

# Switch in Site of Inhibition: A Strategy for Structure-Based Discovery of Human Topoisomerase II $\alpha$ Catalytic Inhibitors

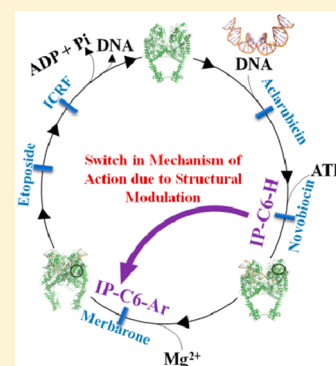
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## Supporting Information

**ABSTRACT:** A study of structure-based modulation of known ligands of hTopoII $\alpha$ , an important enzyme involved in DNA processes, coupled with synthesis and in vitro assays led to the establishment of a strategy of rational switch in mode of inhibition of the enzyme's catalytic cycle. 6-Arylated derivatives of known imidazopyridine ligands were found to be selective inhibitors of hTopoII $\alpha$ , while not showing TopoI inhibition and DNA binding. Interestingly, while the parent imidazopyridines acted as ATP-competitive inhibitors, arylated derivatives inhibited DNA cleavage similar to merbarone, indicating a switch in mode of inhibition from ATP-hydrolysis to the DNA-cleavage stage of catalytic cycle of the enzyme. The 6-aryl-imidazopyridines were relatively more cytotoxic than etoposide in cancer cells and less toxic to normal cells. Such unprecedented strategy will encourage research on "choice-based change" in target-specific mode of action for rapid drug discovery.



**KEYWORDS:** Human topoisomerase II $\alpha$ , anticancer agent, chemotype-modulation, drug discovery

Toward rapid finding of new chemical entities (NCE), the structural modulation of a known drug or bioactive agent has been recognized as a valuable approach.<sup>1,2</sup> Many times, structurally derived compounds have been found to interact with a new molecular target, rather than an original one. Podophyllotoxin is tubulin polymerization inhibitor,<sup>3</sup> whereas its synthetic derivative etoposide is a topoisomerase II-inhibitor (TopoII). In contrast, the structural modulation leading to identification of a new agent that interacts with the same target at different binding site is rare in literature. The examples of such approach are the development of allosteric modulators of the G-protein coupled receptors<sup>4</sup> and structural modulation of ROR $\alpha$  inverse agonistic tertiary sulfonamides, leading to agonistic effects.<sup>5</sup>

DNA topoisomerase maintains the DNA topology and thus plays a crucial role in various DNA processes.<sup>6</sup> About 50% of chemotherapeutic regimens use at least one drug that targets these enzymes.<sup>7</sup> There are several hTopoII $\alpha$ -targeting anticancer drugs. Etoposide, teniposide, doxorubicin, and mitoxantrone form ternary complex with the enzyme and DNA and are called hTopoII $\alpha$  poisons. The hTopoII $\alpha$  catalytic inhibitors (Scheme 1a) hamper the enzyme–DNA cycle in the stages other than ternary complex formation.<sup>8</sup> Topoisomerase II poisons were found to be responsible for triggering secondary leukemias by causing certain chromosomal translocations.<sup>8</sup> In contrast TopoII catalytic inhibitors are known to modulate the cytotoxic effects of the poisons and the alkylating agents and

overcome the multidrug resistance (MDR).<sup>9</sup> Many catalytic inhibitors are now clinically used in combination therapy, such as aclarubicin,<sup>10</sup> MST-16,<sup>11</sup> ICRF-187,<sup>12</sup> suramin,<sup>13</sup> and novobiocin.<sup>14</sup> Merbarone has a unique feature of being the only agent that causes inhibition of TopoII-mediated cleavage of DNA without affecting protein–DNA binding.<sup>15</sup>

In a rational structural modulation of a known hTopoII $\alpha$  catalytic inhibitor class of compounds, imidazo-pyridines/pyrazines,<sup>16,17</sup> our initial docking studies revealed an astonishing important feature. The C2-biaryl and C6-aryl derivatizations implied the possibility of a switch in binding with hTopoII $\alpha$  from the ATP-hydrolysis to DNA cleavage stage of the catalytic cycle. This prompted us to consider a study of detailed exploration/understanding of molecular interactions of the hTopoII $\alpha$  with DNA or known ligands and structure-based rational modulations of the ligands. The present work illustrates an unprecedented target-specific ligand-structural modulation approach that provides "choice-based change" in the mode of inhibition of an enzyme, hTopoII $\alpha$ .

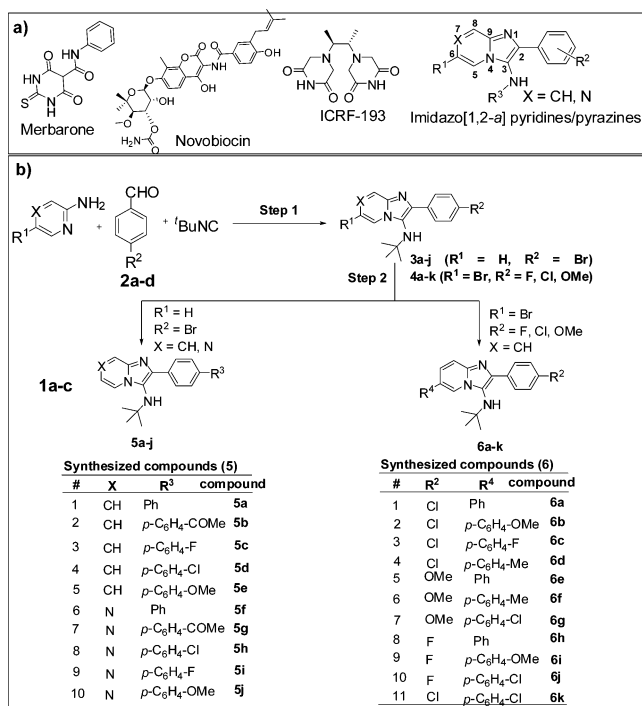
In the preparation of target compounds (**5** and **6**, Scheme 1b) by arylation at C2-aryl and C6 of imidazo[1,2-*a*]-pyridines/pyrazines, 2-(4-bromophenyl) substituted imidazo-pyridines/pyrazines (**3**) and 6-bromo-imidazopyridines (**4**) as respective

**Received:** February 1, 2015

**Accepted:** February 23, 2015

**Published:** February 23, 2015

**Scheme 1. (a) Representative hTopoII $\alpha$  Catalytic Inhibitors;<sup>16</sup> (b) Synthesis of Imidazo-pyridine/pyrazine Derivatives (5 and 6)<sup>a</sup>**



<sup>a</sup>Reagents and conditions: Step 1: ZrCl<sub>4</sub> (10 mol %), *n*-BuOH, 50 °C, 3–4 h.<sup>18,19</sup> Step 2: R<sub>3</sub>/R<sub>4</sub>-B(OH)<sub>2</sub> (1.3 equiv), Pd(PPh<sub>3</sub>)<sub>4</sub> (10 mol %), TBAB (1 equiv), Na<sub>2</sub>CO<sub>3</sub> (5 equiv), DMA-H<sub>2</sub>O (1:1), 105 °C, 3–4 h.<sup>20</sup>

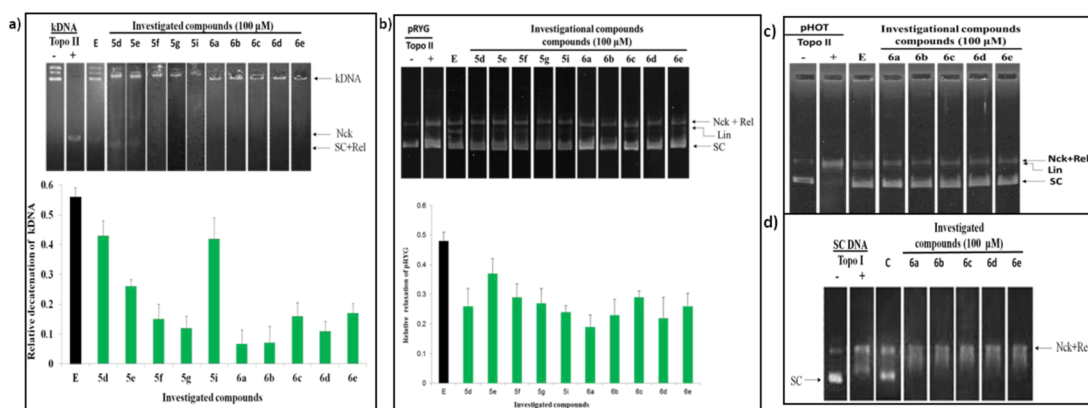
precursors were considered. Compounds 3 were prepared by a ZrCl<sub>4</sub>-catalyzed Ugi–Strecker-type multicomponent reaction of 2-aminopyridine/pyrazine with 4-bromobenzaldehyde and *tert*-butyl isocyanide.<sup>18</sup> Compounds 4 were synthesized using 5-bromo-2-aminopyridine. Pd-catalyzed Suzuki coupling of various boronic acids with compounds 3 and 4 produced imidazo[1,2-*a*]pyridines/pyrazines (5a–j and 6a–k), respectively, in good yields.<sup>20</sup>

The hTopoII $\alpha$ -inhibitory activities of synthesized compounds were investigated by *in vitro* ATP-dependent decatenation and relaxation assays in agarose gel electrophoresis.<sup>16</sup> In agarose gel, catenated kinetoplast DNA (kDNA)

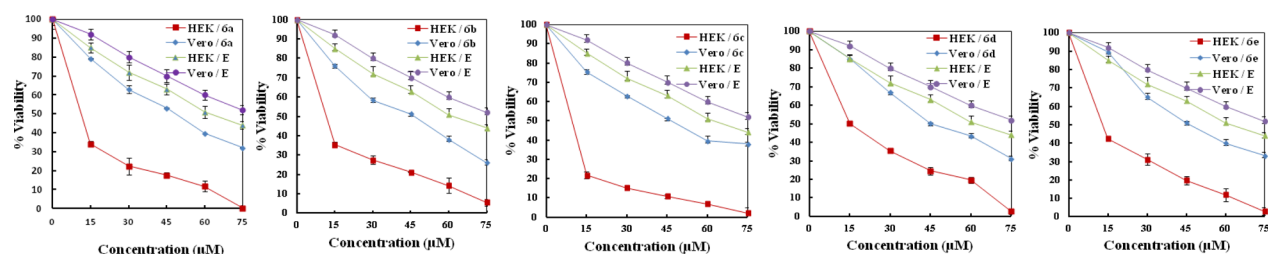
cannot enter due to its large size and appears at the top; whereas the decatenated products (nicked (Nck), relaxed (Rel), and supercoiled (SC) DNA) move easily into the gel.<sup>16,21</sup> Interestingly, compared to etoposide, negligible decatenated products were observed in the presence of compounds 5f, 5g, and 6a–e, and relatively less decatenation occurred for compounds 5d, 5e, and 5i (Figure 1a). In the relaxation assay, variably relaxed topoisomers of negatively supercoiled DNA (pRYG) were observed, which migrated relatively slow as compared to the supercoiled form in agarose gel (Figure 1b). The hTopoII $\alpha$ -relaxation inhibitory activities of the compounds 5d–g, 5i, and 6a–e were found to be in accordance with their hTopoII $\alpha$  decatenation activities. The results of decatenation and relaxation assay indicated that C6-arylated imidazopyridine derivatives 6a–e were potent inhibitors and that compound 6a was most potent (IC<sub>50</sub> was 55.37  $\mu$ M; Figure S1).

Topoisomerase II poisons such as etoposide stabilizes the enzyme–DNA complex and thus generates linear DNA (Lin). In our study of cleavage complex assay, the Lin form of the DNA was observed for etoposide, but not for investigated compounds 6a–e (Figure 1c), which indicated their hTopoII $\alpha$  catalytic inhibition property not as poison. Human topoisomerase I (hTopoI)-mediated relaxation assay<sup>22</sup> for compounds 6a–e showed their nonsignificant activity for inhibition of the enzyme (Figure 1d). This indicated that the compounds were selective for catalytic inhibition of hTopoII $\alpha$ .

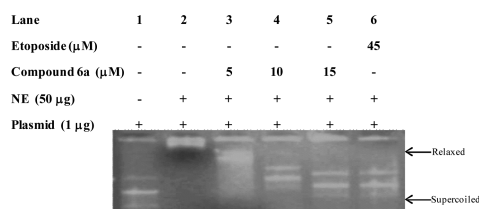
To investigate the cytotoxicity of compounds (6a–e), a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed using representative human embryonic kidney cancer (HEK 293) and normal monkey kidney epithelial (Vero) cell lines.<sup>16,23</sup> With increasing concentration of the investigated compounds, the cell viability of HEK 293 significantly decreased in comparison to Vero cells. Compounds killed 50% HEK 293 cells (LC<sub>50</sub>) at 10, 10, 8, 15, and 13  $\mu$ M, respectively (Figure 2). Interestingly, more than 30% cells survived even after exposure to 75  $\mu$ M concentration of compounds 6a–e in Vero cells; whereas etoposide showed LC<sub>50</sub> at 55  $\mu$ M for HEK 293 cells and at 75  $\mu$ M for Vero cells. To investigate the topoisomerase II inhibitory properties of the compounds directly in cancer cells, we studied an electrophoretic assay using nuclear lysate of HEK 293 cells (Figure 3). The untreated lysate showed the highest topoisomerase activity (lane 2), and the increasing movement of DNA entering into the gel was noted with increasing concentration (5–15  $\mu$ M) of compound 6a (lane 3–5), which revealed an increasing



**Figure 1.** (a) Decatenation assay, (b) relaxation assay, (c) cleavage complex assay, and (d) Topo I-mediated relaxation assay of imidazo-pyridines/pyrazines.



**Figure 2.** MTT assay: The signs  $\blacksquare$ ,  $\blacklozenge$ ,  $\blacktriangle$ , and  $\bullet$  represent cell survival for HEK-293/investigated, Vero/investigated, HEK-293/etoposide, and Vero/etoposide, respectively.



**Figure 3.** Inhibition of topoisomerase-activity by compound **6a** in HEK 293 cells.

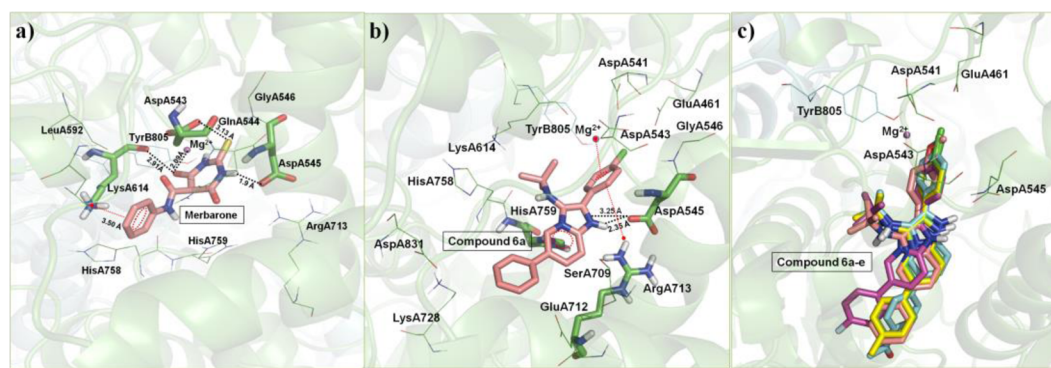
inhibitory activity of hTopoII $\alpha$ -mediated relaxation of DNA. In comparison with etoposide (45  $\mu\text{M}$ , lane 6), the inhibitory-activity of compound **6a** was found to be significant. We then studied to find out the stage at which these novel compounds inhibited the catalytic cycle of hTopoII $\alpha$ . The catalytic inhibitor that binds with DNA changes its conformation while binding and thus prevents it from interacting with topoisomerase II in the first step of the catalytic cycle (e.g., aclarubicin, a DNA intercalating agent). The absorbance study in DNA binding assay and the DNA intercalation assay revealed that there was little or no significant binding affinity of the compound **6a** with DNA (see Supporting Information for details, Figure S2).

From quantum chemical studies, these imidazopyridine derivatives were established to be basic in nature (see Supporting Information, Tables S1 and S2). Therefore, their N1-protonated forms were considered for molecular modeling studies. A primary structural analysis supported by the in vitro ATPase inhibition assay (see Supporting Information for details, Figure S3) indicated that these molecules cannot occupy any binding site in the ATPase domain (neither the ATP binding site nor the ICRF binding site). Accordingly, docking studies at other possible binding sites were performed, which revealed a binding domain close to etoposide binding

site in the central domain of hTopoII $\alpha$  (PDB ID: 4FM9).<sup>24</sup> However, these compounds are not hTopoII $\alpha$  poisons like etoposide.<sup>25</sup> Hence, the interactions of these compounds are expected to be different from that of etoposide. Literature search reveals that merbarone (a catalytic inhibitor of hTopoII $\alpha$ ) occupies an interaction domain overlapping with that of etoposide.<sup>15</sup> This is supported by the fact that merbarone can competitively impede the inhibition of cleavage complex formation by etoposide.<sup>15</sup> To the best of our knowledge, molecular recognition interactions of merbarone are not yet reported in the literature. The important components of the catalytic machinery of the enzyme ( $\text{Mg}^{2+}$ , TOPRIM, and TyrB805) are located in the central domain (Figure S4) near the etoposide/merbarone-binding pocket.<sup>26,27</sup>

The results from molecular docking of merbarone in hTopoII $\alpha$  (emodel<sup>28</sup> score  $-64.17$ ) showed that merbarone formed metal coordination bond (through carbonyl oxygens) with  $\text{Mg}^{2+}$  (2.09 Å), hydrogen bonding interaction with Asp543 (3.13 Å), Asp545 (1.90 Å), and Lys614 (2.91 Å), and  $\text{NH}\cdots\pi$  interactions with Lys614 (distance 3.50 Å) (Figure 4). The binding is further stabilized by interaction with polar residues (Gln544, Arg713, His758, and His759) and hydrophobic residues (Gly546, Ile577, Leu592, and Tyr805). This set of interactions with catalytically important components of hTopoII $\alpha$  may be considered as the molecular recognition interactions for merbarone. In order to understand the in vitro competitive binding of merbarone and etoposide, a comparative molecular docking analysis was undertaken (see Supporting Information for details, Figure S5).

The molecular docking of compounds **6a–e** revealed that they could interact at merbarone-binding site and showed interactions similar to that of merbarone (Figure 4), which are with  $\text{Mg}^{2+}$ , Asp541, Asp543, and Asp545 (of chain A) and



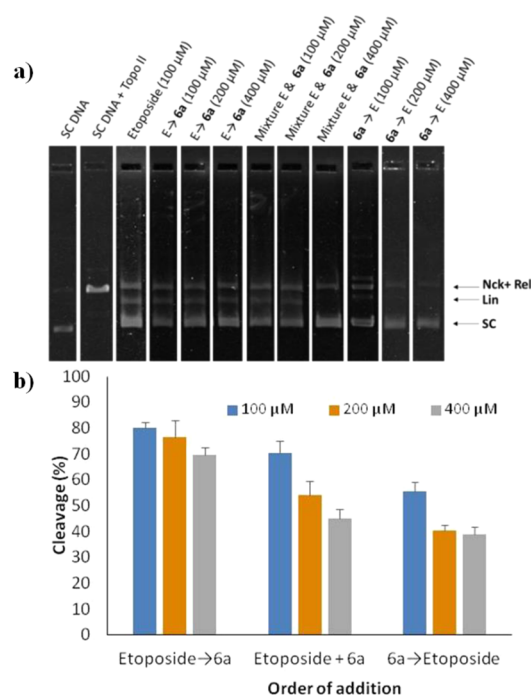
**Figure 4.** (a) Merbarone, (b) compound **6a**, and (c) alignment of compounds **6a–e** in the merbarone-binding pocket of hTopoII $\alpha$ . Red dashed lines represent the  $\pi$ - $\pi$ , cation- $\pi$ , and  $\text{NH}-\pi$  interactions. Black dashed lines are hydrogen bonding interactions.  $\text{Mg}^{2+}$  is shown as a nonbonded sphere (magenta). Residues that are involved in hydrogen bonding are shown in stick presentation. For clarity, only polar hydrogens are displayed.



Tyr805 (of chain B). Additionally, NH $\cdots\pi$  interactions with Arg713 and His759 also contribute. Further stabilization of the complex is contributed by the polar residues (Lys614, Ser709, Glu712, Lys728, His758, and Asp831) and the hydrophobic residues (Gly546, Ile549, Gly760, Ile577, and Leu592). The molecules **6a–e** showed cation $\cdots\pi$  interaction with Mg<sup>2+</sup> via C6-aryl-substitution. The docking scores (emodel) of the active compounds are quite comparable to that of merbarone, and also a good correlation with the relative hTopoII $\alpha$ -inhibitory activities was observed (Table S3). Further, a molecular docking analysis of the neutral imidazo-pyridines/pyrazines revealed a poor correlation with the biological activity (see Supporting Information, Table S4). The parent imidazo-pyridines (nonarylated, Figure S6) were known to interact with hTopoII $\alpha$  at the ATPase domain (evident by molecular modeling and in vitro studies).<sup>16</sup> With the interactions involved in the merbarone-binding site as explored in this study, it was found that the parent nonarylated imidazo-pyridines could also have similar interactions (Table S5). Therefore, it can be assumed that the C<sub>6</sub> nonarylated imidazo-pyridines preferably bind at ATP-binding domain but can also be accommodated in the merbarone-binding site. However, their arylated analogues selectively bind only at the merbarone-binding site (see SI for structure–activity relationships of these series of molecules). In support of these molecular modeling results, we performed an in vitro assay<sup>15</sup> for the attenuation of etoposide-enhanced hTopoII $\alpha$ -mediated DNA cleavage using compound **6a** and a representative compound of previously reported<sup>16</sup> C<sub>6</sub>-nonarylated imidazo-pyridines, 4-(3-(*tert*-butylamino)imidazo[1,2-*a*]pyridin-2-yl)benzoic acid (IP-C6-H, **7a**, Figure S6). It was observed that the DNA cleavage decreased with an increase in the concentration (100 to 400  $\mu$ M) of compound **6a** (Figure 5). This inhibition is dependent on the order of sequential addition of compounds. Adding compound **6a** before etoposide significantly reduced the formation of the cleavage complex. These observations are in accordance with the reported<sup>15</sup> effects of merbarone toward attenuation of etoposide-enhanced hTopoII $\alpha$ -mediated DNA cleavage.

Interestingly, compound **7a** showed no such effect at 100  $\mu$ M and a small effect at 200 and 400  $\mu$ M concentrations (Figure S7). Thus, compound **7a** has preferential binding at the ATPase domain of hTopoII $\alpha$ , although some interaction at merbarone-binding site also occurs at increased concentration. Taken all together, these results provide an interesting and unique observation, i.e., a switch in the site of inhibition from ATP-hydrolysis to DNA cleavage stage in catalytic cycle of hTopoII $\alpha$  with a rational structural modulation, i.e., arylation of imidazo-pyridine.

In conclusion, the exploration/understanding of molecular interactions of the hTopoII $\alpha$  with known ligands, structure-based rational modulations of imidazo-pyridines/pyrazines, synthesis, and relevant in vitro evaluations have led to the establishment of a strategy of “choice-based change” in catalytic mode of inhibition of hTopoII $\alpha$  and development of the new potent and specific inhibitors and cytotoxic agents. A set of residues (Mg<sup>2+</sup>, TOPRIM, TyrB805, and ArgB804) important for interaction in merbarone-binding domain has also been explored for the first time. This work will prompt for rational structural modulation studies of known hTopoII $\alpha$ -based drugs/agents toward “choice-based change” in mode of action for rapid drug discovery. The concept and the results are also useful in the bacterial (DNA-gyrase) and leishmanial topoisomerase II enzyme-based research.



**Figure 5.** (a) Attenuation of etoposide-enhanced hTopoII $\alpha$ -mediated DNA cleavage<sup>15</sup> by compound **6a**. The order of addition of inhibitors was as follows: etoposide  $\rightarrow$  **6a** (etoposide first followed by compound **6a**), etoposide + **6a** (etoposide and compound **6a** simultaneously), and **6a**  $\rightarrow$  etoposide (compound **6a** first followed by etoposide). (b) Quantification of the results.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Details of synthetic procedure, characterization, and analytical data, HPLC study (>95% purity), assay protocols, and molecular modeling methodology. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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<sup>†</sup>These authors contributed equally to this work.

### 💰 Funding

We gratefully acknowledge financial support from DBT, DST, and CSIR, Government of India, New Delhi, for this investigation. N.T. and V.C. are thankful to CSIR for their fellowships.

### 📝 Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

hTopoII $\alpha$ , human topoisomerase II $\alpha$ ; MDR, multidrug resistance; TBAB, tetra-*n*-butylammonium bromide; DMA, *N,N*-dimethylacetamide; kDNA, kinetoplast DNA; Nck, nicked; Rel, relaxed; Lin, linear; SC, supercoiled; TAE, Tris-acetate-EDTA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MER, merbarone; ANP, phosphoaminophosphonic acid-adenylate ester; ICRF-187, (+)-1,2-Bis(3,5-dioxopiperazinyl-1-yl)propane/dexrazoxane; rmsd,

root-mean-square deviation; IEFPCM, integral equation formalism polarizable continuum model

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