The data obtained indicate that Mdm2 inhibitors are a promising platform for the development of multitarget drugs that can overcome tumor resistance by inhibiting the P-glycoprotein efflux transporter. The results obtained in cells that have the native balance of drug resistance and sensitivity showed the ability of a part of the cell population to both actively throw out xenobiotics and to lose this ability using P-gp inhibitors. From our point of view, this suggests the effectiveness of using either single target or multitarget P-gp inhibitors at the early stages of the development of hyperproliferative diseases to overcome primary resistance and prevent the development of secondary resistance.

Acknowledgments

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Computer modeling is suitable for search for P-gp inhibitors among Mdm2 inhibitors

P-gp interaction site and binding modes for substrates/inhibitors are identified

Mdm2 inhibitors are promising platform for multitarget drug development

Rhodamine 123 allows quantitative evaluating P-gp inhibitors on non-resistant cells