

research article

Discovery of 'click' 1,2,3-triazolium salts as potential anticancer drugs

Ivana Steiner¹, Nikolina Stojanovic¹, Aljosa Bolje², Anamaria Brozovic¹, Denis Polancec³, Andreja Ambriovic-Ristov¹, Marijana Radic Stojkovic⁴, Ivo Piantanida⁴, Domagoj Eljuga⁵, Janez Kosmrlj², Maja Osmak¹

¹ Division of Molecular Biology, Ruđer Bošković Institute, Zagreb, Croatia

² Faculty of Chemistry and Chemical Technology, University of Ljubljana, Slovenia

³ Department for Translational Medicine, Children's Hospital Srebrnjak, Zagreb, Croatia

⁴ Division of Organic Chemistry and Biochemistry, Ruđer Bošković Institute, Zagreb, Croatia

⁵ Department for Oncoplastic and Reconstructive Surgery, University Hospital for Tumors, University Clinical Hospital Centre Sisters of Mercy, Zagreb, Croatia

Radiol Oncol 2016; 50(3): 280-288.

Received 23 November 2015

Accepted 17 March 2016

Correspondence to: Maja Osmak, Ph.D., Division of Molecular Biology, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia. Phone: + 385 1 456 0939; Fax: + 385 1 456 1 177; E-mail: maja.osmak@irb.hr and Janez Košmrlj, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Večna pot 113, SI-1000 Ljubljana, Slovenia. Phone: +386 1 479 8558; E-mail: janez.kosmrlj@fkkt.uni-lj.si

Ivana Steiner and Nikolina Stojanović: equal contributing authors

Disclosure: No potential conflicts of interest were disclosed.

Background. In order to increase the effectiveness of cancer treatment, new compounds with potential anticancer activities are synthesized and screened. Here we present the screening of a new class of compounds, 1-(2-picolyl)-, 4-(2-picolyl)-, 1-(2-pyridyl)-, and 4-(2-pyridyl)-3-methyl-1,2,3-triazolium salts and 'parent' 1,2,3-triazole precursors.

Methods. Cytotoxic activity of new compounds was determined by spectrophotometric MTT assay on several tumour and one normal cell line. Effect of the selected compound to bind double stranded DNA (ds DNA) was examined by testing its influence on thermal stability of calf thymus DNA while its influence on cell cycle was determined by flow cytometric analysis. Generation of reactive oxygen species (ROS) was determined by addition of specific substrate 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA).

Results. Parent triazoles were largely inactive, while some of the triazolium salts were highly cytotoxic for HeLa cells. Triazolium salts exhibited high cell-type dependent cytotoxicity against different tumour cells. Selected compound (4-(4-methoxyphenyl)-3-methyl-1-(2-picolyl)-1H-1,2,3-triazolium hexafluorophosphate(V) (2b) was significantly more cytotoxic against tumour cells than to normal cells, with very high therapeutic index 7.69 for large cell lung carcinoma H460 cells. Additionally, this compound was similarly cytotoxic against parent laryngeal carcinoma HEP-2 cells and their drug resistant 7T subline, suggesting the potential of this compound in treatment of drug resistant cancers. Compound 2b arrested cells in the G1 phase of the cell cycle. It did not bind ds DNA, but induced ROS in treated cells, which further triggered cell death.

Conclusions. Our results suggest that the 'click' triazolium salts are worthy of further investigation as anti-cancer agents.

Key words: triazoles; triazolium salts; anticancer activity; cell cycle; ROS

Introduction

Cancer is one of the major causes of death in developed countries.¹ Despite the fact that several decades of investigations and massive funding have been devoted to the cancer research, the de-

crease in cancer mortality is relatively modest.² Based on numerous different genes implicated in sustained proliferative signalling, evading growth suppression, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis, cancers exhibit

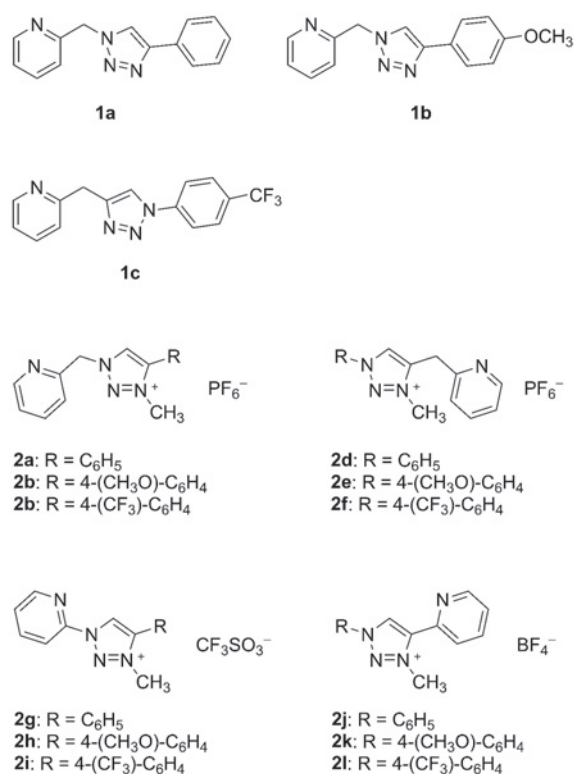


FIGURE 1. The structures of triazoles 1 and triazolium salts 2.

considerable genetic complexity and genome instability, which generates the genetic diversity.^{2,3} Consequently, amongst all diseases, cancer is one of the most difficult to treat and cure, because it is not a single disease but rather consists of numerous different types and subtypes.

Targeted therapy with novel drugs directed at specific molecular pathways opens promising new avenues to improve the efficacy of therapy. However, in spite of the initial enthusiasm, the clinical application of such target oriented anticancer drugs did not fulfil the expectations. Although the targeted therapy has been successful, it is still limited to some very specific types of tumours.³ Hence, in order to increase the effectiveness of cancer treatment, new compounds with potential anticancer activities have to be synthesized and screened.

Modern synthetic chemistry is powered by facile synthetic protocols that allow for a rapid generation of compound scaffolds. Catalyzed azide-alkyne cycloaddition reaction producing 1,2,3-triazoles ('click' triazoles) is one of such example.^{4,5} Moreover, this chemistry paves the way to a variety of derivatives including 1,3,4-trisubstituted 1,2,3-triazolium salts ('click' triazolium salts).⁶ 1,2,3-Triazole has drug-like properties⁷ and its de-

derivatives are recognized for their broad range of biological activities, including antiviral, antibacterial, antifungal, anti-inflammatory and analgesic, anti-convulsant, antiparasitic, antidiabetic, antiobesitic, antihistaminic, antineuropathic, antihypertensive, and anticancer activities⁸, presenting a promising group of potential anticancer drugs. Despite the fact that there are numerous examples on cytotoxic activities of compounds with a triazole subunit⁹⁻¹⁵, to our knowledge, only one such report exists for the 1,2,3-triazolium salts. Namely, Shrestha and Chang reported an interesting anticancer activity of the compounds having triazolium ring fused to 1,4-naphthoquinone.¹⁶ No cytotoxic activity of the 1,3,4-trisubstituted 1,2,3-triazolium salts has been reported to date.

We recently developed a strategy for the 'click' triazolium salts synthesis, and prepared a library of twelve isomeric and homologous pyridine tethered 1,2,3-triazoles (1), which were then subjected to the selective N-methylation into the 3-methyl-1,4-disubstituted 1,2,3-triazolium salts 2a – 1.^{17,18} The library constituents differed in the 1,4-substitution pattern consisting of phenyl, 4-methoxyphenyl and 4-(trifluoromethyl)phenyl functionalized 1-(2-picolyl) (2a – c), 4-(2-picolyl) (2d – f), 1-(2-pyridyl) (2g – i) and 4-(2-pyridyl) (2j – l) isomers (Figure 1).

The unique properties of this class of compounds, including the charge and hydrogen bonding ability, prompted us to evaluate their anticancer activity and to gain more insight into the mode of action that underlies their antiproliferative activity.

Materials and methods

Triazoles and triazolium salts

Triazoles 1 and triazolium salts 2 were prepared as described previously.^{17,18} Compounds 1b, 1f, 2b, 2c, 2f – i and 2l were dissolved in ethanol while compounds 1a, 2a, 2d, 2e, 2j and 2k were dissolved in DMSO. The solutions were stored at -20°C, and diluted to the appropriate concentrations with growth medium just before use.

Cell culture

Human cervical carcinoma HeLa and laryngeal carcinoma HEP-2 cells were obtained from cell culture bank (GIBCO BRL, Invitrogen, Grand Island, NY, USA). HEP-2 cell line was recently recognized and categorized by ATCC as human laryngeal carcinoma cell line cross-contaminated with HeLa cells. The development of HEP-2 subline resist-

ant to carboplatin (7T) has been published previously.¹⁹ These cells are cross-resistant to anticancer drug cisplatin, transplatin, mitomycin C and the natural compound curcumin as well.¹⁹⁻²¹ Large cell lung carcinoma H460 cells and colorectal carcinoma HCT-116 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Normal human skin fibroblasts were isolated from the upper arm of a 7-years-old female donor at the Neurochemical Laboratory, Department of Chemistry and Biochemistry, School of Medicine, University of Zagreb. They were used for the cytotoxicity assay at 32 and 36 population doublings. All cell lines were grown as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) in a humidified atmosphere of 5% CO₂ at 37°C and were sub-cultured every 3–4 days.

Cytotoxicity assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cytotoxic activity of triazoles and triazolium salts was determined by MTT assay²², modified as described. Cells were seeded into 96-well tissue culture plates (3 × 10³ cells/0.18 mL medium/well). The next day different concentrations of triazoles or triazolium salts were added (0.02 mL) to each well and each concentration was tested in quadruplicate. Following 72 h incubation at 37°C, the medium was aspirated, and 20 µg of the MTT dye (Sigma-Aldrich) /0.04 mL medium/well was added. Three hours later, formazan crystals were dissolved in DMSO (0.17 mL/well), the plates were mechanically agitated for 5 min and the optical density at 545 nm was determined on a microtiter plate reader (Awareness Technology Inc, Palm City, FL, USA). To examine the effect of reactive oxygen species (ROS) scavengers on survival of 2b treated cells, the same procedure was used as described above, except that two hours prior to addition of 2b, 5 mM of N-acetyl-cysteine (NAC, Sigma-Aldrich), or 1mM tempol (Santa Cruz Biotechnology, Dallas, TX, USA) was added in wells. Experiment was repeated at least three times.

Colony-forming assay

For determination of colony formation 1000 cells were seeded in 6 cm dish. The next day the cells were pretreated either with 5 mM NAC or with 1 mM (HEp-2 cells) or 0.125 mM (H460 cells) tem-

pol. Two hours later different concentrations of 2b were added in dishes with or without ROS scavengers. The effect of antioxidants on colony formation alone was determined as well. After ten days of continuous treatment, the colonies were washed with PBS, fixed with methanol, stained with Giemsa-crystal violet and counted. Untreated samples were used for determination of plating efficiency. Each concentration was tested in triplicate. Experiment was repeated at least three times.

Cell cycle analysis

Human large cell lung carcinoma H460 cells were seeded into 6-well tissue culture plates (10⁵ cells/2 mL medium/well) and treated with 29.7 and 110 µM of 2b on the following day for 24, 48 and 72 hours. Thereafter, both adherent and floating cells were collected, washed with PBS and fixed overnight in 70% ethanol at -20°C. Fixed cells were treated with RNase A (0.1 mg/mL, Sigma-Aldrich) for 1 h at room temperature and afterward stained with propidium iodide (50 µg/mL, Sigma-Aldrich) for 30 min in the dark. The DNA content was analysed by flow cytometry (FACS Calibur, Becton Dickinson, Mountain View, CA, USA). Data were analysed with ModFitLTTM program (Verity Software House Inc., Topsham, ME, USA). Experiment was repeated more than three times.

Determination of DNA binding

The calf thymus (ct)-DNA was purchased from Sigma-Aldrich, dissolved in Na-cacodylate buffer, $I = 0.05 \text{ mol/dm}^3$, pH = 7, additionally sonicated and solution filtered through a 0.45 µm filter. Polynucleotide concentration was determined spectroscopically as the concentration of phosphates by $\epsilon_{260 \text{ nm}} = 6600 \text{ dm}^3/\text{mol}^1 \text{ cm}^{-1}$. Thermal denaturation curves for ct-DNA and its complexes with studied compounds were determined in Na-cacodylate buffer, $I = 0.05 \text{ mol/dm}^3$, pH = 7 by following the absorption change at 260 nm as a function of temperature, as previously described.^{23,24} The absorbance of the compound was subtracted from each curve and the absorbance scale was normalized. Measured T_m values are the midpoints of the transition curves determined from the maximum of the first derivative and checked graphically by the tangent method. The DT_m values were calculated subtracting T_m of the free nucleic acid from T_m of the complex. Every ΔT_m value reported here was an average of at least two measurements. The error in DT_m is $\pm 0.5^\circ\text{C}$.

TABLE 1. IC₅₀ values of triazolium salts and some parent triazoles against cervical carcinoma HeLa cells

Cmpd.	IC ₅₀ (μM) ^a
1a	> 100
1b	> 100
1f	> 100
2a	91.6 ± 3.9
2b	57.0 ± 12.9
2c	> 100
2d	> 100
2e	55.4 ± 9.4
2f	– ^b
2g	– ^c
2h	88.9 ± 7.5
2i	54.4 ± 14.7
2j	54.9 ± 3.5
2k	> 100
2l	– ^c

^a IC₅₀ is the concentration of the triazoles and triazolium salts inducing 50% cell growth inhibition after 72 h incubation. The results are shown as mean values of at least three experiments (± SD).

^b Triazolium salt precipitated promptly after the addition to the growth medium and thus the cytotoxicity could not be measured accurately.

^c The range of concentrations 10 – 1000 μM reduced survival from about 60 to 40%, and therefore the exact IC₅₀ was difficult to determine.

Induction of reactive oxygen species (ROS)

Generation of ROS was determined by addition of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) (Invitrogen). Briefly, logarithmically growing H460 cells were incubated with 10 mM CM-H₂DCFDA for 1 hour according to manufacturer's instructions. Afterward, cells were incubated with or without different concentrations of 2b during indicated time periods. After trypsinization and centrifugation, the cells were fixed in cold 80% methanol. Shortly before measurement, they were centrifuged and resuspended in PBS. The fluorescence of the product, developed by removal of the acetate groups from CM-H₂DCFDA by intracellular esterases and oxidation, was measured by flow cytometry on BC Navios instrument (Beckman Coulter, Inc., Miami, FL, USA).

To further examine whether toxicity of 2b is coupled with formation of ROS, two ROS scavengers were used: NAC, a drug that has been known for

years to directly reduce the level of ROS²⁵ or the new ROS scavenger tempol.²⁶ Their effect was determined by MTT assay or colony-forming assay as described in *Cytotoxicity assay*.

Statistical analysis

All data were analysed by unpaired Student's t-test, and expressed as the mean ± standard error of the mean. Data were considered significant when P values were lower than 0.05, and in the figures these are designated as * = P < 0.05 or ** = P < 0.01. Experiments were repeated at least three times.

Results and discussion

Cancer is the second leading cause of death in developed countries.¹ Primary or acquired drug resistance and heavy side-effects strongly limit the effectiveness of classical chemotherapy. The success of advanced, target-oriented cancer therapy is at present limited only to the special types of cancers.³ This provides a great impetus for investigation of new compounds with potential anticancer activities. 1,2,3-Triazoles are very important class of heterocycles, which have been well-recognized for their broad range of biological activities, including anticancer activity.⁸⁻¹⁶

This and the fact that no cytotoxic activity of the unfused 1,3,4-trisubstituted 1,2,3-triazolium salts has yet been reported encouraged us to examine the cytotoxic activity of compounds 2a – l along with some selected parent 1,4-disubstituted triazoles 1a, 1b and 1f.

Antiproliferative effects of triazoles and triazolium salts

The effect of tested triazoles and triazolium salts was first evaluated in HeLa cells, the cell model system that we previously found suitable for screening of new compounds.^{27,28} The results are collected in Table 1.

It appears that in the picolyl series of the triazolium cations (2a – f) the aryl substituent modulated the cytotoxicity against the tumour cells, with the electron donating 4-methoxyphenyl group being greater as compared to the electron neutral phenyl and the electron withdrawing 4-(trifluoromethyl)-phenyl groups. In this series of the compounds the pyridine ring was separated from the triazole core by a methylene bridge and thus less likely influences the electron density at the latter. The situation dras-

tically changes in the pyridyl series (2g – l) were the pyridine ring was attached directly to the triazole, now playing an important role in the cytotoxicity. In 2g – l the triazole core was functionalized with pyridine in two different ways, either through the triazole N1 nitrogen atom as in 2g – i, or through the triazole C4 carbon atom (2j – l). Interestingly, in the former (2g – i), the electron deficient 4-(trifluoromethyl)phenyl group (2i) increased the cytotoxicity whereas in the latter series of compounds the member with the electron neutral phenyl group (2j) was identified as the most active.

Apparently, no clear structure-activity correlation, based purely on the electronic considerations of the triazolium cations, could be drawn at this point. Noteworthy, different chemical reactivity patterns of the four isomeric triazolium cations have been previously observed in some chemical transformations, with the structure-reactivity relation still remaining to be fully understood.²⁹ It is reasonable to expect that in a far more complex system such as the living cell, the structure-activity relation is likely to be a result of combined stereo-electronic effects and could be addressed after considerably larger library of derivatives have been assayed.

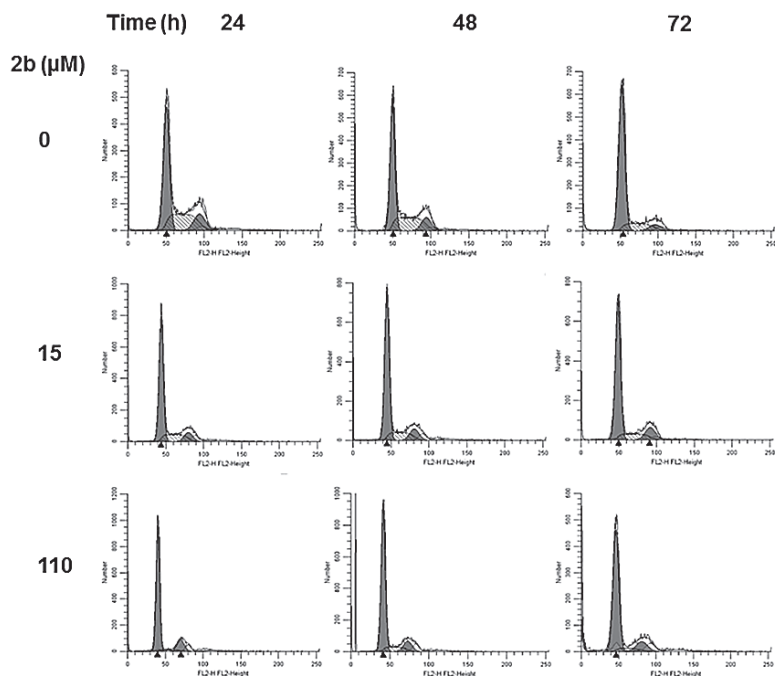


FIGURE 2. Effects of 2b on the cell cycle of H460 cells. Logarithmically growing H460 cells were treated with 29.7 and 110 μM of 2b for indicated period of time. Afterwards they were harvested for cell cycle analysis measured by FACS as described in Materials and methods. Representative data of three experiments are shown.

TABLE 2. Cytotoxic activity of 2b against different cell lines

Cell line	IC ₅₀ (μM) ^b	T.I. ^c
HeLa	57.0 \pm 12.9	4.01
HEp-2	87.0 \pm 28.5	2.63
7T	111.7 \pm 7.6	2.05
H460	29.7 \pm 4.5	7.69
HCT-116	51.9 \pm 7.2	4.40
Fibroblasts	228.5 \pm 5.6	–

HeLa = cervical carcinoma cells; HEp-2 = laryngeal carcinoma cells; 7T = carboplatin and cisplatin-resistant HEp-2 subline; H460 = large cell lung carcinoma cells; HCT-116 = colorectal carcinoma cells; Fibroblasts = normal primary fibroblasts. The results are shown as mean values of three experiments (\pm SD).

^b IC₅₀ is the concentration of 2b that induces 50% cell growth inhibition after 72 h incubation.

^c T.I. refers to therapeutic index, calculated from the ratio of cytotoxicity (IC₅₀) on normal fibroblasts and cytotoxicity (IC₅₀) on tumour cells.

In order to compare the results with those for the triazolium salts 2, three parent triazoles 1a, b, f were also selected for the biological screening. None of them exhibited cytotoxic activity against HeLa cells with IC₅₀ below 100 μM .

To shed more light on the mechanisms responsible for the cytotoxic effect of above examined compounds, we decided to proceed further with compound 4-(4-methoxyphenyl)-3-methyl-1-(2-picolyl)-1H-1,2,3-triazolium hexafluorophosphate(V) (2b) as a representative compound. First, we explored its antiproliferative activity on several tumour cell lines from different origin, as well as on the normal human fibroblasts. The results are shown in Table 2. Compound 2b strongly inhibited the growth of all examined tumour cell lines and this effect was cell-type specific. Human large cell lung carcinoma H460 cells were the most sensitive toward 2b (and were selected for further studies), while HEp-2 cells were most resistant. Difference in sensitivity between most sensitive H460 and most resistant 7T cells (for IC₅₀ value) was almost 4 times.

Drug resistance is the major cause of failure in successful treatment of cancer patients.^{30,31} It is based on the variety of complex mechanisms.³⁰⁻³⁵ The compounds that might be efficient against drug-resistant cells could be of great help in improvement of cancer treatment. Therefore we also included carboplatin, cisplatin and mitomycin C resistant 7T subline of HEp-2 cells in our study. As shown in Table 2, both, parental HEp-2 and drug-resistant 7T cells are similarly sensitive to 2b compound, suggesting a potential future application of

TABLE 3. Effect of compound 2b on the cell cycle of H460 cells

Conc. (μM)	24 h				48 h				72 h			
	G1	S	G2/M	subG1	G1	S	G2/M	subG1	G1	S	G2/M	subG1
0	54	31	15	2	59	26	15	2	73	13	14	2
29.7	61	24	15	2	61	26	13	5	65	17	18	6
110	78	9	13	3	78	9	13	15	79	5	16	35

H460 cells were treated for the indicated time period with 2b, stained with propidium iodide and analysed by flow cytometry. Cell cycle distribution was assessed as described in the Materials and methods section.

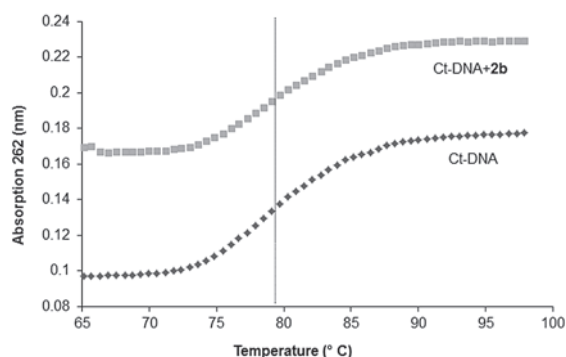


FIGURE 3. DNA as possible target of compound 2b. Thermal denaturation curves of ct-DNA ($c(\text{ct-DNA}) = 2 \times 10^{-5}$ M, $r_{[\text{compound}]/[\text{ct-DNA}]} = 0.3$) at pH 7.0 (sodium cacodylate buffer, $I = 0.05$ M) upon addition of compound 2b. Error in DTm values: $\pm 0.5^\circ\text{C}$.

2b compound in clinical treatment of cisplatin and carboplatin resistant tumours. For comparison, 7T cells were 3.3 fold more resistant to cisplatin as shown previously.¹⁹

One of the most requested characteristics of potential anticancer drug is its higher efficacy against tumour than the normal cells. In this study all examined tumour cell lines were more sensitive to compound 2b than normal cell line, with the therapeutic index higher than 2 (Table 2). Specifically, for H460 cells it was particularly high, 7.69, fulfilling the request of this essential characteristic for a potential anticancer compound.

Effect of compound 2b on the cell cycle of H460 cells

To gain more insight into the mode of action that underlies the antiproliferative activity of 2b, we investigated its effect on the cell cycle in H460 cells. The flow cytometric analysis is presented in Figure 2, and Table 3. They show that compound 2b arrested the cells in the G1 phase of the cell cycle in dose-dependent manner, even after 24 hours of treatment. At later time points a dose- and time-dependent increase was detected in a fraction of

cells with reduced DNA content (subG1), which represents the apoptotic cells subG1 fraction. These results suggest that 2b induces apoptosis in treated cells.

DNA as possible target of compound 2b

According to their structure, triazolium salts resemble the structures of DNA minor groove binders like those studied by Chenoweth and Dervan³⁶, that show precise recognition of the DNA sequence by thermodynamically controlled “H-bond based reading” of pre-designed heterocycle-polyamide molecules. Some non-condensed heterocyclic molecules also proved to be DNA intercalators.^{8,37} Therefore we studied the interactions of 2b with double strand (ds) DNA. Thus, in thermal denaturation experiment compound 2b was mixed with ct-DNA in a ratio $r_{[\text{compound}]/[\text{ct-DNA}]} = 0.3$, at which any DNA binding mode should give a measurable change in DNA melting point transition. However, in the thermal denaturation experiment no measurable change in DNA melting point transition was observed, and no influence on the thermal stability of ct-DNA (Figure 3), indicating that at biologically relevant conditions (pH 7, $c(\text{compound}) = 6 \times 10^{-6}$ M) stable non-covalent complex with ds DNA was not formed, suggesting that DNA was not the target of 2b compound.

Formation of ROS by compound 2b

Although 2b does not bind to DNA, alternative mechanisms of 2b action and cytotoxicity were examined. Literature data indicate that diverse compounds can induce cell damage due to formation of ROS.^{38,39} ROS may irreversibly oxidize DNA, nucleic acids, proteins, and lipids, thereby representing the primary source of damage in biological systems that may eventually lead to cell death.^{40,41} Accordingly, we directly measured the induction of ROS formation following the treatment with 2b. For this, we stained the cells with 10 mM CM-

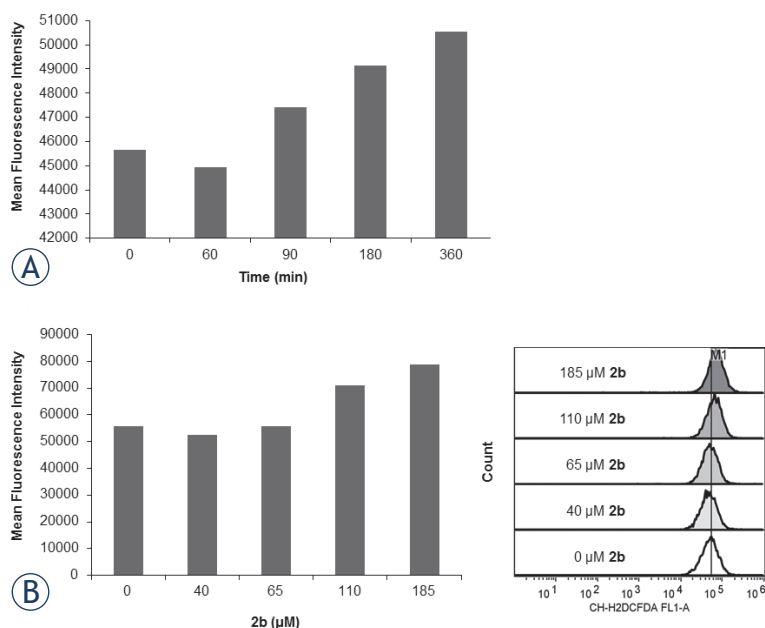


FIGURE 4. Formation of ROS by 2b in H460 cells. Logarithmically growing H460 cells were stained for 1 hour with 10 mM CM-H₂DCFDA and then either treated with 110 μM 2b during indicated time points (A) or treated with indicated concentrations of 2b for 180 min (3 hours) (B). Afterward ROS formation was determined by flow cytometry as described in Materials and methods section. Dose-dependent formation of ROS was additionally presented by cell count and fluorescence intensity of CM-H₂DCFDA. M1 line is positioned to designate MFI value of the non-treated sample (white histogram) compared to signals obtained upon cell treatment with indicated concentrations of 2b.

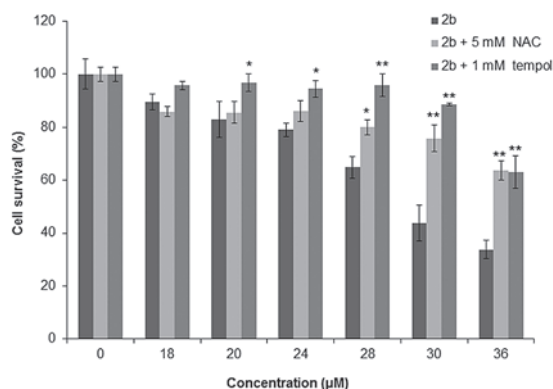


FIGURE 5. The effects of ROS scavenger on survival of H460 cells treated with 2b determined by MTT assay. H460 cells were seeded and next day pretreated for 2 hours with 5 mM of NAC or 1 mM tempol. Afterwards different concentrations of 2b were added. The cell survival was determined 72 hours later by MTT assay as described in Materials and methods section. Each point represents the mean \pm SD of at least three independent experiments. All data are expressed as the average percentage of survival values relative to an untreated control \pm SD or samples treated with antioxidants alone. The significance in differences is indicated (*, $P < 0.05$; **, $P < 0.01$).

H₂DCFDA for one hour and then treated them either with 110 μM 2b during different time points (Figure 4A) or with different concentrations of 2b during 3 hours (Figure 4B). For better illustration of ROS formation upon 2b treatment during 3 hours we presented additionally results as CM-H₂DCFDA fluorescence intensity compared to cell number (count). As shown in Figure 4, 2b induced ROS in time- and dose-dependent manner. Dose-dependent skewing of signals toward higher fluorescence intensity with increased concentration of compound is noticeable (Figure 4B). In order to confirm the formation of ROS and validity of detection we incubated the cells in each experiment with 0.01% H₂O₂ for 30 min (data not shown).

To approve this result and additionally examine the possible role of 2b-induced formation of ROS in cell toxicity, we pretreated for 2 hours H460 cells with two different ROS scavengers: NAC and tempol. As shown in Figure 5, the pretreatment of cells with either NAC or tempol increased survival of 2b treated cells compared to the cells treated only with 2b, indicating that 2b induced ROS and that the cytotoxicity of 2b can be reduced by addition of ROS scavenger. To confirm the data that we obtained by MTT assay (Figure 5), we have done additional experiments using colony-forming assay and chronic exposure to 2b with ROS scavenger. The pre-treatment of HEP-2 cells (a less responsive cell line to 2b compound) with either NAC or tempol increased the survival of 2b treated cells compared to the survival of cells treated only with 2b (Figure 6A). Similar results were obtained with the most sensitive cell line to 2b compound, *i. e.* H460 cells treated with NAC. Again, the pretreatment with this ROS scavenger increases the survival of cells as compared to the survival of cells treated only with 2b. However H460 cells were highly sensitive to tempol. The highest non-toxic concentration of tempol for chronic treatment of H460 cells was 0.125 mM, which is 8 times lower concentration than could be applied for HEP-2 cells (see Figure 6B). It is possible that this concentration of tempol was too low to scavenge ROS induced by 2b. Perhaps, higher concentration could protect the cells from toxic effect of 2b, but higher concentration used during 10 days incubation was too toxic for H460 cells. H460 and HEP-2 cells differ in their p53 status: H460 has wild type p53, while HEP-2 cells have mutated p53. While p53 has important role in cell response to oxidative stress and apoptosis⁴², we can assume that wild type p53 can be involved in increased H460 sensitivity to chronic treatment with tempol.

Thus, experiments in which the cells were pretreated with ROS scavengers suggest that 2b indeed induced ROS in treated cells and that ROS induction was involved in cytotoxicity, but that induction of ROS is not the only mechanism of action of the selected compound 2b.

Conclusions

In conclusion, pyridine tethered 'click' triazolium salts have been tested for their anticancer activity for the first time. As revealed on human cervical carcinoma HeLa cells, selected compound (4-(4-methoxyphenyl)-3-methyl-1-(2-picolyl)-1*H*-1,2,3-triazolium hexafluorophosphate(V) (2b), exhibits high cytotoxicity. Its antiproliferative activity was cell type dependent, being mostly cytotoxic against large cell lung carcinoma H460 cells. It is of utmost importance that 2b was significantly more cytotoxic against tumour cells than normal cells, having very high therapeutic index, such as 7.69 for H460 cells. Additionally, this compound was similarly cytotoxic against parental laryngeal carcinoma HEP-2 cells and their drug resistant 7T subline which is, having in mind the importance of inhibitory effect of drug resistance on the success of cancer treatment, a very valuable result. Compound 2b arrested tumour cells in the G1 phase of the cell cycle and induced programmed cell death. This compound does not form a complex with ds DNA, but rather induced ROS in treated cells which further triggers cell death. In short, our results suggest that the 'click' triazolium salts are simple to make compounds that are worth of further investigation as anticancer agents. Work is in progress to design and examine an extended library of their analogues.

Acknowledgments

This study was supported by the Ministry of Science, Education and Sport of the Republic of Croatia (Projects 098-0982913-2748, 098-0982913-2850 and 098-0982914-2918). The Ministry of Education, Science and Sport, Republic of Slovenia, the Slovenian Research Agency is gratefully acknowledged for the financial support (Project P1-0230; Young Researcher Grant to A.B.). Financial support from Joint Projects BI-HR/12-13-028 and BI-HR/14-15-007 is also acknowledged. We thank Ms. Snježana Juler and Ana Tupek for technical assistance.

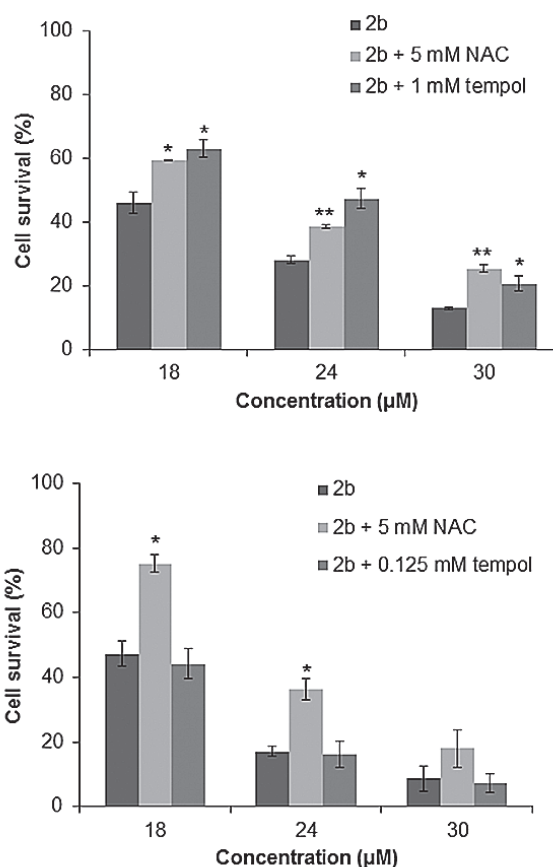


FIGURE 6. The effect of ROS scavenger on survival of HEP-2 (A) and H460 (B) cells treated with 2b determined by colony-forming assay. HEP-2 and H460 cells were seeded and next day pretreated for 2 hours with 5 mM of NAC or 1 mM (HEP-2 cells) or 0.125 mM (H460 cells) tempol. Afterwards different concentrations of 2b were added. Ten days later the colonies were counted. Non-treated cells and cells treated with antioxidants alone were used as controls. Each point represents the mean \pm SD of at least three independent experiments. All data are expressed as the average percentage of survival values relative to an untreated control \pm SD or samples treated with antioxidants alone. The significance in differences is indicated (*, $P < 0.05$; **, $P < 0.01$).

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin* 2012; **62**: 10-29.
2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-74.
3. Duffy MJ. The war on cancer: are we winning? *Tumour Biol* 2013; **34**: 1275-84.
4. Kolb HC, Sharpless KB. The growing impact of click chemistry on drug discovery. *Drug Discov Today* 2003; **8**: 1128-37.
5. Košmrlj J. *Click Triazoles*, 1 edn, vol. 28. Berlin Heidelberg: Springer-Verlag; 2012.
6. Aizpurua JM, Fratila RM, Monasterio Z, Perez-Esnaola N, Andreieff E, Irastorza A, et al. Triazolium cations: from the "click" pool to multipurpose applications. *New J Chem* 2014; **38**: 474-80.
7. Massarotti A, Aprile S, Mercalli V, Del Grosso E, Grosa G, Sorba G, et al. Are 1,4- and 1,5-disubstituted 1,2,3-triazoles good pharmacophoric groups? *Chem Med Chem* 2014; **9**: 2497-508.

8. Zhou CH, Wang Y. Recent researches in triazole compounds as medicinal drugs. *Curr Med Chem* 2012; **19**: 239-80.
9. da Silva EN, Jr., Cavalcanti BC, Guimaraes TT, Pinto Mdo C, Cabral IO, Pessoa C, et al. Synthesis and evaluation of quinonoid compounds against tumor cell lines. *Eur J Med Chem* 2011; **46**: 399-410.
10. Ahmed N, Konduru NK, Ahmad S, Owais M. Design, synthesis and antiproliferative activity of functionalized flavone-triazole-tetrahydropyran conjugates against human cancer cell lines. *Eur J Med Chem* 2014; **82**: 552-64.
11. Chinthala Y, Kumar Domatti A, Sarfaraz A, Singh SP, Kumar Arigari N, Gupta N, et al. Synthesis, biological evaluation and molecular modeling studies of some novel thiazolidinediones with triazole ring. *Eur J Med Chem* 2013; **70**: 308-14.
12. Majeed R, Sangwan PL, Chinthakindi PK, Khan I, Dangroo NA, Thota N, et al. Synthesis of 3-O-propargylated betulinic acid and its 1,2,3-triazoles as potential apoptotic agents. *Eur J Med Chem* 2013; **63**: 782-92.
13. Ou L, Han S, Ding W, Jia P, Yang B, Medina-Franco JL, et al. Parallel synthesis of novel antitumor agents: 1,2,3-triazoles bearing biologically active sulfonamide moiety and their 3D-QSAR. *Mol Divers* 2011; **15**: 927-46.
14. Glowacka IE, Balzarini J, Wroblewski AE. The synthesis, antiviral, cytostatic and cytotoxic evaluation of a new series of acyclonucleotide analogues with a 1,2,3-triazole linker. *Eur J Med Chem* 2013; **70**: 703-22.
15. Sambasiva Rao P, Kurumurthy C, Veeraswamy B, Santhosh Kumar G, Poornachandra Y, Ganesh Kumar C, et al. Synthesis of novel 1,2,3-triazole substituted-N-alkyl/aryl nitro derivatives, their anti-inflammatory and anticancer activity. *Eur J Med Chem* 2014; **80**: 184-91.
16. Shrestha JP, Chang CW. Safe and easy route for the synthesis of 1,3-dimethyl-1,2,3-triazolium salt and investigation of its anticancer activities. *Bioorg Med Chem Lett* 2013; **23**: 5909-11.
17. Bolje A, Urankar D, Kosmrlj J. Synthesis and NMR analysis of 1,4-disubstituted 1,2,3-triazoles tethered to pyridine, pyrimidine, and pyrazine rings. *Eur J Org Chem* 2014; **36**: 8167-81.
18. Bolje A, Kosmrlj J. A selective approach to pyridine appended 1,2,3-triazolium salts. *Org Lett* 2013; **15**: 5084-7.
19. Osmak M, Bizjak L, Jernej B, Kapitanovic S. Characterization of carboplatin-resistant sublines derived from human larynx carcinoma cells. *Mutat Res* 1995; **347**: 141-50.
20. Rak S, Cimbora-Zovko T, Gajski G, Dubravcic K, Domijan AM, Delas I, et al. Carboplatin resistant human laryngeal carcinoma cells are cross resistant to curcumin due to reduced curcumin accumulation. *Toxicol In Vitro* 2013; **27**: 523-32.
21. Brozovic A, Vukovic L, Polancac DS, Arany I, Koberle B, Fritz G, et al. Endoplasmic reticulum stress is involved in the response of human laryngeal carcinoma cells to carboplatin but is absent in carboplatin-resistant cells. *PLoS One* 2013; **8**: e76397.
22. Mickisch G, Fajta S, Keilhauer G, Schlick E, Tschada R, Alken P. Chemosensitivity testing of primary human renal cell carcinoma by a triazolium based microculture assay (MTT). *Urol Res* 1990; **18**: 131-6.
23. Piantanida I, Palm BS, Cudic P, Zinic M, Schneider HJ. Interactions of acyclic and cyclic bis-phenanthridinium derivatives with ss- and ds-polynucleotides. *Tetrahedron* 2004; **60**: 6225-31.
24. Mergny JL, Lacroix L. Analysis of thermal melting curves. *Oligonucleotides* 2003; **13**: 515-37.
25. Arooma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of N-acetylcysteine: its reaction with hydrogen-peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radical Biol Med* 1989; **6**: 593-7.
26. Mitchell JB, DeGraff W, Kaufman D, Krishna MC, Samuni A, Finkelstein E, et al. Inhibition of oxygen-dependent radiation-induced damage by the nitroxide superoxide dismutase mimic, tempol. *Arch Biochem Biophys* 1991; **289**: 62-70.
27. Burja B, Cimbora-Zovko T, Tomic S, Jelusic T, Kocevar M, Polanc S, et al. Pyrazolone-fused combretastatins and their precursors: synthesis, cytotoxicity, antitubulin activity and molecular modeling studies. *Bioorg Med Chem* 2010; **18**: 2375-87.
28. Cimbora-Zovko T, Brozovic A, Piantanida I, Fritz G, Virag A, Alic B, et al. Synthesis and biological evaluation of 4-nitro-substituted 1,3-diaryltriazenes as a novel class of potent antitumor agents. *Eur J Med Chem* 2011; **46**: 2971-83.
29. Bolje A, Hohloch S, Urankar D, Pevec A, Gazvoda M, Sarkar B, et al. Exploring the scope of pyridyl- and picolyl-functionalized 1,2,3-triazol-5-ylidenes in bidentate coordination to Ruthenium(II) cymene chloride complexes. *Organometallics* 2014; **33**: 2588-98.
30. Garraway LA, Janne PA. Circumventing cancer drug resistance in the era of personalized medicine. *Cancer Discovery* 2012; **2**: 214-26.
31. Zahreddine H, Borden KLB. Mechanisms and insights into drug resistance in cancer. *Front Pharmacol* 2013; **4**: 28.
32. Stewart DJ. Mechanisms of resistance to cisplatin and carboplatin. *Crit Rev Oncol Hematol* 2007; **63**: 12-31.
33. Fojo T. Multiple paths to a drug resistance phenotype: mutations, translocations, deletions and amplification of coding genes or promoter regions, epigenetic changes and microRNAs. *Drug Resist Updat* 2007; **10**: 59-67.
34. Heffeter P, Jungwirth U, Jakupec M, Hartinger C, Galanski M, Elbling L, et al. Resistance against novel anticancer metal compounds: differences and similarities. *Drug Resist Updat* 2008; **11**: 1-16.
35. Ambriovic-Ristov A, Osmak M. Integrin-mediated drug resistance. *Curr Signal Transduct Ther* 2006; **1**: 227-37.
36. Chenoweth DM, Dervan PB. Allosteric modulation of DNA by small molecules. *Proc Natl Acad Sci US* 2009; **106**: 13175-9.
37. Zhao M, Ratmeyer L, Peloquin RG, Yao SJ, Kumar A, Spychala J, et al. Small changes in cationic substituents of diphenylfuran derivatives have major effects on the binding-affinity and the binding mode with RNA helical duplexes. *Bioorg Med Chem* 1995; **3**: 785-94.
38. Brozovic A, Stojanovic N, Ambriovic-Ristov A, Brozovic Krijan A, Polanc S, Osmak M. 3-Acetyl-bis(2-chloro-4-nitrophenyl)triazene is a potent antitumor agent that induces oxidative stress and independently activates the stress-activated protein kinase/c-Jun NH2-terminal kinase pathway. *Anticancer Drugs* 2014; **25**: 289-95.
39. Brozovic A, Ambriovic-Ristov A, Osmak M. The relationship between cisplatin-induced reactive oxygen species, glutathione, and BCL-2 and resistance to cisplatin. *Crit Rev Toxicol* 2010; **40**: 347-59.
40. Cross CE, Halliwell B, Borish ET, Pryor WA, Ames BN, Saul RL, et al. Oxygen radicals and human disease. *Ann Intern Med* 1987; **107**: 526-45.
41. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007; **39**: 44-84.
42. Vousden KH, Ryan KM: p53 and metabolism. *Nat Rev Cancer* 2009; **9**: 691-700.