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Mannich Bases of 1,2,4-Triazole-3-thione Containing Adamantane Moiety: Synthesis, Preliminary Anticancer Evaluation, and Molecular Modeling Studies

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Abstract

A series of 18 novel *N*-Mannich bases derived from 5-adamantyl-1,2,4-triazole-3-thione was synthesized and characterized using NMR spectroscopy and X-ray diffraction technique. All derivatives were evaluated for their anticancer potential against four human cancer cell lines. Several tested compounds exerted good cytotoxic activities on K562 and HL-60 cell lines, along with pronounced selectivity, showing lower cytotoxicity against normal fibroblasts MRC-5 compared to cancer cells. The effects of compounds **5b**, **5e** and **5j** on the cell cycle were investigated by flow cytometric analysis. It was found that these compounds cause the accumulation of cells in the subG1 and G1 phases of the cell cycle and induce caspase-dependent apoptosis, while the anti-angiogenic effects of **5b**, **5e** and **5j** have been

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confirmed in EA.hy926 cells using a tube formation assay. Further, the interaction of Bax protein with compound **5b** was investigated by means of molecular modeling, applying the combined molecular docking/molecular dynamics approach.

Keywords**:** triazoles, Mannich bases, adamantane, anticancer activity, molecular modeling

Introduction

The chemistry of 1,2,4-triazoles and their fused heterocyclic derivatives has received considerable attention owing to their synthetic and biological importance. Among them, thione-substituted 1,2,4-triazole ring systems have been well studied due to a wide variety of their biological activities, such as antibacterial,[1] antifungal,[2] anticancer,[3] anti-inflammatory,[4] and hypoglycemic[5] properties. Additionally, 1,2,4-triazoles are important precursors for other heterocyclic compounds, such as Mannich bases,[6] thioureas,[7] Schiff bases,[8] triazolothiadiazoles, triazolothiadiazines[9] etc. Some derivatives of 4,5-substituted-1,2,4-triazole-5-thiones were synthesized and found to be good cytotoxic agents against thymocytes.[10] Anticancer screening studies for a series of Mannich bases of 3-substituted 4-(5-nitro-2-furfurylidene)amino-1,2,4-triazole-5-thiones were performed against a panel of 60 cell lines showing the GI_{50} values in the range of 10-100 μM.[3]

The adamantyl group is known to positively regulate the therapeutic index of many compounds, and seven of its derivatives found use in current clinical practice, in treatment of viral infections, neurodegenerative disorders, acne vulgaris and type 2 diabetes mellitus.[11] RARγ-selective adamantyl-based compound induces G0/G1 cell cycle arrest and apoptosis in human breast carcinoma and human leukemia cell lines in a p53-independent fashion.[12] It is also found to be a potent growth inhibitor of cell lung cancer (NSCLC) lines with IC_{50} values in the range of <0.13 – 0.53 μM.[13]

The scientific literature regarding anticancer activity of compounds which contain both adamantoyl and 1,2,4-triazole moieties is very scarce. One of such compounds, 4-(1-adamantyl)-5-[2-(3-hydroxynapthyl)]-2*H*-1,2,4-triazole-3(4*H*)-thione was evaluated for its *in vitro* anticancer activity against MCF-7 breast cancer cell line giving IC_{50} value of 8.15 μM.[14]

Most common chemotherapeutic drugs, doxorubicin, cisplatin and 5-fluorouracil, used for treating a wide range of cancers, induce reduced cancer cell division and growth. Chemotherapy does not distinguish between a cancer and normal cells and destroys not only the fast-growing cancer cells but also healthy cells in the body. Therefore, it is of most importance to design and perform biological screening of new pharmacophores, which could potentially enhance the selectivity of cytotoxic agents against cancer cell lines and decrease their toxic effects towards normal cells. Numerous newly synthesized compounds which possess excellent cytotoxic activity, display very low cancer selectivity. Having in mind these facts, in this paper we examined cytotoxicity, cancer selectivity and plausible mechanism of action of 18 novel *N*-Mannich bases of 5-adamantyl-1,2,4-triazole-3-thione, which displayed a pronounced selectivity showing IC_{50} values for MRC-5 significantly higher in comparison to cancer cell lines. In addition, molecular action mode of these compounds was investigated through western blot analysis of key proteins involved in apoptosis, anti-angiogenic activity and molecular docking studies.

Methods and Materials

Physical measurements and methods

Melting points were determined on a Mel-Temp capillary melting points apparatus, model 1001 and are uncorrected. Elemental (C, H, N, S) analysis of the samples was carried out in the Center for Instrumental Analysis, Faculty of Chemistry, Belgrade. IR spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer with a KBr disc. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 200 MHz spectrometer. Crystal structures were determined by single-crystal X-ray crystallography. Diffraction measurements were performed on an Oxford Diffraction Gemini S diffractometer. Structures were solved by using the *SHELXT*,[15] and refined with the *SHELXL*.[16] Details of preparation and structure determinations are given in the Supporting information.

In vitro cytotoxicity assay

Human cervical adenocarcinoma HeLa, human chronic myelogenous leukemia K562, human acute promyelocytic leukemia HL-60, human lung carcinoma A549 and normal human lung fibroblasts MRC-5 were grown in RPMI 1640 medium (Sigma Aldrich). Nutrient medium was supplemented with 10% fetal bovine serum, L-glutamine, and penicillin-streptomycin (Sigma Aldrich). Medium for HL-60 cells was supplemented with 20% fetal bovine serum. HeLa (2000 cells per well), K562, A549 and MRC-5 (5000 cells per well) and HL-60 (7000 cells per well), were seeded in 96-well plates. The tested compounds were added to the cells 20 h later (for adherent cell lines) or 2 h later (for cell lines that grow in a suspension). The nutrient medium was added to control cells. Final concentrations of compounds were 6.25 μM, 12.5 μM, 25 μM, 50 μM and 100 μM. Cisplatin was used as a positive control. After 72 h treatment, the survival of cells was measured by MTT test, as described earlier.[17,18] More details of determination of cell survival are given in the Supporting information.

Cell cycle analysis

Chronic myelogenous leukemia K562 cells were seeded into a 6-well plates ($2.5x10^5$ cells/well), and after 2 h treated with compounds, with the exception of control cells. Tested concentrations corresponded to IC_{50} and $2IC_{50}$ values. After 24 h treatment, the cells were collected, and fixed as described elsewhere.[19] Cell cycle distribution was analyzed after incubation of cells with RNase and propidium iodide. The statistical significance of differences in percentages of cells at specific cell cycle phases between control samples and samples exposed to tested compounds was evaluated using Repeated Measures ANOVA with Tukey's multiple comparison test. The p-values less than 0.05 were regarded as statistically significant.

Morphological analysis of cell death mode

K562 cells were seeded into 6-well plates in 2 mL of complete nutrient medium ($2x10^5$ cells/well). After 2 h, the cells were treated with $2IC_{50}$ concentrations of the investigated compounds. After 24 h treatment, the cells were collected by centrifugation and stained with acridine orange/ethidium bromide mixture (3 μg/ml AO and 10 μg/ml EB in phosphate-buffered saline), and visualized under a fluorescence microscope (Carl Zeiss PALM MicroBeam with Axio Observer.Z1 using AxioCam MRm).

Western blot analysis

Whole cell lysates of K562 cells (2×10^6) treated with compounds **5b**, **5e** and **5j** (IC_{50} respectively) for 24h were prepared by homogenization of harvested cells in buffer containing 0.1% Triton X-100 (Sigma Aldrich) and triple protease inhibitor (Roche Applied Science). Following sonication (3x10 s) using UP 50H Ultraschallsrozessor (Hielscher GmbH) and centrifugation (30 min/20 000xg) of lysates, protein concentration of the supernatant was determined by BCA assay (Thermo Scientific Pierce). Samples were separated on an SDS-PAGE and transferred to a PVDF membrane as described previously.[20] To block non-specific binding, the membranes were incubated for 1h at room temperature (RT) in 5% non-fat dry milk/TBST (150 mM NaCl, 50 mM Tris, pH 7.4, and 0.05% Tween20). The following antibodies were used for overnight incubation at 4 °C in 2% blocking solution: rabbit anti-caspase 3 (Santa Cruz Biotechnology), rabbit anti-cleaved caspase 3 (Cell Signaling), rabbit anti-caspase 8 (Santa Cruz Biotechnology), mouse anti-caspase 9 (Santa Cruz Biotechnology) and mouse anti-Bax (1:300, Santa Cruz Biotechnology). After several rinses in TBST, the membranes were incubated for 1h in the appropriate Horse Radish Peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology). HRP-immunoreactive bands were visualized using enhanced chemiluminescence (ECL, GE Healthcare) and film exposure (Kodak Biomax). Each blot was subsequently re-probed with goat anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibody (Santa Cruz Biotechnology), as a control for protein load.

Tube formation assay

To examine the *in vitro* antiangiogenic activity of selected compounds **5b**, **5e** and **5j** on human umbilical vein endothelial EA.hy926 cells, the tube formation assay was performed. Briefly, 24-well plates were coated with Corning® Matrigel® Basement Membrane Matrix, (Corning, 356234). EA.hy926 cells were seeded onto the coated wells ($4x10^4$ cells per well), with or without the investigated compounds. Concentrations of the compounds **5b**, **5e** and **5j** corresponded to IC_{20} values (15 μM) previously determined for this cell line for 24h treatment. After 24 h of incubation, the cells were photographed under the inverted microscope.

Preparation of enzymes crystal structures

Molecular docking, homology modeling and molecular dynamics protocols were conducted using experimentally available Bax crystal structure (PDB ID: **1F16**),[21] Bax crystal structure in complex with $Bcl\text{-}_{XL}$ (PDB ID: **3PL7**)[22] and Bcl-2 (PDB ID: **2XA0**),[23] or Bax with C terminus truncated in complex with CHAPS (PDB ID: **4BH8**),[24] retrieved from the Protein Data Bank. Details of molecular docking studies are given in the Supporting information.

Results and Discussion

Chemistry

The synthetic pathway for the targeted novel *N*-Mannich bases of 5-adamantyl-1,2,4-triazole-3-thione is presented in Scheme 1. The synthesis starts from a commercially available 1-adamantanecarboxylic acid **1** which was converted into 1-adamantanecarbonyl chloride **2** in the reaction with thionyl chloride in the presence of catalytic amounts of *N*,*N*-dimethylformamide (DMF). The corresponding acylthiosemicarbazide **3** was obtained with very high purity by stirring acid chloride **2** with

thiosemicarbazide in dry tetrahydrofuran (THF) at room temperature. The intramolecular cyclization of acylthiosemicarbazide **3** was performed by addition of 2M aqueous solution of NaOH and refluxing for 3 hours. After refluxing and cooling, the clear solution was acidified using 2M HCl until pH 1 was reached followed with the formation of the precipitate of the 5-adamantyl-1,2,4-triazole-3-thione **4**. The final compounds **5a-r** were prepared in a Mannich reaction which included stirring of the equimolar concentrations of 5-adamantyl-1,2,4-triazole-3-thione **4**, formalin and corresponding primary aromatic amine in THF for two days. Then, the solvent was evaporated, and EtOH was added to the residue. The compounds **5a-r** were obtained with excellent purity excluding any need for further purification.

The structure of all synthesized compounds was determined by means of ^{1}H and ^{13}C NMR spectroscopy (see Supporting information, Figures S1-S38).

Scheme 1: Reagents and conditions: **a)** $SOCl_2$, DMF, CH_2Cl_2, 2 h, r.t.; **b)** $NH_2NHC(S)NH_2$, THF, 6 h, r.t.; **c)** NaOH, 3h, reflux; HCl; **d)** CH_2O, Ar-NH_2, THF, 48 h, r.t.

Theoretically, the alkylation of 5-substituted-1,2,4-triazole-3-thione can be performed at N2 and N4 atom of triazole ring. Herein, we have obtained a suitable single crystal of **5e** and by means of X-ray crystallography unambiguously demonstrated that the aminomethylation occurs exclusively at N2 atom of triazole moiety (Figure 1, CCDC 1465502–1465503). For more details see Supporting information (cif file and checkcif report).

Figure 1: Molecular structure of **5e**. Molecule *A* of the monoclinic polymorph is chosen as the representative.

Biology

In vitro cytotoxicity

The cytotoxic activity of 5-phenyl-1,2,4-triazole-3-thione was determined, proving this compound to be inactive against K562 cell line showing IC_{50} value of 200.00 μM. In order to determine its influence on the activity, the corresponding phenyl group was substituted with adamantyl group. The cytotoxic activity of the synthesized 5-adamantyl-1,2,4-triazole-3-thione (**4**) was also determined against K562 cells and the IC_{50} value for this compound was 101.38 μM. This increase in the activity, caused by the incorporation of the adamantyl group, has encouraged us to perform the synthesis of the corresponding Mannich bases starting from the intermediate **4** and to investigate their cytotoxic potential. Cytotoxic activity of the newly synthesized triazoles was evaluated against four malignant cell lines (HeLa, K562, HL-60 and A549) as well as against normal, non-transformed MRC-5 cells. The obtained IC_{50} values are shown in Table 1. Most of the compounds that displayed the highest cytotoxic activities against K562 cell line possess a substituent at *ortho*-position of the phenyl ring (**5b**, **5e**, **5h**, **5j**). On the other hand, all the tested compounds showed comparable activity against HL-60 cells, where compounds containing different substituents at *meta*-position (**5c**, **5f**, **5k**) exerted the weakest activity. The electronic effect of the substituent does not have a noticeable influence on the activity, since both electron-donating and electron-withdrawing substituents showed similar effectiveness against HL-60 cancer cells. Some of the active and highly selective compounds against K562 cell line **5b**, **5e** and **5j** were chosen for further examination of the mechanisms of their anticancer activity.

Table 1: The *in vitro* cytotoxic activity of investigated compounds

Cell cycle analysis and fluorescence microscopy

We examined the mechanisms of cytotoxic activity of the selected compounds **5b, 5e** and **5j** by flow cytometric analysis, using propidium iodide to label DNA. Figure 2 shows cell cycle distribution of K562 myelogenous leukemia cells incubated in the absence or presence of investigated compounds. The obtained results demonstrate that after 24 h treatment with IC_{50} and $2IC_{50}$ concentrations of triazoles **5e,** and **5j** the percentages of K562 cells in the G1 cell cycle phase were significantly higher compared with the percentage of cells within G1 phase in the control cell sample. The G1 cell cycle phase accumulation was also observed in K562 cells exposed to compound **5b**, although this increase was not statistically significant. The increase in the G1 phase cell population was accompanied by a decrease in the G2/M phase cell populations. Our results are in accordance with literature data about different triazoles which induce G1 cell cycle phase arrest.[25,26] In addition, treatment of K562 cells with $2IC_{50}$ concentrations of **5b, 5e,** and **5j** led to increase of cells in the subG1 phase in comparison to control cells.

Figure 2: Changes in the cell cycle phase distribution of human myelogenous leukemia K562 cells induced by the compounds **5b**, **5e** and **5j** after 24h treatment (tested concentrations corresponded to IC_{50} (**A**) and $2IC_{50}$ values (**B**)). The results are presented as the mean ± S.D. from two independent experiments. Statistically significant differences between control and treated cells are marked with * ($p<0.05$).

After 24 h treatment with triazoles **5b**, **5e** and **5j,** K562 cells were stained with a mixture of acridine orange and ethidium bromide and analyzed by fluorescence microscopy. As shown in Figure 3, the morphological changes typical for apoptotic cell death were observed in K562 cells treated with $2IC_{50}$ concentrations of compounds. In the non-treated control the K562 cells had normal morphology. In contrast, early apoptotic cells stained green with condensed chromatin in the shrunken nuclei or even fragmented nuclei could be seen in the cell samples exposed to triazoles in addition to orange-red stained late apoptotic cells. The obtained results indicate that **5b, 5e** and **5j** are able to induce apoptotic cell death in myelogenous leukemia K562 cells.

Figure 3: Photomicrographs of acridine orange/ethidium bromide stained control K562 cells, and K562 cells treated with $2IC_{50}$ concentrations of compounds **5b**, **5e** and **5j** for 24 h.

Western blot analysis

Molecular mechanisms underlying cytotoxic activity of the compounds **5b, 5e** and **5j** were further demonstrated by Western blot analysis (Figure 4). Activation of both principal apoptotic signal pathways[27] results in the activation of cysteine aspartic acid-containing proteases (caspases). Caspase-8 and caspase-9 are usually involved in extrinsic or death receptor-mediated pathway and the intrinsic or mitochondria-mediated pathway, respectively, while caspase-3 is downstream of both caspase-8 and caspase-9. The caspases are activated by cleavage, which further activates the downstream substrate molecules.[28]

Figure 4: Induction of caspase-dependent apoptosis by compounds **5b**, **5e** and **5j** at IC_{50} concentrations in K562 myelogenous leukemia cells after 24h treatment. Immunoblotting was performed using anti-caspase 3, anti-cleaved caspase 3, anti-caspase 8, anti-caspase 9 and anti-Bax antibodies. The anti-GAPDH antibody was used as a control for protein load. Besides full-length caspases – procaspases, the fragments of activated caspase-3 (subunits p19 and p17), fragments of activated caspase-8 (the cleaved intermediates p43/p41, the active fragments/subunits p18, p16 and p10) and fragments of activated caspase-9 (subunits p37 and p35) were detected.

The protein levels of full-length caspase-8 and caspase-3 in cells treated with compounds **5b**, **5e** and **5j** were increased in comparison to untreated, control cells. As shown in Figure 4, this increase was accompanied by strong appearance of active fragments. The most intensive effect was recognized for the compound **5j,** while the effects declined for compounds **5b** and **5e**, respectively. Although an increase in protein levels of full-length caspase-9 was also observed in treated cells, it was less prominent comparing to other procaspases examined.

We further examined whether the protein expression levels of Bax were altered following the treatment with compounds **5b, 5e** and **5j**. Bax is a member of the Bcl-2 protein family. As key regulators of apoptosis, these proteins are involved in the control of mitochondrial permeability and, in particular, the release of apoptogenic proteins from this organelle.[29] Western blot analysis showed that Bax expression was substantially upregulated in K562 cells treated with all three compounds tested. Furthermore, a smaller band of ~18-kDa was detected in addition to a 21-kDa band in treated cells. Detected cleavage fragment of Bax protein, p18, has been reported to be produced by calpain and to accelerate apoptosis in numerous tumor cell lines following treatment with various chemotherapeutic or biological agents.[30] In contrast, 18-kDa Bax was not detected in the control cells. We further demonstrated that caspase-dependent apoptosis is involved in compounds **5b**-, **5e**- and **5j**-induced cytotoxicity in K562 cells, and that both intrinsic and extrinsic pathway seem to be involved in induction of apoptosis. The intrinsic pathway is characterized by mitochondrial dysfunction with release of caspase activators, followed by activation of caspase-9 and caspase-3. Mitochondrial-induced apoptosis further requires involvement of the Bcl-2 family, including Bax. Caspase-8, on the other hand, is one of the upstream mediators of cell death and its activation is associated with an increase in TNF-like cytokines, such as TNF-α and Fas ligand (FasL; CD95).[31] Caspase-8 is inactivated in a variety of human cancers, which may promote tumor progression as well as resistance to current treatment approaches.[32,33] A significant increase in its full length and cleaved fragments is consistent with the high cytotoxic efficacy of selected compounds. In addition, augmentation of apoptosis in treated cells can also be attributed to up-regulation of calpain-produced Bax fragment. Previous studies have demonstrated that 18-kDa Bax, a characteristic feature of Bax activation, is a more potent inducer of apoptosis than 21-kDa Bax.[34] Further research confirmed that interruption of Bax cleavage reduces drug-induced apoptosis, while depression of 18-kDa Bax degradation significantly augments drug-mediated apoptosis.[35]

Anti-angiogenic activity

Angiogenesis represents the formation of new blood vessels and has a fundamental role in development, reproduction and repair of the tissue including tumors.[36,37] Dawson et al. have showed that an adamantyl-substituted retinoid-derived molecule effectively reduces human microvascular endothelial (HMVE) cell growth (IC_{50}=0.3 μM) and suggested its potential *in vivo* antiangiogenic activity.[38] Itraconazole, a compound containing 1,2,4-triazole, has been found to possess potent and selective inhibitory activity against multiple key aspects of tumor-associated angiogenesis, both *in vitro* and *in vivo*.[39] One of the most frequently used and best characterized permanent human vascular endothelial cell line in angiogenesis research is EA.hy926.[40] To evaluate the anti-angiogenic potential of the compounds **5b, 5e** and **5j** applied at subtoxic concentrations, a tube formation assay, also known as *in*

vitro angiogenesis assay was performed. As it could be seen in the Figure 5 reduced number of elongated and connected EA.hy926 cells in addition to significantly inhibited formation of tubular and polygonal structures was observed in the cell samples treated with compounds **5b, 5e** and **5j** compared with control cells. The compound **5j** exerted more pronounced anti-angiogenic effect in comparison to **5b** and **5e**. Our results point out the significant anti-angiogenic potential of investigated triazoles **5b**, **5e**, **5j.**

Figure 5: Effects on *in vitro* angiogenesis of EA.hy926 cells. Photomicrographs of control EA.hy926 cells and cells exposed to subtoxic IC_{20} concentrations (15 μM) of compounds **5b**, **5e** and **5j** after 20 h treatment.

The mechanism of 5b pro-apoptotic activity

The treatment of chronic myelogenous leukemia K562 cells with compound **5b** elevated levels of pro-apototic protein Bax, caspase-8 and main effector caspase-3, demonstrating that this triazole triggered both intrinsic and extrinsic apoptotic pathways.[41] In modern apoptosis-based anticancer therapy, one of the recognized approaches is to identify small molecules that activate pro-apoptotic Bcl-2 proteins[42] like Bax. Consistently, **5b**, as confirmed inducer of apoptosis, might exert its anticancer action as small-molecule Bax agonist.[43]

Thus, **5b** proapoptotic mode of action is exerted most likely *via* the displacement model pathway[44] which involves: 1) the Bax activation, as an executioner protein, with agonist, 2) the interaction of Bax-**5b** proapototic complex with anti-apoptotic enzymes Bcl-$_{XL}$ and/or Bcl-2, 3) the extrusion of Bax-**5b** from anti-apoptotic enzymes with BH3 sensitizer proteins like Bad, Bik, Bmf, Bnip3, Noxa or Hrk, 4) the Bax-**5b** penetration into the outer mitochondrial membrane (MOM) and dimerisation, 5) membrane disruption to release intermembrane space (IMS) proteins such as cytochrome c, OMI/HTRA2, SMAC/DIABLO, and endonuclease G,[45] 6) the activation of caspase-3 as an effector of apoptosis.[46] The displacement model steps 1), 2) (see Supporting information), and 4) (see Supporting information) were considered in this report by means of molecular modelling to reveal the pharmacology of **5b**, applying the combined molecular docking/molecular dynamics approach.

Bax agonists target the C-terminal α9 helix.[42,43] To the best of our knowledge there is no available crystal data regarding the binding modes of any Bax agonists, while there is limited structure-based derived information about small-molecule Bax agonists positioning.[42,43] Therefore, the blind docking procedure had been applied in order to outline the **5b** bioactive conformation (Figure 6) using the scoring function available in AutoDock4.2[47] program. During the Bax activation (step 1), **5b** was stabilized within the α9 helix[42] mainly due to the established advantageous hydrophobic interactions with the helix residues. Hence, the adamantyl moiety was involved in the steric clash with Val180 side chain. The *o*-tolyl scaffold was encircled by Ala183 and Ile187 where the hydrophobic interference is constituted *via* the aromatic ring inasmuch as the *o*-methyl group was pointed away from the C-terminal domain. The orientation of adamantyl and *o*-tolyl groups provided some more interesting observations. Both of the functionalities were positioned in front of the Ser184, a key amino acid appointed as a target for post-translational phosphorylation during the prevention of apoptotic process[42] by anti-apoptotic proteins. If this post-translational modification is avoided, Ser184 provides the C-terminal domain stabilization by means of C-terminus helix merging with the outer mitochondrial membrane. Consequently, the pro-apototic protein Bax prevails in effort to induce apoptosis.[48]

Small-molecule Bax agonists induce conformational changes in Bax by blocking Ser184 phosphorylation, facilitating Bax insertion into mitochondrial membranes and forming Bax oligomers.[43] The role of the **5b** as the small-molecule Bax agonist in the induction of apoptosis will be discussed lately

in text but at this point it is crucial to accentuate that adamantyl and *o*-tolyl groups both served as a sort of spatial gate in front of the Ser184 and therefore prevented the amino acid phosphorylation before the actual interaction of Bax with the anti-apoptotic proteins.

The second level of Bax-**5b** complex stabilization was provided by 1,2,4-triazole-3(4*H*)-thione ring where sulfanylidene group as hydrogen-bond (HB) acceptor was narrow to Ser101 forming a strong hydrogen bond (d_{HB} = 2.501 Å), while the HB donor nitrogen at position 4 of heterocyclic ring was in the close proximity to Asp102 (d_{HB} = 1.767 Å). At last, the imino nitrogen at position 1 was electrostatically attracted by the α4 helix residue Asp98 and during the rigid docking simulation this interaction has not disturbed the hydrogen bond between the Asp98 and Ser184.[21] Accordingly, **5b** positioning within the Bax C-terminal domain was characterized by the binding affinity of K_d = -9.34 kcal/mol and inhibition constant of K*i* = 0.76 μM.

Figure 6: The best docked pose of **5b** (agonist colored in blue) as predicted by AutoDock4.2. The Bax α1 helix is colored in red, α2 helix in forest green, α3 helix in conflower blue, α4 helix in magenta, α5 helix in gold, α6 helix in steel blue, α7 helix in olive grab, α8 helix in siena, and α9 helix in orange. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The general stability of Bax-**5b** was confirmed by molecular dynamics study (Figure S41, Supporting information). Thus, the complex was stabile after 0.82 ns by means of RMSD (Figure S41A) and RMSF values (ligand interactions in range from 0.597 to 1.638 Å; Figure S41B). The energy decomposition procedure for Bax-**5b** complex outlined that calculated binding affinity of **5b** (Table 2) correctly predicted the possibility of agonist to interact with the Bax C terminus. The low value of binding energy of **5b** (ΔG_{bind} = -51.78 kcal/mol) suggests that the agonist is easily bound to the α9 helix. As expected, hydrophobic interactions were dominant for **5b** binding (ΔE_{vdw} = -54.13 kcal/mol) while the significant contribution was perceived for electrostatic interactions, too (ΔE_{ele} = -33.52 kcal/mol). The relatively low value for the solvation energy (ΔE_{solv} = 2.33 kcal/mol) showed that **5b** was stable but no so tightly bound to the C terminus. In that manner, **5b** suffered conformational perturbation immediately after the start of simulation (0.01 ns) in manner that hydrogen bonds between the sulfanylidene group and Ser101 as well as between nitrogen at position 4 and Asp102 were broken and 1,2,4-triazole-3(4*H*)-thione ring was rotated towards Asp98. This advantageous alignment of 1,2,4-triazole-3(4*H*)-thione was supported by the establishment of new hydrogen bond between the nitrogen at position 4 and Asp98-Met99 peptide bond carbonyl oxygen, which arouse after 0.149 ns and remained stabile until the end. The disagreement between the pose obtained by molecular dynamics (**5b-MD**) and the best docked one (**5b-BD**) however cannot be related to false docking outcome, but can be attributed to applied rigid docking protocol where no flexibility for amino acids was allowed. The RMSD between the **5b-BD** and **5b-MD** amounted 1.748 Å, suggesting that both **5b-BD** and **5b-MD** are actually borderline solutions of **5b** physiologically conformation and the active structure itself is in-between the **5b-MD** and **5b-BD**. As a result of docking/dynamics coupled simulation, Asp98 was estranged from Ser184 and kept away from latter residue due to hydrophobic repulsion between the **5b** sulfanylidene group and Asp98 side chain methylene group initially observed after 0.28 ns of simulation and occurred during the whole period of stabilization. This predicted conformational change for protein is vital for the Bax pro-apoptotic efforts inasmuch as it resulted is the degeneration of Asp98-Ser184 hydrogen bond[21] occurring in agonist-free Bax structure. Particularly, if this hydrogen bond remains stabile, Bax may actually inhibit itself even before the interaction with anti-apoptotic proteins since it becomes incapable to invade the mitochondrial membrane *via* the C-terminus.[21,49] On the other hand, the elimination of Ser184-Asp98 hydrogen bond promotes the dissociation of the Bax C-terminal helix from the hydrophobic groove through major conformational change, making it more accessible to mitochondrial membrane.[21,49] In that manner, **5b** initially exerts pro-apoptotic potential by forcing the Asp98 away from Ser184 and facilitates Bax-mediated apoptotic event.

Table 2: Binding free energies and individual energy terms of ATP-rTopIIα complex with various inhibitors

Utilizing the molecular modeling approach, it was decidedly proven that Bax-**5b** complex as apoptosis initiator. The remaining steps of the displacement model pathway still remain poorly understood by means of luck of experimental support, but for the purpose of **5b** pro-apoptotic potential understanding the contribution in the form of molecular modeling (Supporting information) was given for steps 2) and 4), outlining the interaction of Bax-**5b** with anti-apoptotic proteins Bcl-$_{XL}$ and Bcl-2 and Bax-**5b** facilitation of MOM disturbance-based apoptosis, respectively.

An additional discussion relating to molecular docking and molecular dynamics studies of Bax-5b-Bcl-$_{XL}$/Bax-5b-Bcl2 complexes formation is presented in Supporting information.

Conclusion

A novel class of 1,2,4-triazole *N*-Mannich bases containing adamantane moiety has been synthesized. The confirmation of the structure of these compounds and determination of the exact nitrogen atom at which Mannich reaction occurs were obtained by X-ray diffraction technique. The investigated compounds showed moderate to good cytotoxic activities against four malignant cell lines, with K562 and HL-60 cells being significantly more sensitive. The low toxicity of tested derivatives towards normal MRC-5 cell line indicated their prominent selectivity in the anticancer action. The selected active compounds **5b**, **5e** and **5j** induced accumulation of K562 cells in subG1 and G1 phase. Western blot analysis demonstrated that selected compounds exert their cytotoxic activity through the caspase-dependent apoptosis and upregulation of Bax expression levels. Also, it was found, using a tube formation assay, that tested derivatives exhibit antiangiogenic effects. Finally, the molecular modeling study revealed, applying the combined molecular docking/molecular dynamics approach, that compound **5b** has a role in protection of Bax Ser184 from phosphorylation in order to facilitate the proapoptotic event.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1. Procedure for the preparation of **5a-r**, spectral data, crystal structure determination details and structural data for **5e**, details of biological experiments and molecular docking studies.

Figure S1-S38. ^{1}H and ^{13}C NMR spectra of **4** and **5a-r**.

Table S1-S4. Crystallographic data for **5e**.

Figure S39 and S40. Hydrogen bonding in the orthorhombic and monoclinic form, respectively.

Figure S41-S45. Molecular docking and molecular dynamics studies of the interaction of **5b** with Bax.

Appendix S2. CIF and CheckCIF files containing crystallographic information and full structural check regarding compound **5e**.

Figure caption list:

Scheme 1: Reagents and conditions: **a)** $SOCl_2$, DMF, CH_2Cl_2, 2 h, r.t.; **b)** $NH_2NHC(S)NH_2$, THF, 6 h, r.t.; **c)** NaOH, 3h, reflux; HCl; **d)** CH_2O, Ar-NH_2, THF, 48 h, r.t.

Figure 1: Molecular structure of **5e**. Molecule *A* of the monoclinic polymorph is chosen as the representative.

Figure 2: Changes in the cell cycle phase distribution of human myelogenous leukemia K562 cells induced by the compounds **5b**, **5e** and **5j** after 24h treatment (tested concentrations corresponded to IC_{50} (**A**) and $2IC_{50}$ values (**B**)). The results are presented as the mean ± S.D. from two independent experiments. Statistically significant differences between control and treated cells are marked with * ($p<0.05$).

Figure 3: Photomicrographs of acridine orange/ethidium bromide stained control K562 cells, and K562 cells treated with $2IC_{50}$ concentrations of compounds **5b**, **5e** and **5j** for 24 h.

Figure 4: Induction of caspase-dependent apoptosis by compounds **5b**, **5e** and **5j** at IC_{50} concentrations in K562 myelogenous leukemia cells after 24h treatment. Immunoblotting was performed using anti-caspase 3, anti-cleaved caspase 3, anti-caspase 8, anti-caspase 9 and anti-Bax antibodies. The anti-GAPDH antibody was used as a control for protein load. Besides full-length caspases – procaspases, the fragments of activated caspase-3 (subunits p19 and p17), fragments of activated caspase-8 (the cleaved intermediates p43/p41, the active fragments/subunits p18, p16 and p10) and fragments of activated caspase-9 (subunits p37 and p35) were detected.

Figure 5: Effects on *in vitro* angiogenesis of EA.hy926 cells. Photomicrographs of control EA.hy926 cells and cells exposed to subtoxic IC_{20} concentrations (15 μM) of compounds **5b**, **5e** and **5j** after 20 h treatment.

Figure 6: The best docked pose of **5b** (agonist colored in blue) as predicted by AutoDock4.2. The Bax α1 helix is colored in red, α2 helix in forest green, α3 helix in conflower blue, α4 helix

in magenta, α5 helix in gold, α6 helix in steel blue, α7 helix in olive grab, α8 helix in siena, and α9 helix in orange. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1: The *in vitro* cytotoxic activity of investigated compounds

	IC_{50}±SD [μM]				
	HeLa	**K562**	**HL-60**	**A549**	**MRC-5**
5a	51.19±0.40	35.19±1.71	22.74±2.70	75.24±10.32	≈100
5b	61.09±4.92	25.42±0.89	21.91±1.47	72.90±1.95	≈100
5c	76.78±1.17	40.35±0.57	30.30±1.67	84.46±1.71	≈100
5d	49.73±2.30	26.89±1.21	25.16±3.27	85.77±0.82	93.38
5e	66.82±8.11	27.71±2.46	21.02±2.76	94.04±0.13	>100
5f	66.42±6.35	30.62±2.85	34.09±2.51	92.90±3.78	>100
5g	63.21±9.32	30.48±0.72	24.26±2.27	≈100	>100
5h	82.84±1.78	24.26±0.17	22.66±3.48	96.54±4.89	>100
5i	>100	63.84±2.08	24.73±0.23	>100	>100
5j	64.01±2.09	27.05±5.35	23.76±2.63	87.98±3.10	87.66
5k	54.60±0.84	32.98±5.14	28.10±3.85	97.94±2.92	≈100
5l	78.71±7.61	41.68±1.84	26.26±4.58	>100	>100
5m	77.25±0.37	32.40±0.10	23.32±3.93	>100	>100
5n	71.18±1.10	36.98±2.54	26.87±2.12	90.05±12.86	≈100
5o	42.01±1.30	29.20±4.88	25.82±0.45	55.49±1.36	55.11
5p	74.17±0.41	36.70±0.54	25.76±1.44	83.28±10.82	≈100
5q	54.98±6.79	33.94±3.08	23.32±1.84	83.36±5.34	≈100
5r	58.68±3.62	35.13±0.46	23.56±0.72	96.25±2.14	>100
cisplatin	2.24±0.26	5.45±0.18	3.37±0.06	14.42±0.70	9.24±0.18

Table 2: Binding free energies and individual energy terms of ATP-rTopIIα complex with various inhibitors

Complex	ΔE_{ele} (kcal/mol)	ΔE_{vdw} (kcal/mol)	ΔG_{solv} (kcal/mol)	$-T\Delta S$ (kcal/mol)	ΔG_{bind} (kcal/mol)
Bax-**5b**	-33.52±0.31	-54.13±0.46	2.33±0.43	-33.54±0.24	-51.78±0.34
Bax-**5b**-Bcl-$_{XL}$	-34.39±0.41	-67.59±0.46	6.38±0.52	-34.32±0.32	-61.28±0.22
Bax-**5b**-Bcl2	-34.26±0.46	-67.41±0.61	3.71±0.72	-37.54±0.15	-60.42±0.25

[a]ΔE_{ele} - electrostatic energy difference. [b]ΔE_{vdw} - van der Waals energy difference. [c]ΔG_{solv} - solvation free energy. [d]ΔS - the entropy change. [e]ΔG_{bind} - predicted binding free energy.