

Benzothiazole derivatives as human DNA topoisomerase II α inhibitors

Cigdem Kaplan-Ozen · Betul Tekiner-Gulbas ·
Egemen Foto · Ilkay Yildiz · Nuran Diril ·
Esin Aki · Ismail Yalcin

Received: 7 January 2013 / Accepted: 5 March 2013
© Springer Science+Business Media New York 2013

Abstract Benzothiazole derivatives resembling the structure of DNA purine bases were tested to determine their topoisomerase inhibition activities. Based on DNA topoisomerase I and II relaxation assay results, all 12 derivatives acted as human topoisomerase II α inhibitors, whereas only two compounds inhibited Calf thymus topoisomerase I. 3-amino-2-(2-bromobenzyl)-1,3-benzothiazol-3-ium 4-methylbenzenesulfonate (BM3) was observed to be the most effective human topoisomerase II α inhibitor with the lowest IC₅₀ value of 39 nM. The mechanistic studies suggested that BM3 was neither a DNA intercalator nor a topoisomerase poison, it was only a DNA minor groove-binding agent. BM3 initially bound to the DNA topoisomerase II α enzyme, then to DNA. As a result, the tested benzothiazole derivatives were obtained as strong topoisomerase II α inhibitors. The benzothiazole tosylated salt form BM3 was found as the most effective topoisomerase II α inhibitor. BM3's mechanisms of action might be its direct interaction with the enzyme. BM3's minor groove-binding

property might also contribute to this action. Hence, BM3 could be a good candidate as a new anticancer agent.

Keywords Benzothiazole derivatives · Human topoisomerase II α · DNA binding · Intercalation · Catalytic inhibitor · Anticancer drugs

Introduction

DNA topoisomerases (topo I and II) are enzymes that regulate topological states of DNA double helix in both prokaryotes and eukaryotes during DNA processing reactions. The basic role of the two types of topoisomerases is solving the DNA constraint problems (Wang, 2002). Due to their central role in DNA topology maintenance, mammalian DNA topoisomerases might be proper targets for anticancer drugs (Nelson *et al.*, 1984). Topoisomerase inhibitors could be divided into two main groups such as poisons and catalytic inhibitors. Topoisomerase poisons stabilize the DNA-enzyme cleavable complex and cause DNA strand breaks. Catalytic inhibitors block one or more steps of the catalytic cycle.

Benzothiazoles are heterocyclic compounds and they structurally look like DNA purine bases (Castelli *et al.*, 2001). They have been investigated for antitumor, antimicrobial, antifungal, and antimalarial activities (Soni *et al.*, 2010; Saeed *et al.*, 2010).

In 1996, Shi and his colleagues found that 2-(4-aminophenyl) benzothiazole derivatives inhibited proliferation of human breast cancer cell lines, MCF7 and MDA 468. In the same study, the antiproliferative effects of 3'-methyl-, 3'-bromo-, 3'-iodo-, and 3'-chloro-substituted benzothiazole derivatives on ovarian, lung, and kidney cancer cell lines were also examined (Shi *et al.*, 1996). As a new

Electronic supplementary material The online version of this article (doi:10.1007/s00044-013-0577-5) contains supplementary material, which is available to authorized users.

C. Kaplan-Ozen · E. Foto · N. Diril
Biology Department, Molecular Biology Section,
Faculty of Science, Hacettepe University, 06800 Ankara, Turkey

C. Kaplan-Ozen (✉)
BilGen Genetics and Biotechnology Center, Department
of Molecular Biology and Genetics, Bilkent University,
06800 Ankara, Turkey
e-mail: cozen@bilkent.edu.tr

B. Tekiner-Gulbas · I. Yildiz · E. Aki · I. Yalcin
Pharmaceutical Chemistry Department, Faculty of Pharmacy,
Ankara University, Ankara, Turkey

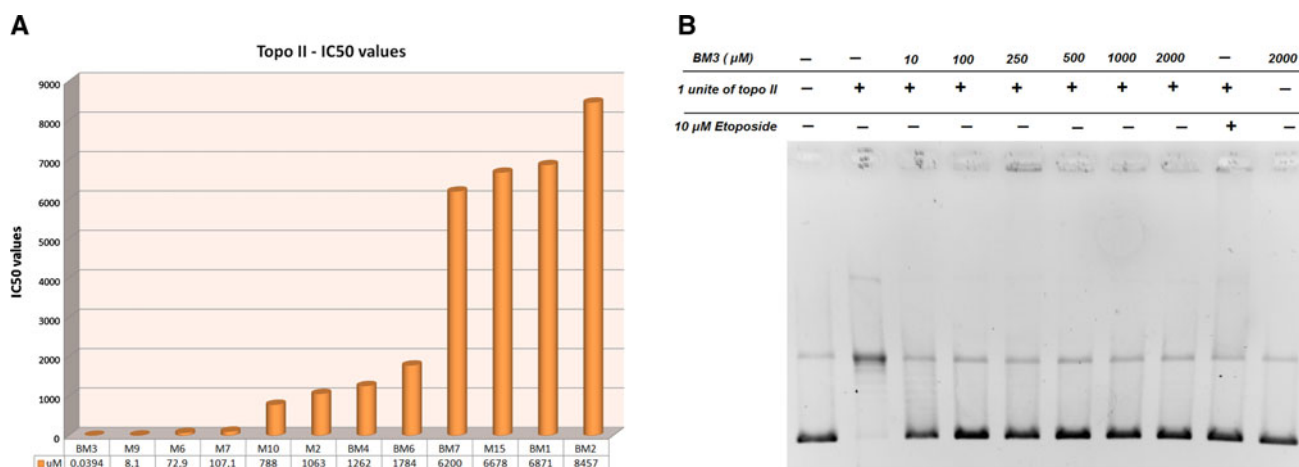


Fig. 1 Topo II α inhibition results of the benzothiazoles. **a** Graphical demonstration of IC₅₀ values for topo II α inhibition of the compounds, **b** 1 % agarose gel result after 1-h topo II α relaxation

anticancer drug candidate in phase-I clinical trials, 2-(4-amino-3 methylphenyl)-5-fluorobenzothiazole (5F203) has a potential and selective effect on a series of tumor cell lines (Bradshaw *et al.*, 2002). However, the antitumor mechanisms of action of these derivatives in cancer cells have not been clear yet.

Benzothiazole-containing compounds were shown to have stronger cytotoxic activities on breast cancer cell lines compared to non-cancer cells (Solomon *et al.*, 2009). After 24-h treatment of the most effective benzothiazole to MCF7, cell cycle progression was measured by cytometry. It was found that the most effective benzothiazole derivative arrested MCF7 cells at G2/M phases of the cell cycle. This arrest could be thought of as a result of topoisomerase II α (topo II α) inhibitory properties. It was reported that topo II α expression level increased about 10-fold at a late S phase and G2/M, while the other types of topoisomerases remained at the same expression level during the cell cycle (Cortes *et al.*, 2003). Increased expression level of topoisomerase II α could cause a great risk of DNA damage due to its breaking-religating ability on both strands of the DNA. If the topoisomerase is inhibited with a benzothiazole derivative, it is highly possible that DNA damage occurs triggering G2/M arrest.

Li *et al.* reported that a new benzothiazole derivative showed strong cytotoxicity against a liver cancer cell line (Hep G2) leading to apoptosis (Li *et al.*, 2010). They suggested that apoptosis in this cell line was signaled by the reactive oxygen species (ROS) pathway. Prior to this, it was shown that inhibitors of topoisomerases induced ROS apoptotic pathway (Kawiak *et al.*, 2007; Mend and Ding, 2007). It is highly possible that the inhibitory activity of benzothiazoles on topoisomerase II α also triggers ROS-mediated apoptosis.

Benzothiazoles, benzimidazoles, and benzoxazines are members of benzazoles. Our group previously defined other benzazoles which had strong eukaryotic topo II inhibition

assay with or without different concentration of BM3; FI indicates supercoiled form of plasmid, FII nicked form, FIV relaxed form

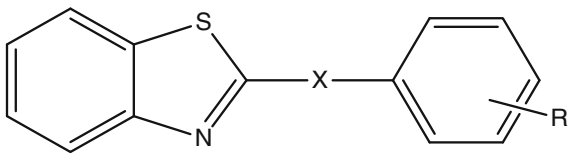
properties (Pinar *et al.*, 2004; Tekiner-Gulbas *et al.*, 2006). Especially 2-phenoxyethylbenzothiazole (IC₅₀ value of 11.4 μ M) among the tested series was found to be more active than the reference drug etoposide (IC₅₀ 21.8 μ M). In the present study, we used 12 different benzothiazole derivatives which were previously synthesized (Tekiner-Gulbas *et al.*, 2008; TUBITAK/TBAG-U/76 (103T089), Supplementary doc 1) by our group to examine their DNA topoisomerase I and II inhibitory potentials in a cell-free system (Table 1) and discuss the structure–activity relationships.

The main goal of this study was to discover the benzothiazole-based new anticancer drug candidates. In this respect, we investigated inhibitory properties of 12 benzothiazole derivatives on eukaryotic DNA topoisomerase I and II. For screening of these derivatives, the relaxation assay was used. Their 50 % inhibitory concentrations were calculated using topoisomerase enzyme inhibition percentages from the relaxation assay, and the most effective molecular structure was determined. BM3 was found to be the strongest topoisomerase II α inhibitor. BM3's mechanism of action on human topoisomerase II α was examined by several in vitro methods such as cleavage assay, electrophoretic mobility shift assay (EMSA), T4 DNA ligase and topoisomerase I DNA unwinding assays, polymerase chain reaction, restriction endonuclease assay, and DNA protection assay.

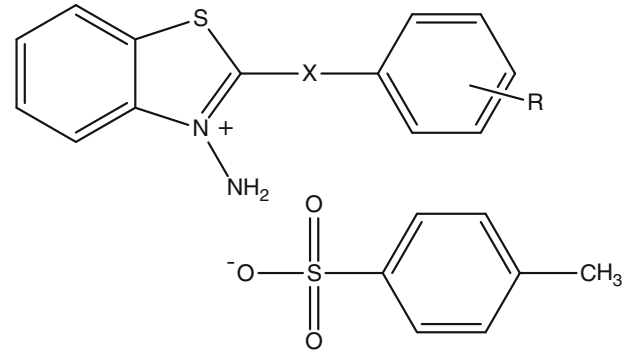
Results and discussion

Benzothiazole derivatives inhibited the catalytic activity of both topo I and II α

The benzothiazole derivatives described in this study were examined as two groups: 2-substituted benzyl and/or phenyl benzothiazole derivatives and their 3-aminobenzothiazol-3-

Table 1 General structure of main and tosylated benzothiazole derivatives with IC₅₀ values for catalytic activity of topo I and II


Compounds	X	R	Topo I IC ₅₀	Topo II IC ₅₀
M2	CH ₂	2-F	ND	1.063 mM
M6	CH ₂	4-CH ₃	ND	72.93 μM
M7	CH ₂	4-Cl	ND	107.1 μM
M9	CH ₂	4-Br	ND	8.1 μM
M10	CH ₂	2-Br	ND	788 μM
M15	–	2-Br	ND _a	6.678 mM



<i>N</i> -amino tosylated salts	X	R	Topo I IC ₅₀	Topo II IC ₅₀
BM6	CH ₂	2-F	3.04 mM	1.784 mM
BM1	CH ₂	4-CH ₃	ND	6.871 mM
BM7	CH ₂	4-Cl	ND	6.2 mM
BM2	CH ₂	4-Br	ND	8.457 mM
BM3	CH ₂	2-Br	8.9 mM	39.4 nM^a
BM4	–	2-Br	ND	1.262 mM
Positive controls				
CPT			80 μM	
Etoposide				10 μM

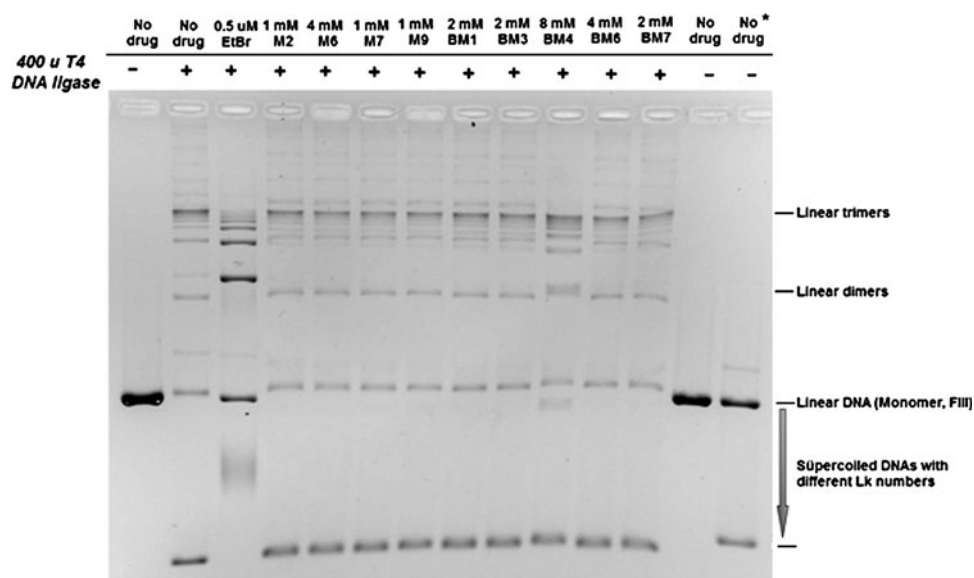
ND Not detected

^a Most effective compound

ium 4-methylbenzenesulfonate salt forms (Table 1). IC₅₀ values for topo I inhibition were found to be higher than 3 mM (Table 1) for these benzothiazoles. All of the tested compounds inhibited human topoisomerase II α , while they showed very low inhibitory activities on calf thymus topo I. As seen in Table 1, compound M9 showed the lowest IC₅₀ value (8.1 μM) among the main compounds for human topo II α even more effective than a standard drug, Etoposide. The *N*-amino tosylated salt form (BM3) of 2-bromobenzyl benzothiazole (M10) was found to be the most effective derivative among the other salt forms with an IC₅₀ value of 39.4

nM (Fig. 1a, b) and was significantly more active than etoposide as well. These results strongly supported that topo II α could be the main target for this compound. It was reported that more than half of 20 different benzothiazole derivatives inhibited topo II α and the most effective compound had an IC₅₀ value of 71.7 μM (Collins *et al.*, 2009). If we compare the results to ours, we could state that BM3 was 1,820 times more effective than the most active derivative found. Our experimental data indicated that the two tested benzothiazole derivatives (BM3 and M9) exhibited lower inhibitory concentrations than the positive topo II α inhibitor etoposide

Fig. 2 Intercalative potentials of the benzothiazoles. T4 DNA ligase unwinding assay was carried out with 9 different benzothiazoles and ethidium bromide. 0.5 μg linear pBR322 plasmid DNA was religated by 400 units of T4 DNA ligase in the presence of different compounds for 1 h at 37 $^{\circ}\text{C}$. Gel was migrated for about 18 h at room temperature



($\text{IC}_{50} = 10 \mu\text{M}$), which has been found as a topo II α poison. We did not see any cleavage action of these compounds. It could be concluded that benzothiazole derivatives inhibited topo II α , but its mode of action is different from etoposide.

Human topoisomerase II α inhibition mechanisms of BM3

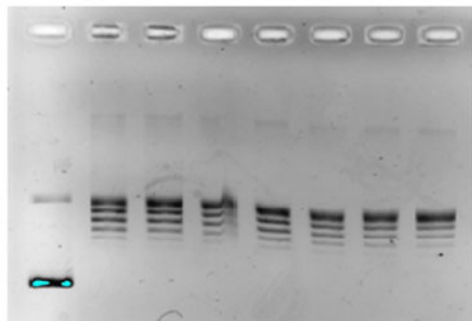
The outcome of our study results suggested that BM3 was not a topo II α poison, but it was a strong catalytic inhibitor of human topoisomerase II α enzyme. In this respect, we decided to investigate the topo II α catalytic inhibition mechanism of BM3. We basically examined the mechanism of topo II α inhibition. However, the topo I relaxation assay results implied that there might be relatively nicked plasmid accumulation. Therefore, we chose 9 suspicious compounds from the tested series and studied their poison effects by cleavage assay and intercalative effects (by T4 DNA ligase unwinding assay). However, we found neither poison nor intercalative effects except BM3 and BM4 (Fig. 2, Supl. Figs. 1 and 2) which did change Lk numbers of DNA in a way different from EtBr. So, we thought that these two chemicals were not intercalators, but they still interacted with DNA in other ways. Indeed, we confirmed this idea by the DNA topo I unwinding assay. Again, both of them changed the Lk numbers of the DNA (Figs. 3, 4). It was reported that several DNA intercalator agents could hold topo I cleavable complex (Pommier *et al.*, 2002), but we did not find any poison effects for both of these compounds. We believed that especially high concentrations of these compounds changed the Lk number of the plasmid because of their accumulation on DNA molecule. For instance, just high concentrations of BM3 (Supplementary data) and BM4 (8–10 mM) changed the conformation of

plasmid, while low concentrations at the μM range had no effect on the topoisomerases or the Lk number. If there were strong interactions between the compounds and DNA like EtBr, we could have seen certain enzyme inhibitions. So, the compounds probably accumulate on DNA without binding it. Störl *et al.* reported that 6 well-known DNA minor groove-binding agents could change the Lk number like the intercalators did, but in a different way (Störl *et al.*, 1993). The ability of BM3 and BM4 to change the Lk number depends on how many of them bind per 1 base pair of DNA. Therefore, the compounds might be DNA minor groove-binding agents rather than intercalators. It was previously reported that cyanine dyes with benzothiazoles and benzimidazoles (TO-PRO-1 and TO-PRO-2) bound to DNA through the minor groove instead of intercalation (Sovenyhazy *et al.*, 2003). They also that showed each groove had a certain number of dye compounds bound to it. This situation could be explained as these compounds could accumulate on DNA minor grooves and they could give different results depending on their concentrations. It was reported that the agents without intercalation properties could still bind on DNA minor grooves to inhibit enzymes that manipulate DNA (Störl *et al.*, 1993; Reddy *et al.*, 1999; Racane *et al.*, 2010). For example, it was shown that the agents that induce structural changes on DNA were active on topo I and II catalysis and DNA–protein interactions in vitro (Bailly *et al.*, 1999).

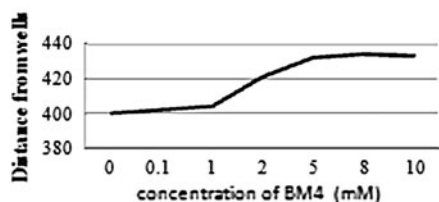
BM3 is a catalytic inhibitor, neither topo I nor topo II α poison

The activity of BM3 was to stall the DNA–enzyme complex that caused DNA strand breaks. However, there were

BM4 (mM)	-	-	0.1	1	2	5	8	10
Sc DNA (0.1 µg)	+	+	+	+	+	+	+	+
Topo I (2 U)	-	+	+	+	+	+	+	+



Mobility changes of most relaxed DNA bands



	0	0.1	1	2	5	8	10
Mobility	400	402	404	421	432	434	433

Fig. 3 DNA unwinding ability of BM4. The upper part shows that topoisomerase I unwinding assay was carried out with 2 unites of topo I, 0.1 µg of supercoiled (Sc DNA) pBR322 plasmid DNA with or without BM4 (0.1, 1, 2, 5, 8 and 10 mM). The Lower part shows that a graphical demonstration for mobilities of more relaxed DNA bands after different concentration of BM4

no results implying that BM3 could have this activity on both topo enzymes.

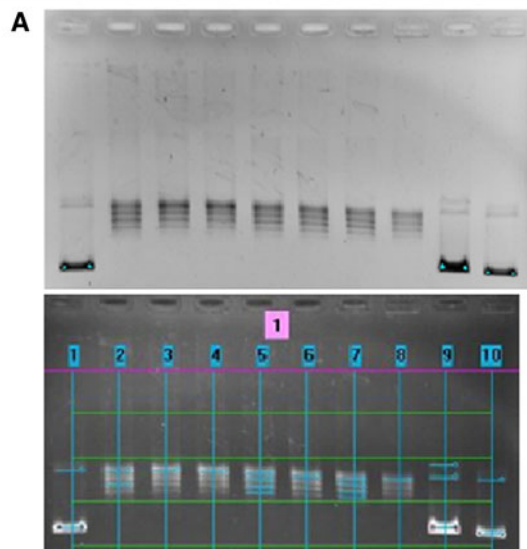
BM3 interrupts DNA-enzyme binding

When topo II α enzyme was preincubated with BM3 in a reaction buffer without ATP, the covalent DNA-binding capacity of the topo II α was reduced dramatically (Fig. 5). This result suggested that BM3 interacted with DNA-binding site of the enzyme or its catalytic site.

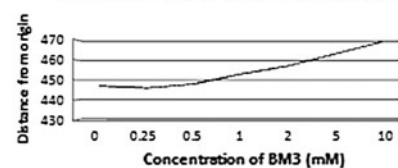
BM3's minor groove-binding ability protects DNA from DNase I digestion

DNase I enzyme binds to the minor groove of the double-stranded DNA and cuts one strand rapidly. Especially agents that bind to the DNA minor groove prevent DNase I digestion. BM3 was tested by the DNase I digestion assay to understand its DNA protection and DNA-binding abilities at same time. As expected, it was found that high concentrations (1 and 5 mM) of BM3 protected DNA (Fig. 6a, d). 100 µM BM3 showed only a partial protection. Like we

BM3 (mM)	-	-	0.25	0.5	1	2	5	10	-	10
0.5 µM EtBr	-	-	-	-	-	-	-	-	+	-
Sc DNA (0.1 µg)	+	+	+	+	+	+	+	+	+	+
Topo I (2 U)	-	+	+	+	+	+	+	+	+	-



Mobility changes of most relaxed DNA bands



	0	0.25	0.5	1	2	5	10
Mobility	447	446	448	453	457	463	469

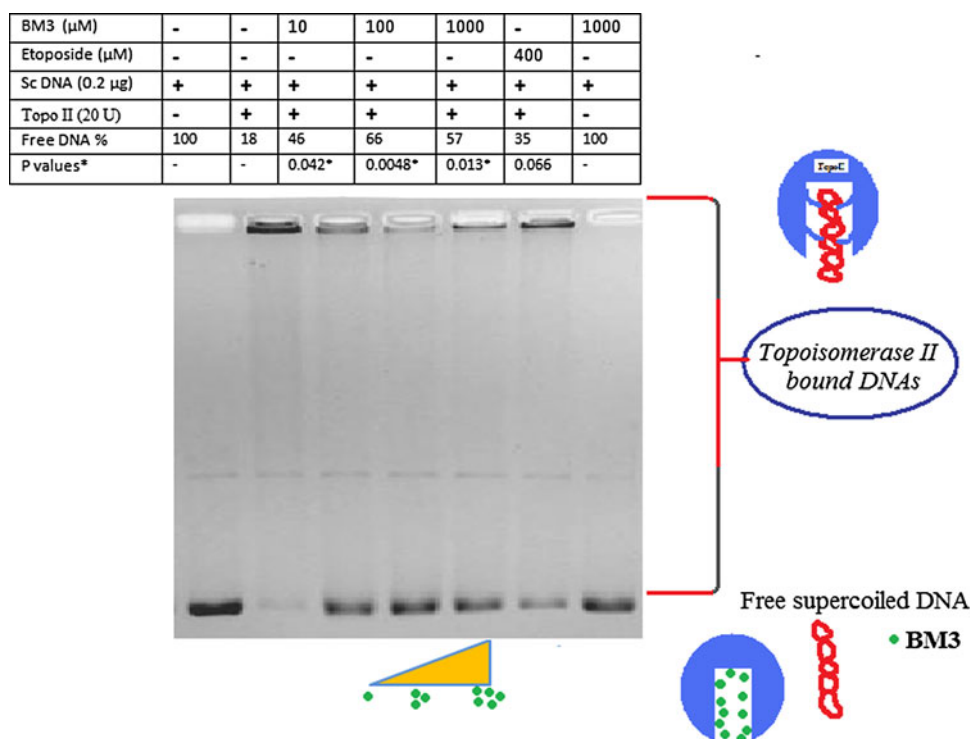
Fig. 4 DNA unwinding ability of BM3. **a**Topoisomerase I unwinding assay was carried out with 2 unites of topo I, 0.1 µg of supercoiled (Sc DNA) pBR322 plasmid DNA with or without BM3 (0.25, 0.5, 1, 2, 5, and 10 mM) and 0.5 µM EtBr. **b** A graphical demonstration for mobilities of more relaxed DNA bands after different concentration of BM3

discussed above, BM3 most probably accumulates on DNA minor grooves, depending on its amount, which indicated that a low concentration of BM3 could not be sufficient to affect DNA minor groove-binding enzymes like DNase I.

Preincubation of BM3 with DNase I or DNA implied that the compound bound either DNA or DNase I enzyme. Then, the DNA digestion pattern of BM3 was analyzed. Without the preincubation step, less DNA protection was observed in both experiments. BM3 was incubated with DNA or DNase I enzyme before the reaction for 1 h (Fig. 6b, e). Figure 6a clearly showed that BM3 protected DNA against DNase I digestion. Furthermore, when BM3 was preincubated with DNA, DNA protection was significantly higher than DNase I preincubation conditions. This result suggested that BM3 bound to DNA.

We also checked DNA protection properties of M10, which was the non-salt form (or main form) of BM3 to

Fig. 5 DNA-Enzyme interaction was interrupted by BM3. Electrophoretic mobility shift assay was carried out in the absence of ATP with different concentrations of BM3 (10, 100, 1,000 μM). 0.2 μg supercoiled pBR322, 20 unites of topo II α were used for 1 h at 37 $^{\circ}\text{C}$



compare DNA-binding abilities of these two compounds. Interestingly, we found that *N*-amino tosylated salt derivative, BM3, was more protective than its main compound M10 (Fig. 6c). In this regard, the *N*-tosyl group of the compound seemed to be an important part for DNA minor groove interaction of BM3.

Particular DNA sequence preference

Six different type II restriction endonuclease enzymes producing sticky or blunt-ended DNA fragments were chosen for investigation of BM3 sequence preference (Table 2). It was found that BM3 inhibited *Bam*HI and *Hind*III restriction endonucleases, while it did not affect the other enzymes when a 1-h incubation period was used (Fig. 7a, b). This result showed that BM3 could bind the 5'GGATCC3' palindromic recognition site of *Bam*HI or 5'AAGCTT3' sequence of *Hind*III. The recognition sites of these two enzymes share the property that there are 3 purines followed by 3 pyrimidines at the ends of the sequences. When we used a longer incubation period, the inhibition potential of BM3 was decreased. We confirmed this theory by cutting DNA by *Bam*HI and *Hind* III enzymes at the same time (double digestion) for at least 2 h of incubation (Fig. 7d). There was a detectable decrease in the inhibitory effect of BM3 on *Eco*RI-*Bam*HI (Fig. 7c) or *Hind*III-*Bam*HI double digestions. This implied that binding of BM3 to these (5'GGATCC3' or 5'AAGCTT3') sequences was reversible. So, with this finding, we

provided more evidence for the non-covalent DNA-binding activity of BM3. It is known that the compounds that are carrying the benzothiazole ring system have anticancer and DNA-binding abilities. The researchers used *Bam*HI for a restriction endonuclease digestion (RED) assay and they found the GC-rich binding ability of benzothiazole derivatives (Kamal *et al.*, 2010). When we tested BM3 by the RED assay for six different restriction endonucleases, we found similar results for *Bam*HI besides *Hind*III. Furthermore, it was shown that benzothiazole derivatives protect 83 % of DNA from hydroxyl radical breakages and they completely protect DNA from gamma ray breakages (Prouillac *et al.*, 2009).

BM3 interrupts in vitro replication

PCR was used to confirm the DNA-binding activity of BM3. The experimental data suggested that a high concentration (5 mM) of BM3 blocked in vitro replication. In the presence of BM3, the activity of *Taq* polymerase decreased in a concentration-dependent manner. BM3 did inhibit the PCR reaction, but this inhibitory effect was less profound than doxorubicin or ethidium bromide (100 and 96 % of the reaction, respectively). It could be seen in Fig. 8 that 0.1, 1, and 5 mM of BM3 inhibited 8, 45, and 60 % of the reaction, respectively, while 0.4 mM etoposide, 10 $\mu\text{g}/\text{mL}$ CPT, and 0.5 mM mAMSA inhibited 46, 56, and 40.6 % of the reaction, respectively. These findings suggested that BM3 was more effective than known

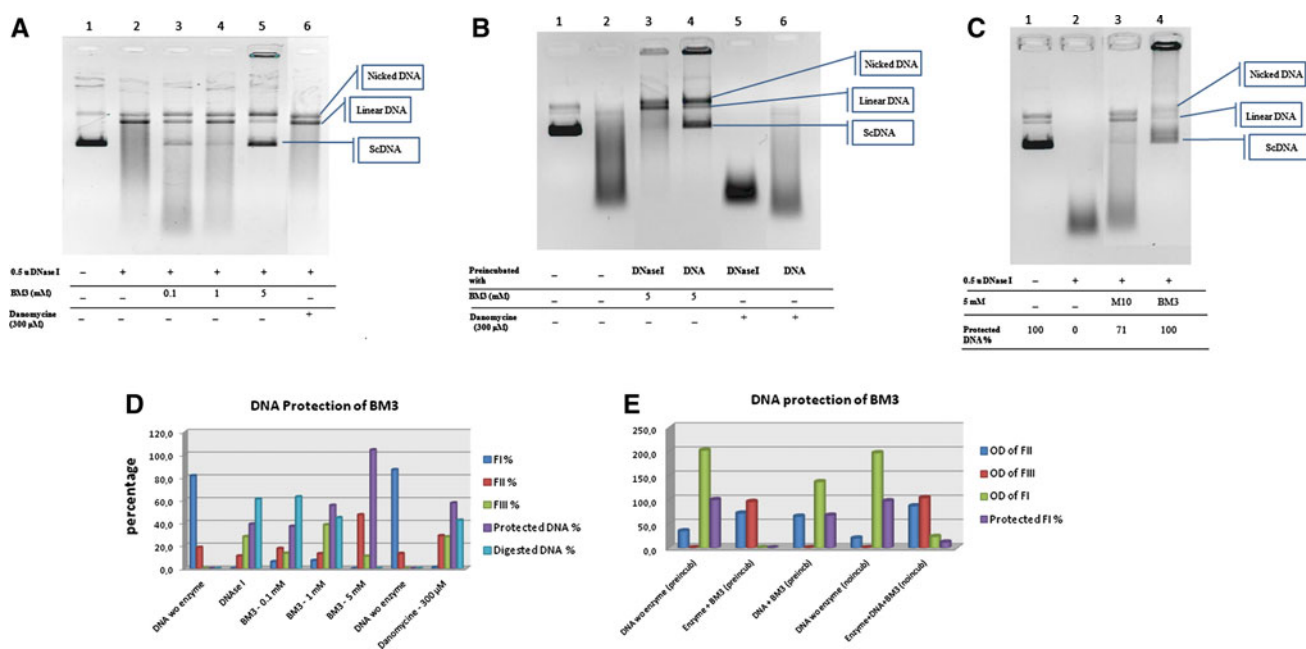


Fig. 6 DNA protection activities of the compounds. 0.5 unite of DNase I and 0.5 µg supercoiled pBR322 plasmid DNA, 300 µM daunomycine (minor groove binder) were used for all DNase I digestion experiments. **a** Effect of different concentrations of BM3 (0.1.1 and 5 mM). **b** 5 mM BM3 was preincubated with DNA or

DNase I for 1 h at 37 °C before the reaction. **c** Comparing protection abilities of BM3 and M10. **d** Graphical demonstration of DNA protection percentage of different concentrations of BM3. **e** Graphical demonstration of affects of different preincubations of BM3 before DNase I digestion reaction

Table 2 Information about restriction endonuclease enzymes used in the study

Enzyme name	Enzyme unites	Cut position on pBR322 plasmid DNA	Recognition sites
<i>EcoRI</i>	1	1	5'-G↓AATTC-3' 3'-CTTAA↑G-5'
<i>BamHI</i>	3	375	5'-G↓GATCC-3' 3'-CCTAG↑G-5'
<i>HindIII</i>	1	29	5'-A↓AGCTT-3' 3'-TTCGA↑A-5'
<i>PvuII</i>	3	2,064	5'-CAG↓GTC-3' 3'-GTC↑CAG-5'
<i>ScaI</i>	3	3,844	5'-AGT↓TCA-3' 3'-TCA↑AGT-5'
<i>VspI</i>	3	3,537	5'-AT↓TAAT-3' 3'-TAAT↑TA-5'

topoisomerase inhibitors such as etoposide, amsacrine (mAMSA) (topo IIα inhibitor), and camptothecin (topo I inhibitor), but it was less effective than DNA-binding agents (EtBr, doxorubicin).

Conclusion

This study was aimed to screen different benzothiazole-derived potential topo I/IIα inhibitors to find their associated IC₅₀ values. It was found that all tested 2-(substituted-phenyl/

benzyl)benzothiazole derivatives were strong DNA topo IIα inhibitors instead of topo I. Among the tested benzothiazoles, only 3-amino-2-(2-bromobenzyl)-1,3-benzothiazol-3-ium 4-methylbenzenesulfonate (BM3) showed the most potent inhibition against human topo II α enzyme with an IC₅₀ of 39 nM, even if it was found to be more effective than the standard etoposide. Therefore, the mechanism of topo II α inhibition of BM3 was investigated. According to further studies, it could be stated that BM3 was a catalytic inhibitor of topo IIα, neither a poison of topoisomerases nor an intercalator. Moreover, the results of EMSA stated that BM3

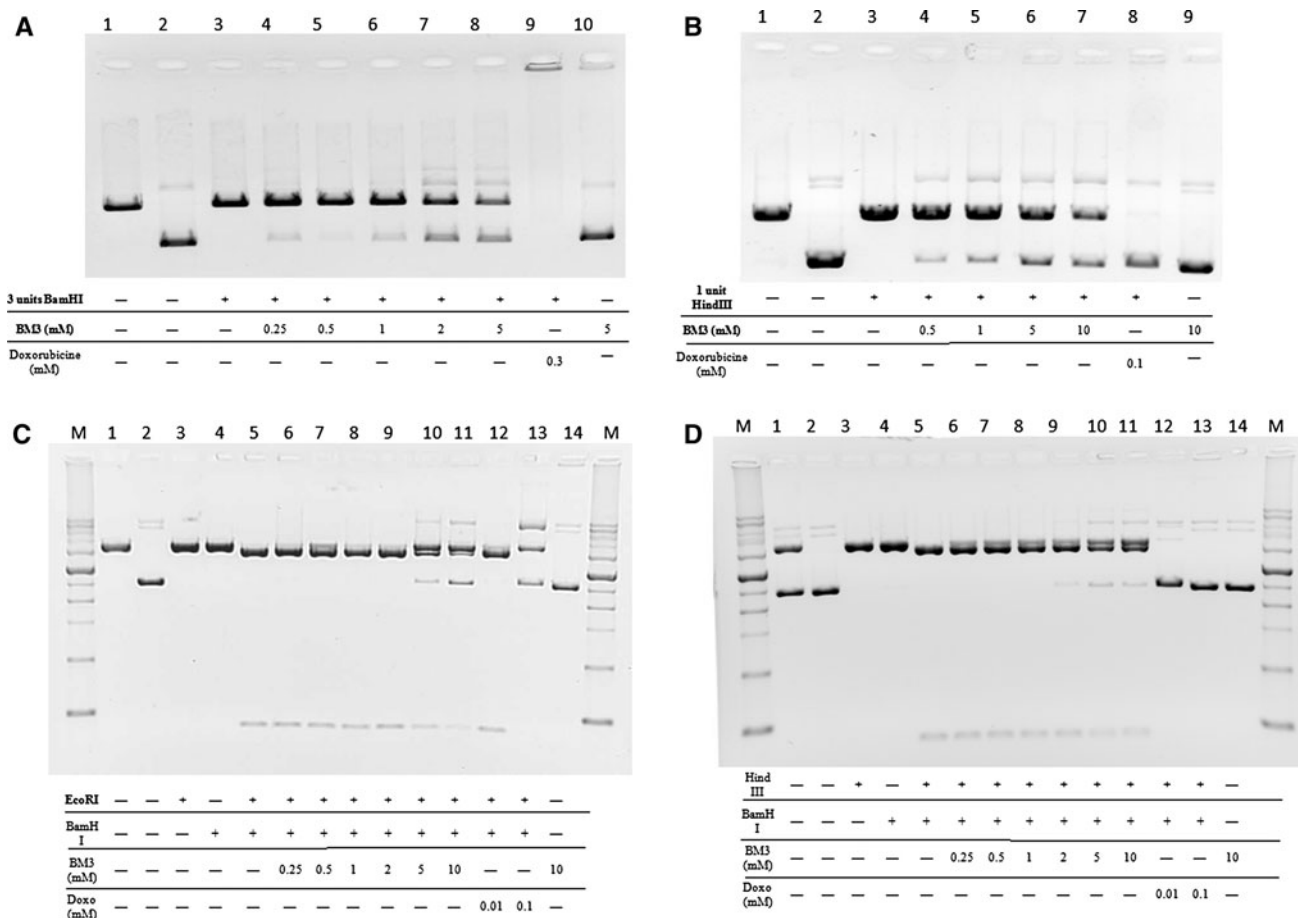
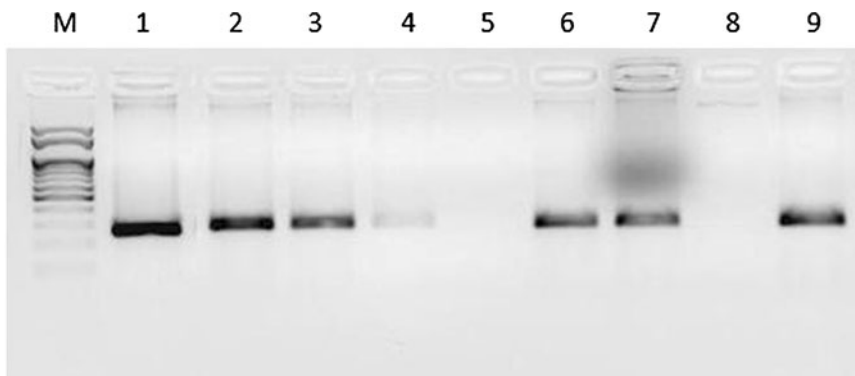


Fig. 7 RED assay results of BM3, 0.5 µg supercoiled pBR322 DNA was used for all RED assay experiments. *Bam*HI (a) and *Hind*III (b) inhibition properties of BM3 were tested. *Eco*RI-*Bam*HI (c) and

*Hind*III-*Bam*HI (D) double digestions of DNA were carried out with different concentrations (0.25, 0.5, 1, 2, 5, 10 mM) of BM3

Fig. 8 Effect of BM3 on in vitro replication. PCR reaction mix was prepared firstly, then all drugs were added into the reaction except Taq polymerase enzyme which was added to the reaction just before the PCR machine was started. Band density of the PCR product was used to calculate inhibition percentages. *indicates there is a significant difference (Student's *t* test, $p < 0.05$)



	Mrk	PCR product (147 bp)	BM3 100µM	BM3 1 mM	BM3 5 mM	Doxo 300µM	ETOP 400µM	CPT 10µg/µl	EtBR 30µM	mAMSA 500µM
Average OD	-	210	193	116	45	0	114	92	8,3	125
Std dev.	-	17	21	47	35	0	10	6	12	12
P values	-	-	0,13	0,01*	0,0004*	7xE-5*	0,001*	0,0001*	0,0002*	0,0026*
Inhibition %	-	0	8	45	60	100	45,7	56,3	96,1	40,6

played a preventable role in the DNA-binding ability of topo II α . It could be concluded that this blockage of BM3 was found to be due to direct interaction of BM3 with topo II α , but not to its interaction with DNA. However, higher doses of BM3 can also accumulate on minor grooves of DNA and this might be in a sequence-specific manner.

The National Cancer Institute (NCI) pointed out that benzothiazoles had anticarcinogenic activity with an unknown mechanism that has not been discovered yet. In here, we provided certain evidence that the inhibitory activity on topoisomerases of benzothiazoles showed through a different mechanism than commercial anticancer drugs (daunomycin, doxorubicin, etoposide, camptothecin). Furthermore, this study could bring up a new anticancer drug candidate, BM3, with a new anticarcinogenic mechanism. Up to now, there have been several benzothiazole-based anticancer drug candidates with unknown mechanisms of action. We believe that the main target of benzothiazoles could be topo II α in cancer cells. Moreover, the non-intercalative DNA-binding activity of BM3 and BM4 provides hints to design new non-toxic DNA-binding compounds.

Experimental procedures

Materials

Reagents for electrophoresis, camptothecin, etoposide, *Eco*RI restriction endonuclease, and the other chemicals were obtained from Sigma (Munich, Germany). Calf thymus DNA topo I and recombinant human DNA topo II α were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). Supercoiled pBR322 plasmid DNA, 6 \times loading buffer, and the other restriction enzymes were purchased from Fermentas (Prizma Lab, Ankara, Turkey). DMSO (molecular biology grade) was obtained from Merck KgaA (Darmstadt, Germany). Proteinase K was obtained from Genaxis (Hessen, Germany). Bovine pancreas DNase I, T4 DNA ligase, and Taq DNA polymerase enzymes were obtained from New England BioLabs (NEB, UK). M13mp18 single-stranded DNA was also purchased from NEB. All benzothiazole derivatives were dissolved in DMSO and freshly prepared just before the experiments. DMSO compatibility tests were carried out for all experiments and 1 % DMSO was used.

Plasmid DNA relaxation by topo I and topo II α

Relaxation assay was performed using methods described in the literature (Hsiang *et al.*, 1985; Halligan *et al.*, 1985). Camptothecin (CPT) and etoposide were used as positive controls for topo I and for topo II α , respectively.

Topo I- and II α -mediated DNA cleavage assay

Cleavage assays were performed using methods described in the literature (Halligan *et al.*, 1985; Umemura *et al.*, 2003). CPT and etoposide were used as positive controls for topo I and for topo II α , respectively.

Electrophoretic mobility shift assay (EMSA)

To reveal the effect of the tested compound on the interaction between topo II α enzyme and its DNA substrate, EMSA was used in the absence of ATP (adenosine 5'-triphosphate). The experimental protocol was carried out as Genaxis described in the literature (Syrovest *et al.*, 2000; Meng *et al.*, 2001). Etoposide was used as a positive control. All experiments were carried out at least three times. The averages of supercoiled DNA bands were used to calculate % of free DNA.

Intercalation potential

DNA topo I and T4 DNA ligase unwinding assays were performed as described in the literature (Meng *et al.*, 2001). Ethidium bromide (0.5 μ M) was used as a positive intercalator.

DNA protection (DNA minor groove binding)

In order to reveal the DNA minor groove-binding potential of the tested compounds (BM3, M10), CPT and etoposide were used as positive controls for topo I and for topo II α , respectively, and the degree of protection of input DNA from DNase I digestion was examined. DNase I enzyme normally cuts all types of DNA randomly. It binds to the minor groove of its substrate (Kochanek *et al.*, 1993). We therefore assumed that if our compound had an ability to bind to the minor groove of DNA, it protected the DNA from digestion of DNase I. The experimental procedure was as follows: 20 μ L of reaction buffer containing 10 mM Tris-HCl, 2.5 mM MgCl₂, 0.5 mM CaCl₂, pH 7.6 with 0.05 % BSA, 0.5 μ g supercoiled pBR322 plasmid DNA, and the test compound was incubated at 37 $^{\circ}$ C for 1 h without DNase I. To start the digestion, 0.05 units of bovine pancreas DNase I were added and the reaction mixture was incubated for 5 min. 25 mM final concentration of Na₂EDTA was used for stopping the DNase I activity. Then, 6 \times DNA loading buffer was added into the mixture. 15 μ L of sample was loaded onto 1 % agarose gel (containing 0.5 μ g/mL EtBr) in TAE (Tris Acetate EDTA, pH8.0), pH 8.3. DNA samples were electrophoresed at 40 V for 1.5 h. Agarose gels were rinsed for 30 min. CPT and etoposide were used as positive controls for topo I and for topo II α , respectively, with water and were

photographed under the UV light by KODAK MI imaging system. Daunomycin was used as a minor groove binder.

Specific DNA sequence protection

In order to find out whether the BM3 DNA binding was specific or not, another experimental protocol with type II restriction endonuclease (RE) enzymes was designed. We chose 6 different types of RE enzymes that create sticky or blunt ends and recognize homogenous or heterogeneous DNA sequences. In brief, 0.5 μg supercoiled pBR322 DNA and the test compound were preincubated at 37 °C for 1 h. Then, RE enzyme was added to the reaction and the mixture was incubated for 1 h (2 h for double digests) at 37 °C. Reactions were stopped either by heat inactivation or phenol extraction. DNA samples were separated by 1 % agarose gel electrophoresis with EtBr at 45 V for 1.5 h. We used daunomycin or doxorubicin (doxo) as positive controls.

In vitro replication blocking

We performed polymerase chain reaction with universal primers (forward: 5'-GTTTTCCAGTCACGACGTTGTA-3'; reverse: 5'-TTGTGAGCGGATAACAATTC-3') of M13mp8 phage DNA. BM3 was added to complete the PCR reaction mix, and amplification of DNA was performed on a PCR machine. Amsacrine (mAMSA, 500 μM), doxo (300 μM), CPT (10 $\mu\text{g}/\mu\text{L}$), etoposide (Etop, 400 μM), and ethidium bromide (EtBr, 30 μM) were used as positive and negative controls for the experiments.

Analysis of data

Comparisons of the optical densities of the DNA bands with or without the tested compounds were used to find inhibitory percentages (Sobhani *et al.*, 2002). With these percentages, IC_{50} values of the compounds were calculated using the Sprobit analysis program. According to the literature, a compound with 50 % inhibitory effect on topoisomerases could be accepted as an inhibitor (Cho *et al.*, 2000). The remaining supercoiled DNA band intensities were used for evaluation of the relaxation assay and for calculating inhibitory percentages. The average of the supercoiled DNA intensities from three independent experiments without enzyme was accepted as 100 % inhibition. If topo did not relax all supercoiled DNA, we got the difference between control supercoiled DNA (without enzyme) and the remaining supercoiled DNA (without compound). We compared these values with the remaining supercoiled DNA of the compounds. For cleavage assay evaluation, we used nicked DNA band intensities for topo I and linear DNA intensities for topo II. We calculated all band intensities

(nicked + linear (if present) + supercoiled + relaxed) and compared these intensities to calculate the cleavage percentage of DNA of the compounds. For DNA unwinding assays, we compared DNA band patterns of compounds to a well-known intercalator, ethidium bromide. In addition, for the topo I unwinding assay, we also used DNA migration values from the origin of the agarose gel wells. Enzyme-bound DNA bands always migrated more slowly than free DNA on the agarose gel. That was why we compared bound and free DNA intensities for EMSA assay evaluations. The calculation of percentage DNase I inhibition of the compounds was done using the remaining DNA band intensities. It was assumed that the total uncut pBR322 plasmid DNA band intensities were 100 % (the control). After the digestion of DNase I with a compound, we compared the total band intensities with the control. Protection from either single or double restriction endonuclease digestions was determined by comparing DNA band patterns with or without the compound. In vitro replication prevention was also determined mathematically. Averages of the band intensities of PCR products were calculated to reach percentages. For all experimental data, we also calculated standard deviations. Student's *t* test was used to compare the results of EMSA and in vitro replication assays ($p < 0.05$ or $p < 0.1$).

Acknowledgments We thank TÜBİTAK (The Scientific and Technological Research Council of Turkey) for supporting this research as a 105T081 project. We are also very grateful to Dr. Felipe Cortés and his research group in Seville, Spain, for their help in optimization of topo II experiments.

References

- Bailly C, Qu X, Anizon F, Prudhomme M, Riou J-F, Chaires JB (1999) Enhanced binding to DNA and topoisomerase I inhibition by an analog of antitumor antibiotic rebeccamycin containing an amino sugar residue. *Mol Pharmacol* 55:377–385
- Bradshaw TD, Chua MS, Browne HL, Trapani V, Sausville EA, Stevens MFG (2002) In vitro evaluation of amino acid prodrugs of novel antitumor 2-(4-amino-3-methylphenyl) benzothiazoles. *Br J Cancer* 86:1348–1354
- Castelli M, Malagoli M, Lupo L, Riccomi TR, Casolari C, Cermelli C, Zanca A, Baggio G (2001) Antiviral and antiproliferative activity in vitro of some new benzimidazole derivatives. *Pharmacol Toxicol* 88:67–74
- Cho KH, Pezzuto JM, Bolton JL, Steele VE, Kelloff GJ, Lee SK, Constantinou A (2000) Selection of cancer chemopreventive agents based on inhibition of topoisomerase II activity. *Eur J Cancer* 36:2146–2156
- Collins TR, Hammes GG, Hsieh TS (2009) Analysis of the eukaryotic topoisomerase II DNA gate: a single-molecule FRET and structural perspective. *Nucleic Acids Res* 37(3):712–720
- Cortés F, Pastor N, Mateos S, Dominguez I (2003) Roles of DNA topoisomerases in chromosome segregation and mitosis. *Mut Res* 543:59–66
- Halligan BD, Edwards KA, Liu LF (1985) Purification and characterization of type II DNA topoisomerase from bovine calf thymus. *J Biol Chem* 260(4):2475–2482

- Hsiang YH, Hertzberg R, Hecht S, Liu LF (1985) Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J Biol Chem* 260(27):14873–14878
- Kamal A, Reddy KS, Khan MNA, Shetti RVCRCNC, Ramaiah MJ, Pushpavalli SNCVL, Srinivas C, Pal-Bhadra M, Chourasia M, Sastry GN, Juverkar A, Zingde S, Barkume M (2010) Synthesis, DNA-binding ability and anticancer activity of benzothiazole/benzoxazole-pyrrolo[2,1-c][1,4]benzodiazepine conjugates. *Bioorg Med Chem* 18:4747–4761
- Kawiak A, Piosik J, Stasiłojc G, Gwizdek-Wisniewska A, Marczak L, Stobiecki M, Bigda J, Lojkowska E (2007) Induction of apoptosis by plumbagin through reactive oxygen species-mediated inhibition of topoisomerase II. *Toxicol Appl Pharmacol* 223(3):267–276
- Kochanek S, Renz D, Doerfler W (1993) Differences in the accessibility of methylated and unmethylated DNA to DNase I. *Nucleic Acid Res* 21(25):5843–5845
- Li J, Xu M, Su W, Gong X (2010) 3-(4-(Benzo[d]thiazol-2-yl)-1-phenyl-1H-pyrazol-3-yl) phenyl acetate induced Hep G2 cell apoptosis through a ROS-mediated pathway. *Chem Biol Interact* 183:341–348
- Meng L, Ding J (2007) Salvicine, a novel topoisomerase II inhibitor, exerts its potent anticancer activity by ROS generation. *Acta Pharmacol Sin* 28(9):1460–1465
- Meng L, Zhang J, Ding J (2001) Salvicine, a novel DNA topoisomerase II inhibitor, exerting its effects by trapping enzyme–DNA cleavage complexes. *Biochem Pharmacol* 62:733–741
- Nelson EM, Tewey KM, Liu LF (1984) Mechanism of antitumor drugs. Poisoning of mammalian DNA topoisomerase II on DNA by an antitumor drug m-AMSA. *Proc Natl Acad Sci USA* 81:1361–1365
- Pinar A, Yurdakul P, Yıldız İ, Temiz-Arpaçlı Ö, Açıkan NL, Akı-Sener E, Yalçın İ (2004) Some fused heterocyclic compounds as eukaryotic topoisomerase II inhibitors. *Biochem Biophys Res Commun* 317:670–674
- Pommier Y, Kohlhagen G, Laco GS, Kroth H, Sayer JM, Jerina DM (2002) Different effects on human topoisomerase I by minor groove and intercalated deoxyguanosine adducts derived from two polycyclic aromatic hydrocarbon diol epoxides at or near a normal cleavage site. *J Biol Chem* 277(16):13666–13672
- Prouillac C, Vicendo P, Garrigues JC, Poteau R, Rima G (2009) Evaluation of new thiadiazoles and benzothiazoles as potential radioprotectors: Free radical scavenging activity in vitro and theoretical studies (QSAR, DFT). *Free Radic Biol Med* 46:1139–1148
- Racané L, Kralj M, Suman L, Stojković R, Tralić-Kulenović V, Karminski-Zamola G (2010) Novel amidino substituted 2-phenylbenzothiazoles: synthesis, antitumor evaluation in vitro and acute toxicity testing in vivo. *Bioorg Med Chem* 18(3):1038–1044
- Reddy BSP, Sondhi SM, Lown JW (1999) Synthetic DNA minor groove-binding drugs. *Pharmacol Ther* 84:1–111
- Saeed S, Rashid N, Jones PG, Ali M, Hussain R (2010) Synthesis, characterization and biological evaluation of some thiourea derivatives bearing benzothiazole moiety as potential antimicrobial and anticancer agents. *Eur J Med Chem* 45(4):1323–1331
- Shi DF, Bradshaw TD, McCall S, Lelieveld P, Fichtner I, Stevens MFG (1996) Antitumor benzothiazoles. 3.1 synthesis of 2-(4-aminophenyl) benzothiazoles and evaluation of their activities against breast cancer cell in vitro and in vivo. *J Med Chem* 39:3375–3384
- Sobhani AM, Ebrahimi S-A, Mahmoudian M (2002) An in vitro evaluation of human DNA topoisomerase I inhibition by *Peganum harmala* L seeds extract and its β -carboline alkaloids. *J Pharm Pharmaceut Sci* 5(1):19–23
- Solomon VR, Hu C, Lee H (2009) Hybrid pharmacophore design and synthesis of isatin-benzothiazole analogs for their anti-breast cancer activity. *Bioorg Med Chem* 17:7585–7592
- Soni B, Ranawat MS, Sharma R, Bhandari A, Sharma S (2010) Synthesis and evaluation of some new benzothiazole derivatives as potential antimicrobial agents. *Eur J Med Chem* 45(7):2938–2942
- Sovenyhazy KM, Bordelon JA, Petty JT (2003) Spectroscopic studies of the multiple binding modes of a trimethine-bridged cyanine dye with DNA. *Nucleic Acid Res* 31(10):2561–2569
- Störl K, Burckhardt G, Lown JW, Zimmer C (1993) Studies on ability of minor groove binders to induce supercoiling in DNA. *FEBS Lett* 334(1):49–54
- Syrovest T, Büchele B, Gedig E, Slupsky JR, Simmet T (2000) Acetyl-boswellic acids are novel catalytic inhibitors of human topoisomerases I and II α . *Mol Pharmacol* 58:71–81
- Tekiner-Gulbas B, Temiz-Arpaçlı O, Yıldız İ, Akı-Sener E, Yalçın İ (2006) 3D-QSAR study on heterocyclic topoisomerase II inhibitors using CoMSIA. *SAR QSAR Environ Res* 17(2):121–132
- Tekiner-Gulbas B, Filak L, Vasko GA, Egyed O, Yalçın İ, Akı-Sener E, Riedl Z, Hajos G (2008) Synthesis of novel pyrazolo[5,1-b][1,3]benzothiazoles: a new pericyclic pathway. *Heterocycles* 75:2005–2012
- TUBITAK/TBAG-U/76 (103T089) Project, Synthesis of New Aminazoles as Potential Multi-Drug Resistance Inhibitors, 2003–2006, Yalçın İ (Project coordinator)
- Umemura K, Yanase K, Suzuki M, Okutani K, Yamori T, Andoh T (2003) Inhibition of DNA topoisomerases I and II, and growth inhibition of human cancer cell lines by a marine microalgal polysaccharide. *Biochem Pharmacol* 66:481–487
- Wang JC (2002) Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol* 3(6):430–440