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The effect of dimeric bisbenzimidazoles on the activity of DNA repair enzymes TDP1, TDP2, PARP1 and PARP2

N.S. Dyrkheeva [1], I.A. Chernyshova [1], A.F. Arutyunyan [2], A.L. Zakharenko [1], M.M. Kutuzov [1], K.N. Naumenko [1], A.S. Venzel [3], V.A. Ivanisenko [3], S.M. Deyev [4], A.L. Zhuze [2], O.I. Lavrik [1]

[1] Institute of Chemical Biology and Fundamental Medicine of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
[2] The Engelhardt Institute of Molecular Biology of the Russian Academy of Sciences, Moscow, Russia
[3] Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
[4] Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Moscow, Russia

lavrik@1bio.ru

Abstract. Oncological diseases remain a leading cause of pathological mortality worldwide, making the development of anticancer drugs a critical focus in medicinal chemistry. A promising strategy to enhance therapeutic efficacy and reduce chemotherapy-induced toxicity involves the combined inhibition of DNA repair enzymes and topoisomerases. Of particular interest are minor-groove DNA ligands, which exhibit potent inhibition of DNA-dependent enzymes while having low toxicity and mutagenicity. A number of research groups, including ours, are developing inhibitors of DNA repair enzymes that act simultaneously on several targets: tyrosyl-DNA phosphodiesterase 1/2 (TDP1/TDP2), poly(ADP-ribose) polymerase 1 (PARP1)/TDP1, topoisomerase 1 (TOP1)/TDP1. Such bifunctional inhibitors are designed to resolve the problem of tumor cell resistance to known chemotherapy drugs and increase the effectiveness of the latter. In this study, we evaluated the inhibitory activity of 22 minor-groove DNA ligands – bis- and trisbenzimidazoles against four key repair enzymes: TDP1, TDP2, PARP1, and PARP2. Four series of dimeric compounds and their monomeric units were studied. The difference in inhibitory activity of dimeric bisbenzimidazoles depending on the structure of the compound and the enzyme is shown. Our findings reveal distinct structure-activity relationships, with monomeric and dimeric ligands exhibiting potent TDP1 inhibition at micromolar to submicromolar IC_{50} values (half-maximal inhibitory concentration). Notably, dimeric compounds from the $DB_2Py(n)$ and $DB_3P(n)$ series demonstrated superior TDP1 inhibition compared to their monomers. In contrast, all tested compounds showed negligible activity against the other three repair enzymes; so, the compounds demonstrate specificity to TDP1. It should be noted that in this work, in the experiments with TDP1 and TDP2, the effect of the tested compounds as narrow-groove ligands binding to DNA was excluded, and their direct effect on the enzyme was investigated. The results of molecular docking suggest the possibility of direct interaction of active compounds with the active center of TDP1. According to the results of modeling, the inhibitors are located in the binding region of the 3′-end of DNA in the active site of TDP1 and could form stable bonds with the catalytically significant TDP1 residues His263 and His493. These interactions probably provide the high inhibitory activity of the compounds observed in biochemical experiments.

Key words: tyrosyl-DNA phosphodiesterase 1 (TDP1); TDP1 inhibitor; inhibitory activity; TDP2; PARP1; PARP2; DNA-ligands; bisbenzimidazole derivatives

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Влияние димерных бисбензимидазолов на активность ферментов репарации ДНК тирозил-ДНК-фосфодиэстераз 1 и 2 и поли(АДФ-рибоза)полимераз 1 и 2

Н.С. Дырхеева [1], И.А. Чернышова [1], А.Ф. Арутюнян [2], А.Л. Захаренко [1], М.М. Кутузов [1], К.Н. Науменко [1], А.С. Вензель [3], В.А. Иванисенко [3], С.М. Деев [4], А.Л. Жузе [2], О.И. Лаврик [1]

[1] Институт химической биологии и фундаментальной медицины Сибирского отделения Российской академии наук, Новосибирск, Россия
[2] Институт молекулярной биологии им. В.А. Энгельгардта Российской академии наук, Москва, Россия
[3] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
[4] Институт биоорганической химии им. академиков М.М. Шемякина и Ю.А. Овчинникова Российской академии наук, Москва, Россия
lavrik@1bio.ru

Аннотация. Онкологические заболевания остаются одной из главных причин патологической смертности в мире, что определяет дизайн противораковых препаратов как ключевое направление медицинской химии. Комбинация ингибиторов ферментов репарации ДНК с ингибиторами топоизомераз – перспективный подход для усиления противоракового действия и снижения токсичности химиотерапии. Особый интерес представляют узкобороздочные ДНК-лиганды, способные эффективно ингибировать ДНК-зависимые ферменты, обладая при этом низкой токсичностью и мутагенностью. Ряд исследовательских групп, включая нашу, разрабатывает ингибиторы ферментов репарации ДНК, действующие одновременно на несколько взаимосвязанных мишеней {тирозил-ДНК-фосфодиэстеразы 1/2 (TDP1/TDP2), поли(АДФ-рибоза)полимераза 1 (PARP1)/TDP1, топоизомераза 1 (TOP1)/TDP1}. Такие бифункциональные ингибиторы призваны решить проблему резистентности опухолевых клеток к известным химиопрепаратам и повысить эффективность последних. В настоящем исследовании представлены данные скрининга ингибирующей активности 22 узкобороздочных лигандов, взаимодействующих с ДНК, – бис- и трисбензимидазолов – в отношении четырех ферментов репарации: TDP1, TDP2, PARP1 и PARP2. Изучены четыре серии димерных соединений и их мономерных единиц. Показана разница в ингибирующей активности димерных бисбензимидазолов в зависимости от структуры соединения и фермента. Мономерные и димерные бисбензимидазолы эффективно ингибируют активность TDP1 в микромолярном и субмикромолярном диапазоне IC_{50} (концентрация полумаксимального ингибирования). Димерные соединения групп $DB_2Py(n)$ и $DB_3P(n)$ проявили более значительную ингибирующую активность в отношении ферментативной реакции с участием TDP1 по сравнению с мономерами, входящими в их состав. Для всех исследованных соединений была показана низкая ингибирующая способность в отношении остальных трех ферментов репарации ДНК, т. е. наблюдается их специфическое воздействие именно на TDP1. Следует отметить, что в данной работе в экспериментах с TDP1 и TDP2 было исключено действие исследуемых соединений как узкобороздочных лигандов, связывающихся с ДНК, и исследовано их непосредственное воздействие на фермент. По результатам молекулярного докинга можно предположить возможность прямого взаимодействия изучаемых соединений с активным центром TDP1. Согласно результатам моделирования, ингибиторы располагаются в области связывания 3′-конца ДНК с активным центром TDP1 и могут образовывать устойчивые связи с каталитически значимыми остатками активного центра His263 и His493. Эти взаимодействия, вероятно, обеспечивают высокую ингибирующую активность соединений, наблюдаемую в биохимических экспериментах.

Ключевые слова: тирозил-ДНК фосфодиэстераза 1 (TDP1); ингибитор TDP1; ингибирующая активность; TDP2; PARP1; PARP2; ДНК-лиганды; производные бисбензимидазола

Introduction

Nowadays, DNA repair enzymes are actively studied by various researchers to understand the mechanisms of maintaining genetic stability and preventing the development of various diseases. Disruptions in DNA repair systems lead to the accumulation of modified bases, DNA breaks, and other damages, which increase the risk of developing oncological and other diseases. The study of DNA repair system functioning helps to identify the causes of hereditary diseases, neurodegenerative dysfunctions associated with repair defects, and develop new methods for the therapy and prevention of oncological diseases.

In recent years, considerable attention has been paid to DNA repair enzymes as targets for drug development. Researchers are actively searching for new compounds that suppress the activity of DNA repair enzymes to enhance the efficacy of anticancer therapy. Inhibition of enzymes involved in repair increases the effectiveness of antitumor therapy, as this leads to cancer cell death due to the accumulation of DNA damage caused by chemotherapy or radiation therapy. Currently, such repair enzymes as tyrosyl-DNA phosphodiesterases 1 and 2 (TDP1 and TDP2) and poly(ADP-ribose) polymerases 1 and 2 (PARP1 and PARP2) are considered promising targets for drug development (Pommier et al., 2014; Curtin, Szabo, 2020; Zakharenko et al., 2023).

TDP1 is a DNA repair enzyme that participates in the removal of covalent adducts of topoisomerase 1 (TOP1) from DNA, catalyzing the hydrolysis of the phosphodiester bond between the Tyr723 residue of TOP1 and the 3′-phosphate group in the single-strand DNA break generated by TOP1. TDP1 is also capable of removing other DNA-protein adducts located at the 3′-end of DNA and various other damage at the 3′-end of DNA (Comeaux, van Waardenburg, 2014; Kawale, Povirk, 2018). TDP2 catalyzes the hydrolysis of covalent adducts between DNA and the Tyr804 residue of the active center of topoisomerase 2 (TOP2) (Pommier et al., 2010). TDP2 removes covalent adducts from DNA located at the 5′-end of DNA through hydrolysis of the 5′-phosphodiester bond, resulting in the formation of DNA with a free 5′-phosphate (Pommier et al., 2014). TDP1 and TDP2 are capable of taking

over each other's functions to some extent, since TDP1 has low activity in the cleavage of 5′-phosphotyrosyl bonds, while TDP2 has low activity in the cleavage of 3′-phosphotyrosyl bonds (Zeng et al., 2012; Pommier et al., 2014).

Today, topoisomerase inhibitors are widely used in clinical practice as anticancer drugs. The most widely used topoisomerase inhibitors are topotecan and irinotecan, which suppress the activity of topoisomerase 1, as well as etoposide, targeting topoisomerase 2 (Pommier et al., 2010). Their mechanism of action consists in the formation of covalent adducts of topoisomerases with DNA, replication arrest, which ultimately leads to the suppression of cell proliferation. Various researchers have expressed the opinion (Pommier et al., 2014; Zakharenko et al., 2015) that the use of TDP1 and TDP2 inhibitors, which enhance the efficacy of topoisomerase inhibitors, may allow reduction of the dose of these rather toxic drugs and, consequently, the toxicity of therapy. Today, the search for TDP1 inhibitors is actively underway (Zakharenko et al., 2023; Zhang M. et al., 2025). As TDP1 inhibitors, derivatives of natural compounds such as usnic acid, berberines, coumarins, nucleosides, and steroids are particularly notable (Zakharenko et al., 2023), which are effective inhibitors of the purified TDP1 enzyme and topotecan sensitizers in experiments conducted on cellular and mouse cancer models (Zakharenko et al., 2023; Kornienko et al., 2024). Among TDP2 inhibitors, deazaflavins are worth noting, being among the most active inhibitors found to date for this enzyme (Marchand et al., 2016).

The enzymes PARP1 and PARP2 are key regulators of DNA repair and other cellular processes. These enzymes catalyze the DNA-dependent synthesis of the branched polymer poly(ADP-ribose) (PAR) and subsequent ADP-ribosylation of proteins. ADP-ribosylation of proteins is a post-translational modification that is induced in response to DNA damage. PARP1 participates in various DNA repair pathways (Ray Chaudhuri, Nussenzweig, 2017; Lavrik, 2020). PARP2 is also a DNA-dependent PARylation agent and can partially replace PARP1 (Lavrik, 2020; Szanto et al., 2024); therefore, the search for PARP1 and PARP2 inhibitors is an urgent task of modern medicinal chemistry. In clinical practice, such PARP1 and PARP2 inhibitors as olaparib, rucaparib, niraparib, veliparib, and talazoparib are currently approved for use in the treatment of ovarian, fallopian tube, breast, and peritoneal cancer (Kim D.-S. et al., 2021). The inhibitors used today work on the principle of synthetic lethality to destroy cancer cells with defects in the homologous recombination system (for example, with BRCA1/2 mutations), converting single-strand DNA breaks into double-strand breaks that cannot be effectively repaired, leading to cancer cell death. The active sites of PARP1 and PARP2 are very similar (Schreiber et al., 2006; Hoch, Polo, 2019); therefore, the currently known inhibitors most often act on both enzymes, as well as on other enzymes of the PARP family, due to the similarity of their active center that binds nicotinamide adenine dinucleotide (NAD^+) and initiates the synthesis of poly(ADP-ribose), therefore the search for selective inhibitors of each of these enzymes is actively conducted (Johannes et al., 2024). PARP inhibitors approved for clinical use are quite toxic and cause severe side effects, so the search for new inhibitors actively continues (Murai et al., 2014; Kim D.-S. et al., 2021; Johannes et al., 2024).

Small-molecule DNA-binding agents are an extremely promising class of compounds for the search of new inhibitors of repair enzymes. Of particular interest are minor-groove DNA ligands capable of inhibiting DNA-dependent enzymes, while not possessing high toxicity and mutagenicity, and being well soluble in water. Such DNA ligands have a low level of DNA geometry alteration and absence of covalent crosslink formation when forming a complex with DNA (Arutyunyan et al., 2023a).

Our research group has significant experience both in experimental investigation of potential inhibitors at the level of individual protein targets, cells, and animal models (Zakharenko et al., 2023), and in the application of molecular docking and modeling methods to study the mechanisms of interaction of small molecules with target proteins. Effective TDP1 inhibitors have been found that inhibit the recombinant TDP1 enzyme in the submicromolar concentration range. The lead compounds were topotecan sensitizers in experiments conducted on cell cultures and mouse tumor models (Zakharenko et al., 2023; Kornienko et al., 2024). We have developed and investigated inhibitors of PARP1, PARP2, and PARP3 based on conjugates of ADP and morpholino nucleosides using structural modeling of the active sites of these enzymes (Sherstyuk et al., 2019; Chernyshova et al., 2024).

This work presents screening data of twenty-two minor-groove ligands as inhibitors of TDP1, TDP2, PARP1, and PARP2. The studied compounds are bis- and trisbenzimidazole derivatives. Four monomeric compounds – MB_2, MB_2(Ac), MB_2Py(Ac), MB_3 – as well as four series of dimeric derivatives were investigated. The dimeric derivatives were obtained by condensation of monomeric subunits with dicarboxylic acids DB_2P(n), DB_2Py(n), and DB_3P(n), where (n) is the number of methylene units in the linker (Fig. 1).

It was shown that the activity of the compounds varies depending on their structure and the type of enzymatic target. The studied compounds exhibited pronounced inhibitory activity against TDP1, and the observed correlation indicates an increase in inhibitor activity upon introduction of additional binding blocks into its structure, such as a pyrrolecarboxamide fragment for the DB_2Py(n) series, or when using a combination of three benzimidazole blocks in the monomeric subunit. Despite the fact that extremely high IC_{50} values were observed for the DB_3(n) series, this phenomenon can be explained by the high propensity of members of this series of compounds to aggregation, since the introduction of a piperazine fragment into the linker in the DB_3P(n) series led to the obtaining of inhibitors with the lowest IC_{50} values, which indirectly confirms our assumption. In order to elucidate the possible mechanism of their inhibitory action for this enzyme, molecular docking was performed, the results of which suggest the presence of direct interaction between the active compounds and the TDP1 enzyme. According to the constructed binding model, the inhibitors are located in the region of the DNA-binding pocket of TDP1 and are capable of forming stable contacts with the catalytically important amino acid residues His263 and His493. The efficacy of these compounds as TDP1 inhibitors was confirmed by experimental data. The results of the work can be used for the rational design of new, even more effective TDP1 inhibitors.

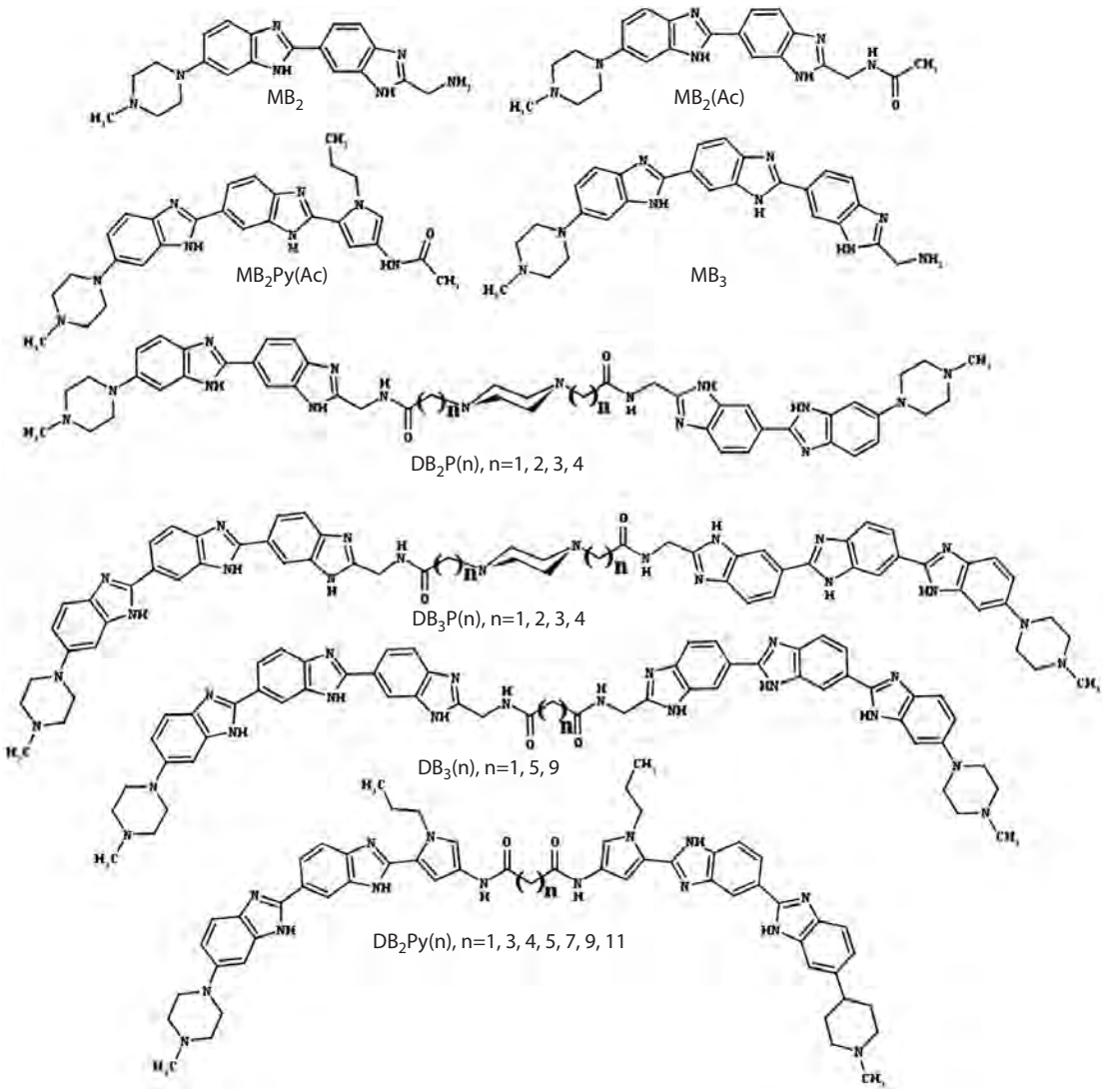

Fig. 1. Structures of bisbenzimidazole derivatives studied in this work.

Materials and methods

Materials and reagents. The studied compounds were synthesized at the Engelhardt Institute of Molecular Biology in the Laboratory of DNA-Protein Interactions according to previously developed methods (Ivanov et al., 2015; Arutyunyan et al., 2023a, b; Susova et al., 2024). The list of IUPAC names of the compounds is provided in the Supplementary Materials[1].

Recombinant human proteins tyrosyl-DNA phosphodiesterase 1 (TDP1) and tyrosyl-DNA phosphodiesterase 2 (TDP2) were expressed in the *E. coli* system, poly(ADP-ribose) polymerase 1 (PARP1) and poly(ADP-ribose) polymerase 2 (PARP2) were expressed in insect cells using a baculovirus expression system and purified as described in (Sukhanova et al., 2004; Sherstyuk et al., 2019; Dyrkheeva et al., 2020, 2021).

The oligonucleotide 5′-FAM-AAC GTC AGG GTC TTC C-BHQ1-3′ was synthesized at the Laboratory of Nucleic Acid Chemistry, Institute of Chemical Biology and Fundamental Medicine (Novosibirsk, Russia), according to (Zakharenko et al., 2015).

[1] Supplementary Tables S1, S2 and Figs S1–S4 are available at: https://vavilov.elpub.ru/jour/manager/files/Suppl_Dyrkheeva_Engl_29_7.pdf

Determination of TDP1 activity. The reaction mixture (200 μl) for real-time fluorescent detection of TDP1 enzyme activity (Zakharenko et al., 2015) contained TDP1 reaction buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 7 mM β-mercaptoethanol), 50 nM oligonucleotide 5′-FAM-AAC GTC AGG GTC TTC C-BHQ1-3′, the test compound at various concentrations, and TDP1 at a final concentration of 1.5 nM. The reaction mixtures were incubated at a constant temperature of 26 °C in a POLARstar OPTIMA microplate fluorometer (BMG LABTECH, GmbH, Ortenberg, Germany). Fluorescence intensity (Ex485/Em520 nm) was measured every minute for 10 min. Mean values of half-maximal inhibitory concentration (IC_{50} – the concentration of the compound that inhibited 50 % of enzyme activity compared to the untreated control well containing only enzyme and substrate) were determined using a dose-response curve of the fluorescence signal level versus inhibitor concentration and calculated using MARS Data Analysis 2.0 (BMG LABTECH). Kinetic curves were obtained in at least three independent experiments and statistically processed in OriginPro 8.6.0 (OriginLab, Northampton, Massachusetts, USA).

Determination of TDP2 activity. For determination of TDP2 enzyme activity, an oligonucleotide 5′-tyrosine-AAC GTC AGG GTC TTC C-FAM-3′ containing a 6-FAM label at the 3′-end and an L-tyrosine residue attached via the phenolic OH group to the 5′-terminal phosphate was used as substrate, synthesized at the Russian-French-Japanese Laboratory of Bionanotechnology of Novosibirsk State University as described in (Dyrkheeva et al., 2021). The substrate at a concentration of 100 nM was incubated with TDP2 at a concentration of 200 nM in the absence or presence of inhibitor (500 μM) for 10 min at 37 °C in buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 7 mM β-mercaptoethanol (Dyrkheeva et al., 2021). The reaction was stopped by addition of PAGE loading buffer (TBE, 10 % formamide, 7 M urea, 20 mM EDTA, 0.1 % xylene cyanol, and 0.1 % bromophenol blue). The samples were then heated at 90 °C for 5 min. The enzymatic reaction products were separated by electrophoresis in 20 % denaturing PAGE with 7 M urea at an acrylamide to bisacrylamide ratio of 19:1. A high-resolution Typhoon FLA 9500 laser scanner (GE Healthcare, Chicago, Illinois, USA) was used for gel scanning and visualization, and the data were analyzed using QuantityOne 4.6.7 software (Bio-Rad Laboratories, Inc., Hercules, California, USA). At least three independent experiments were performed, and statistical processing was carried out using OriginPro 8.6.0 (OriginLab, Northampton, Massachusetts, USA).

Determination of PARP1 and PARP2 activity. For determination of PARP1 and PARP2 enzyme activity in the presence and absence of test compounds, radiolabeled [^{32}P]-NAD$^+$ was synthesized from α-[^{32}P]-ATP according to the protocol (Sherstyuk et al., 2019). The auto-poly(ADP-ribosyl)ation reaction was performed in buffer for PARP1: 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 150 mM NaCl, and 7 mM β-mercaptoethanol, as well as 2 A_{260} units/ml activated DNA, 0.3 mM [^{32}P]-NAD$^+$ at 37 °C. The reaction was initiated by addition of PARP1 to 200 nM and carried out for 2 min.

The buffer for PARP2 contained: 50 mM Tris-HCl, pH 8.0, 3 mM spermine, 150 mM NaCl, and 7 mM β-mercaptoethanol, 2 A_{260} units/ml activated DNA, 0.6 mM [^{32}P]-NAD$^+$ at 37 °C. The reaction was initiated by addition of PARP2 to 600 nM, and the reaction mixtures were incubated for 5 min. The reaction was stopped by placing 5 μl aliquots on Whatman 1 paper filters impregnated with 5 % trichloroacetic acid (TCA). The filters were washed with 5 % TCA four times and air-dried after removal of TCA with 90 % ethanol. The incorporation of the radioactive label into the reaction product was calculated using a Typhoon FLA 9500 scanner (GE Healthcare, Chicago, Illinois, USA). At least three independent experiments were performed.

Molecular modeling. To evaluate the interaction of the studied compounds with the TDP1 enzyme, we performed molecular docking followed by analysis of the resulting complexes. The study included preparation of protein and ligand structures, molecular docking, energy minimization of compounds in the binding site, and assessment of inhibitor affinity using the Vinardo, X-Score, and REF2015 scoring functions.

The crystal structure of TDP1 (PDB ID: 8V0B) was used as the target protein structure. Missing loops in the model were reconstructed based on AlphaFold2 prediction (Jumper et al., 2021) performed in ColabFold (Mirdita et al., 2022) without using multiple sequence alignment (MSA) and using 8V0B as a template.

Hydrogen atoms were then added to the resulting model and charges were calculated using the DockPrep utility in UCSF Chimera (Pettersen et al., 2004). The inhibitor structures were prepared in OpenBabel (O'Boyle et al., 2011): hydrogens were added, partial charges were calculated, and geometry minimization was performed.

Molecular docking was performed using the UCSF DOCK 6.11 software package (Allen et al., 2015). Full-atom flexible docking over the entire protein surface was used. At the first stage of docking, the core fragments of the inhibitors (MB_2(Ac), MB_2Py(Ac)) were positioned, after which full-length molecules were docked with subsequent minimization of their energy in the binding site. Up to nine best conformations by GridScore were requested for each compound. From the nine conformations obtained for each ligand, the structure with the minimum RMSD relative to the optimal conformation of the core fragment was selected. In cases where DOCK6 returned fewer than nine unique conformations (due to clustering, energy filtering, or failure to generate additional conformers), selection was performed from all available conformations (Table S1).

Final assessment of the inhibitors' binding ability to the protein was performed using several independent scoring functions: ContinuousScore from DOCK 6, Vinardo (Quiroga, Villarreal, 2016), X-Score (Wang R. et al., 2002), and REF2015 in the PyRosetta4 environment (Chaudhury et al., 2010; Alford et al., 2017) according to the protocol of Moretti et al. (2016). ContinuousScore is a scoring function in DOCK 6 that accounts for van der Waals interactions, electrostatic interactions, internal ligand energy, and penalties for steric clashes through direct calculation of interatomic distances. Vinardo is a scoring function for docking that accounts for the contribution of hydrogen bonds, hydrophobic and van der Waals interactions, as well as corrections for non-optimal ligand positioning. The X-Score scoring function consists of three components: HPScore, HMScore, and HSScore, based on different empirical principles for assessing ligand-protein affinity. In this study, the averaged X-Score was used, reflecting the influence of hydrophobic, polar, and electrostatic contacts. The full-atom REF2015 scoring function implemented in PyRosetta includes contributions from van der Waals, electrostatic, hydrogen bonding, solvation, and additional atom pair interactions and allows correct ranking of inhibitor positions close in energy.

To validate the molecular docking results and assess the stability of the predicted complex over time, molecular dynamics simulation of the TDP1 complex with the lead compound DB_2Py(1), which had shown the best inhibitory activity, was performed. The simulation was carried out using the OpenMM 8 package (Eastman et al., 2017). A detailed protocol of the molecular dynamics simulation is presented in the Supplementary Materials.

Results

In this work, the ability of four series of small-molecule dimeric DNA ligands DB_2P(n), DB_2Py(n), DB_3(n), DB_3P(n) as well as their monomeric units MB_2, MB_2(Ac), MB_2Py(Ac),

and MB_3 (Fig. 1) to inhibit the activity of recombinant DNA repair enzymes TDP1 and TDP2, PARP1 and PARP2 was studied for the first time (see the Table).

The first group of studied compounds represents dimeric derivatives of the monomeric bisbenzimidazole ligand MB_2, a derivative of the widely studied minor-groove DNA ligand Hoechst 33258, in which the hydroxyphenyl group is replaced by a more hydrophilic aminomethylene fragment – $DB_2P(n)$. As a linker for compounds of this group, 1,4-piperazine-dialkyldicarboxylic acids containing a methylene, ethylene, propylene, or butylene spacer were used (Fig. 1). This series was also supplemented with the monomeric derivative $MB_2(Ac)$, acylated at the aminomethylene fragment, which structurally brings this compound, compared to MB_2, closer to half of the dimeric compound $DB_2P(n)$ and makes it a more appropriate reference for comparison. The $DB_2P(n)$ series differs from other ligand series by the presence of a positively charged 1,4-piperazine introduced into the linker, which improves ligand solubility and may increase ligand affinity for the enzyme.

The next group of compounds are derivatives of the monomeric trisbenzimidazole compound MB_3, which can be considered as a derivative of MB_2 containing one additional benzimidazole fragment, which increases the number of potentially possible hydrogen bonds in the inhibitor-TDP1 complex. Dimeric derivatives of MB_3 are represented by two series of compounds – $DB_3P(n)$, also dimerized with 1,4-piperazinedialkyldicarboxylic acids, and $DB_3(n)$, where n-alkyldicarboxylic acids are used as linkers. The $DB_3(n)$ and $DB_3P(n)$ series are characterized by the presence of trisbenzimidazoles in the structure, and $DB_3P(n)$, also by the presence of 1,4-piperazine in the linker.

The third group of compounds includes derivatives of the monomeric compound $MB_2Py(Ac)$, which is an isosteric analog of MB_3, due to the fact that the pyrrolecarboxamide fragment contained in its structure can act as a hydrogen

Inhibitory activity of test compounds against TDP1, TDP2, PARP1, and PARP2

No.	Compounds	IC_{50} TDP1, μM	TDP2	PARP1	PARP2
			% of residual activity (500 μM)		
1	MB_2	2 ± 1	~100	~100	~100
2	$MB_2(Ac)$	1.5 ± 0.5	~100	~100	~100
3	$DB_2P(1)$	6 ± 4	66 ± 7	57 ± 16	~100
4	$DB_2P(2)$	9 ± 3	44 ± 11	51 ± 15	80 ± 20
5	$DB_2P(3)$	4.1 ± 0.6	36 ± 7	37 ± 10	64 ± 16
6	$DB_2P(4)$	2.3 ± 0.3	44 ± 11	33 ± 13	85 ± 13
7	$MB_2Py(Ac)$	5 ± 2	~100	~100	~100
8	$DB_2Py(1)$	0.25 ± 0.05	55 ± 3	~100	~100
9	$DB_2Py(3)$	0.41 ± 0.09	70 ± 11	~100	~100
10	$DB_2Py(4)$	0.4 ± 0.15	~100	~100	~100
11	$DB_2Py(5)$	0.35 ± 0.13	~100	~100	~100
12	$DB_2Py(7)$	0.28 ± 0.01	~100	~100	~100
13	$DB_2Py(9)$	0.30 ± 0.08	~100	~100	~100
14	$DB_2Py(11)$	0.9 ± 0.1	~100	~100	~100
15	MB_3	0.70 ± 0.05	~100	65 ± 15	~100
16	$DB_3(1)$	>50	70 ± 6	55 ± 13	~100
17	$DB_3(5)$	>50	65 ± 10	62 ± 16	~100
18	$DB_3(9)$	>50	~100	~100	~100
19	$DB_3P(1)$	0.10 ± 0.05	~100	70 ± 12	~100
20	$DB_3P(2)$	0.11 ± 0.01	~100	40 ± 5	~100
21	$DB_3P(3)$	0.20 ± 0.05	~100	47 ± 14	~100
22	$DB_3P(4)$	0.15 ± 0.03	~100	48 ± 15	~100

Note. For IC_{50} values and percentage of residual enzyme activity in the presence of inhibitor, the Table shows mean values ± standard deviation (at least three replicates).

atom donor at the carboxamide nitrogen for hydrogen bond formation, in a position analogous to benzimidazole. Dimeric derivatives are represented by the $DB_2Py(n)$ series containing n-alkyldicarboxylic acids as a linker. This series is represented by a set of compounds containing 1, 3, 4, 5, 7, 9, and 11 methylene units, which allowed for a more accurate assessment of the dependence of the inhibitory activity of compounds on spacer length. The $DB_2Py(n)$ series differs from the $DB_3(n)$ series by the presence, in addition to the bisbenzimidazole structure, of a pyrrolecarboxamide structure, which is a fragment of the AT-specific antibiotic netropsin.

Using the real-time fluorescence analysis method, half-maximal inhibitory concentration (IC_{50}) values of the studied compounds (see the Table) were obtained in the reaction of BHQ1 cleavage from the 3′-end of the oligonucleotide by TDP1, which led to an increase in FAM fluorescence at the 5′-end of the chain (Zakharenko et al., 2015). It should also be noted that a single-stranded oligonucleotide was used as substrate to exclude the binding of dimeric bisbenzimidazoles as minor-groove ligands to the DNA substrate and direct their action toward the enzymatic target.

From the data obtained for the monomeric compounds MB_2 and $MB_2(Ac)$ and their dimeric derivatives $DB_2P(n)$, at n = 1, 2, 3, 4, the IC_{50} values were in the micromolar range, and dimerization did not lead to an increase in the inhibitory activity of the studied compounds. At the same time, for dimers of the monomeric $MB_2Py(Ac)$, which has an IC_{50} value of 5 ± 2 μM, the half-inhibitory concentration parameter value decreased significantly, ranging from 0.25 to 0.90 μM. Similarly, the transition from monomeric MB_3 to the dimeric $DB_3P(n)$ series led to an increase in the inhibitory activity of the compounds, although not as pronounced; however, dimeric derivatives of MB_3 that do not contain a piperazine fragment in the linker – $DB_3(n)$ compounds – showed the lowest level of activity among all the inhibitors tested in this work. The fact that the IC_{50} values for these compounds (see the Table) deviate so strongly from the overall data set is most likely due to the fact that $DB_3(n)$ compounds possess an extended and planar geometry, as well as a rigid linker, which prevents optimal positioning of compounds of this type in the enzyme active site (Fig. 1).

Thus, according to the experimental data, all compounds studied in this work, except for the $DB_3(n)$ group, effectively inhibit TDP1 activity at micromolar and submicromolar concentrations. A structure-activity correlation is observed, consisting of a decrease in concentration to achieve the half-maximal inhibition effect with an increase in the number of blocks containing hydrogen bond donors in the compound. In particular, dimerization is one of the simple approaches to increasing such structures in one molecule, which leads to a nonlinear increase in the binding constant (Neudachina, Lakiza, 2014). A decrease in IC_{50} is also observed upon introduction of a piperazine fragment into the linker structure, which may be due to an increase in the hydrophilicity of the molecules. The results obtained allowed us to establish a structure-activity correlation, as well as to assess the contribution of dimerization to the increase of the inhibitory capacity of the studied compounds.

To study the effect of the studied compounds on TDP2 activity, we tested the ability of this enzyme to remove the tyrosine residue from the 5′-end of the oligonucleotide substrate in the absence and presence of inhibitors, as described in (Dyrkheeva et al., 2021). All compounds of the $DB_2P(n)$ group, as well as $DB_2Py(n)$, at n = 1, 3 and $DB_3(n)$, at n = 1, 5 at a concentration of 500 μM inhibited enzyme activity by approximately 50 %, while all other compounds showed no inhibitory activity (see the Table). Thus, all tested compounds showed a significantly lower propensity to inhibit TDP2 compared to TDP1. Interestingly, the $DB_2P(n)$ group inhibited TDP1 less effectively and TDP2 more effectively than compounds of other groups.

The next step of our work was to test the ability of the studied compounds to inhibit PARP1 and PARP2, that is, their enzymatic activity in the poly(ADP-ribose) (PAR) synthesis reaction, at a rather high concentration range of compounds. All studied compounds showed low efficiency in inhibiting these two enzymes. The most active compounds were those of the $DB_2P(n)$ group, representatives of which with n = 2, 3, 4 reduced the activity of PARP1 and PARP2 at a concentration of 500 μM. Inhibitory action was also observed for compounds of the $DB_3(n)$ and $DB_3P(n)$ series at a concentration of 500 μM, while these compounds exhibited inhibitory activity only in the PAR synthesis reaction catalyzed by PARP1, but not PARP2 (see the Table).

Since, according to the experimental data, all studied compounds, with the exception of the $DB_3(n)$ group, effectively inhibit TDP1 activity, we further performed an *in silico* evaluation of the ability of compounds of the $DB_2P(n)$ and $DB_2Py(n)$ groups to bind to the TDP1 enzyme in order to elucidate the possible molecular mechanism of their inhibitory action. For this purpose, full-atom flexible molecular docking over the entire surface of the TDP1 protein (PDB ID: 8V0B) was performed for $DB_2P(n)$ and $DB_2Py(n)$ compounds.

According to the docking results obtained, it can be assumed that conformations with minimum calculated energy for each inhibitor form interactions in the TDP1 active site, near His263 and His493 residues (Fig. 2*a*), similarly to compound $MB_2(Ac)$ (Fig. S1). An additional analysis of the binding ability of dimeric compounds to TDP1 was performed using the Vinardo, X-Score, and REF2015 scoring functions in the PyRosetta environment (Table S2). The obtained scoring function values suggest high affinity of the studied inhibitors of the $DB_2P(n)$ and $DB_2Py(n)$ groups for TDP1. It should be noted that complete correlation of the parameters obtained by docking (Table S2) with the IC_{50} values found experimentally (see the Table) is not observed, which can be explained by the contribution of hydrophobic linkers, which are difficult to account for in energy calculations.

According to molecular modeling data, compound $MB_2(Ac)$ (Fig. 2*b*), which is the monomeric unit for dimeric derivatives $DB_2P(n)$, may form a hydrogen bond with His263 and a π-cation interaction with His493, which could potentially lead to blocking of the TDP1 catalytic act. In addition to interactions with catalytically active residues, $MB_2(Ac)$ may form hydrophobic contacts with Tyr204 and Ala520, as well as a hydrogen bond with Phe259, which could enhance the inhibitory action of this compound. In contrast to $MB_2(Ac)$, compound $MB_2Py(Ac)$ (Fig. 2*c*) appears to interact with only one catalytic residue – His493 – through hydrogen bond formation. Such a difference in interactions could be the reason for the higher inhibitory activity of $MB_2(Ac)$ compared to

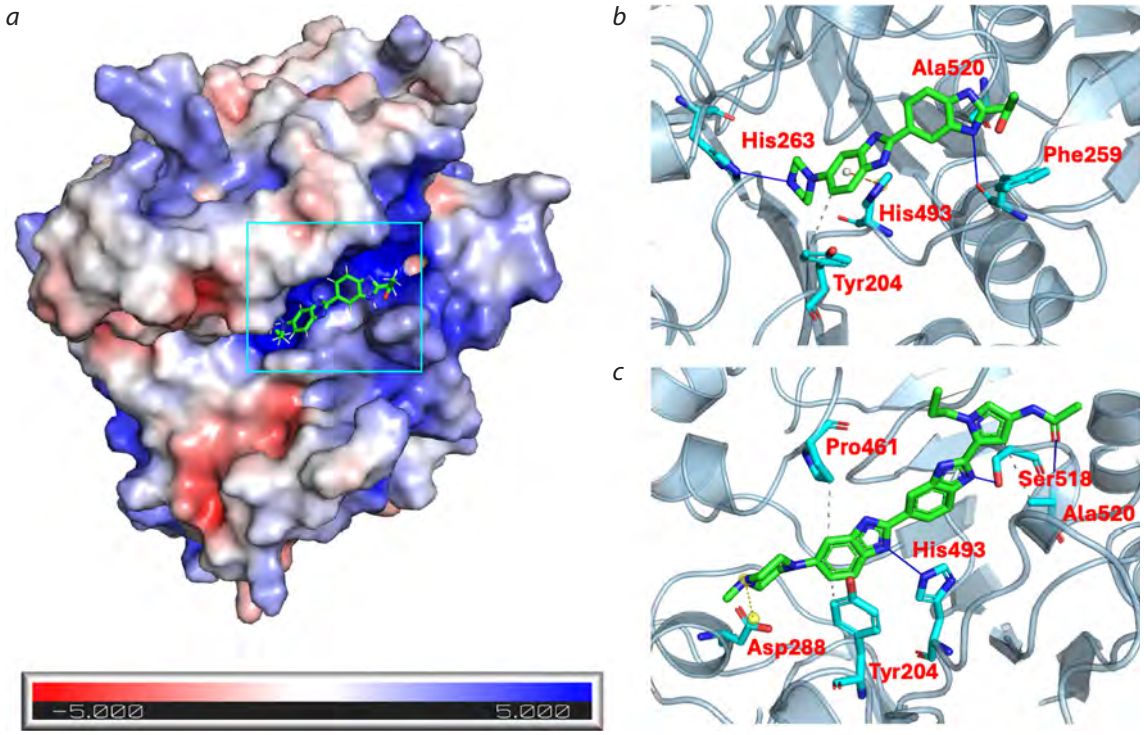

Fig. 2. *a*, Structure of TDP1 (PDB ID: 8V0B) with inhibitor MB_2(Ac) located in the positively charged region of the TDP1 active site. The protein surface is colored according to the electrostatic potential distribution calculated using APBS (Jurrus et al., 2018). The DNA-binding region of TDP1 is highlighted by a rectangular frame. Below is a scale of TDP1 surface electrostatic potential values (in units of kT/e, where kT/e ≈ 25.7 mV at 298 K). Color scale: red indicates negative potential (–5 kT/e), white indicates neutral (0 kT/e), blue indicates positive potential (+5 kT/e). *b*, *c*, Predicted conformations of inhibitors MB_2(Ac) and MB_2Py(Ac) (green) in complex with TDP1 with contacting residues (cyan).

MB_2Py(Ac), which is consistent with experimental data (see the Table).

Analysis of interactions using PLIP (Protein–Ligand Interaction Profiler) (Salentin et al., 2015) for predicted TDP1 complexes with dimeric compounds of the DB_2Py(n) group (Fig. S2) showed that these compounds form a greater number of protein-ligand contacts (hydrogen bonds and hydrophobic interactions) compared to the MB_2Py(Ac) monomer. In particular, compound DB_2Py(1) forms hydrogen bonds with Ser400 and Ser403, as well as hydrophobic interactions with Pro463 – the residues of these amino acids are located in the ligand binding site with the TDP1 active center, which likely contributes to stabilization of the interacting dimer fragment in the enzyme active site. The data obtained from docking analysis, characterizing the larger contact surface area of dimeric DB_2Py(n) compounds with TDP1 compared to the MB_2Py(Ac) monomer, correlate with the decrease in IC_{50} values for dimers, which indicates an increase in the affinity of these compounds for the enzyme active site (see the Table). According to the data obtained, hydrophobic interactions with Pro461 and/or Tyr204 residues localized in the TDP1 active site may also contribute to increasing the inhibitory activity of DB_2Py(n) group compounds.

Analysis of interactions of compounds from the DB_2P(n) group with TDP1 showed that analogous amino acid residues participate in complex formation, with the exception of Tyr204, with which DB_2P(n) compounds, unlike DB_2Py(n), apparently do not interact (Fig. S3). In addition, possible differences in the nature of interactions with the same amino acids were noted. For example, for the Lys519 residue in the case of DB_2P(n) compounds, formation of hydrogen bonds with nitrogen atoms of the piperazine fragment through the N1 atom of the side chain can be assumed. At the same time, two types of interactions with Lys519 are predicted in DB_2Py(n) compounds: a hydrogen bond between the backbone nitrogen atom of Lys519 and the oxygen atom in the pyrrolecarboxamide group (in DB_2Py(1), DB_2Py(4), DB_2Py(7), DB_2Py(9)), as well as a π-cation interaction between pyrrole and the Lys519 side chain (in DB_2Py(3) and DB_2Py(5)) (Fig. S2).

For compound DB_2Py(1), which demonstrated the highest inhibitory activity (lowest IC_{50} value) among the studied derivatives, additional molecular dynamics modeling in the predicted complex with TDP1 was performed. Analysis of the MD trajectory showed that the TDP1-DB_2Py(1) complex maintains stability throughout the simulation time. RMSD values of the ligand were in the range of 1.5–3.0 Å (Fig. S4), which indicates stable binding of DB_2Py(1) in the protein active site without signs of dissociation or significant conformational rearrangements. The data obtained confirm the strength of the formed complex and are consistent with the high biological activity of this compound.

It should be noted that our analysis of molecular contacts, as well as the scoring function values obtained according to molecular docking results, indicate the ability of compounds of both analyzed groups – DB_2P(n) with an aliphatic linker and DB_2Py(n) with a piperazine fragment in the linker – to

form a stable complex with TDP1. Nevertheless, experimental data show differences in their inhibitory activity: compounds with an aliphatic linker demonstrate higher inhibition efficiency compared to compounds containing a piperazine ring. This difference cannot be fully explained based on contact analysis, which suggests a possible difference in the conformational mobility of these groups of compounds. In particular, the inclusion of a piperazine fragment in the central part of the linker apparently restricts its flexibility, which affects the dynamics of inhibitor interaction with the active site, prevents optimal positioning of the inhibitor in the enzyme active site and, consequently, reduces its inhibitory activity.

Discussion

TDP1 plays a key role in eliminating DNA damage located at the 3′-end of DNA, stabilized by anticancer drugs used in clinical practice, such as topotecan and irinotecan, which are derivatives of the natural compound camptothecin (Comeaux, van Waardenburg, 2014; Kawale, Povirk, 2018). Consequently, TDP1 activity may be a possible cause of tumor resistance to TOP1 inhibitors used in the clinic. Currently, searches for combined TOP1 and TDP1 inhibitors are actively underway (Conda-Sheridan et al., 2013; Nguyen et al., 2015; Zhang X.-R. et al., 2018; Hu et al., 2021;Yang et al., 2023).

Furthermore, since it is known that the activities of TDP1 and TDP2 overlap, albeit to a minor extent (Pommier et al., 2014), the ability of these enzymes to perform each other's functions makes the combined use of inhibitors of these two enzymes or the creation of agents capable of simultaneously inhibiting both TDP1 and TDP2 quite promising. Simultaneous suppression of the activity of these two enzymes can be used to enhance the efficacy of a large set of clinically important anticancer drugs, TOP1 and TOP2 inhibitors. Triple TOP1/TDP1/TDP2 inhibitors have also been discovered, which exhibit moderate activity against TDP1 and weak activity against TDP2 (Wang P. et al., 2017). The most effective TDP2 inhibitors to date are deazaflavins, which exhibit synergy with etoposide *in vitro* at non-toxic concentrations (Marchand et al., 2016), and some effective TDP2 inhibitors from other compound classes have also been found (Yang et al., 2021; Zhang Y. et al., 2021).

It is known that the N-terminal domain of TDP1 directly binds to the C-terminal domain of PARP1, and TDP1 undergoes PARylation by PARP1 in order to be recruited to the TOP1-DNA adduct (Das et al., 2014; Lebedeva et al., 2015). PARylation of TDP1 stimulates its recruitment to sites with damaged DNA without affecting the catalytic activity of this enzyme (Chowdhuri, Das, 2021). It has also been shown that PARP1 can interact with TDP1, forming protein-protein contacts (Moor et al., 2015). It was established that the combination of TDP1 knockdown and inhibition of PARP1 activity with rucaparib reduces cell proliferation more significantly than these methods of enzyme function suppression separately (Fam et al., 2013). Therefore, there is a suggestion in the literature that the anticancer effect of TOP1 inhibitors can be significantly enhanced by simultaneous inhibition of PARP1 and TDP1 (Smith et al., 2005; Alagoz et al., 2014; Das et al., 2014; Murai et al., 2014; Elsayed et al., 2016; Matsuno et al., 2018; Jing et al., 2020; Kim J.W. et al., 2020; Chowdhuri, Das, 2021; Flörkemeier et al., 2022). The interaction between PARP1 and TDP1 enzymes has been demonstrated in a number of publications (Das et al., 2014; Moor et al., 2015), which makes the search for dual TDP1 and PARP1 inhibitors relevant.

Previously, we discovered dual TDP1 and TDP2 inhibitors, as well as triple TDP1, TDP2, and PARP1 inhibitors (Dyrkheeva et al., 2021) – usnic acid thioethers that weakly inhibit TDP2 and PARP1; therefore, the search for new compounds capable of acting on two or three functionally interacting targets simultaneously is relevant. In this work, the ability of a series of minor-groove DNA ligands to inhibit TDP1, TDP2, PARP1, and PARP2 enzymes was tested. Effective inhibitors acting on all four enzymes simultaneously were not found, but it was shown that these compounds inhibit TDP1. The DNA ligands studied in this work are capable of inhibiting DNA-dependent enzymes through binding to double-stranded DNA. However, in the present work we showed that they are capable of selectively inhibiting TDP1, since the experiments were conducted in the absence of double-stranded DNA as an alternative target.

The results of molecular docking and analysis of intermolecular interactions suggest that most of the studied compounds of the $DB_2P(n)$ and $DB_2Py(n)$ groups may possess high affinity for the TDP1 enzyme and form stable complexes with its catalytic center. Interactions with key catalytic residues of the TDP1 protein active site were predicted for all compounds.

Conclusion

In this work, a study of the effect of dimeric bis- & tris-benzimidazoles on the activity of DNA repair enzymes – TDP1, TDP2, PARP1, and PARP2 – was conducted. The main results showed that all studied inhibitors, except compounds of the $DB_3(n)$ series, effectively inhibit TDP1. The most active were compounds $DB_2Py(n)$ and $DB_3P(n)$, capable of inhibiting TDP1 in the submicromolar concentration range. The studied compounds demonstrate high selectivity, with minimal effect on the activity of other tested enzymes.

Based on the results of molecular docking, it is proposed that the studied active inhibitors are localized in the region of the DNA-binding pocket of TDP1 and may form stable interactions with the catalytically important residues His263 and His493. These interactions likely underlie the observed high inhibitory activity.

An important result is also the establishment of the structure-activity relationship. Dimerization had a mixed effect on the inhibitory effect: compounds of the $DB_2Py(n)$ and $DB_3P(n)$ series were significantly (by an order of magnitude) more active than the corresponding monomers; in the $DB_2P(n)$ series, the inhibitory activity was influenced not only by dimerization, but also by linker length and the introduction of 1,4-piperazine bearing two positive charges into the linker. The $DB_3(n)$ series was inactive, unlike the monomer. Introduction of the piperazine fragment into the linker in the $DB_3P(n)$ series led to pronounced inhibitory activity compared to $DB_3(n)$ without such a fragment. We propose that the enhancement of the inhibitory effect is related to the introduction of two positive charges into the linker and to the increase in the number of possible contacts of ligands with the enzyme active site.

Overall, based on the results of this work, new strategies for the development of cancer therapy may be proposed. The

obtained data also highlight the potential of dimeric bis- & tris-benzimidazoles as safe and effective tools for targeted regulation of DNA repair enzymes.

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