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Brief Article

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Phenylpyrazolo[1,5-*a*]quinazolin-5(4*H*)-one: a suitable scaffold for the development of non-camptothecin Topoisomerase I (Top1) inhibitors.

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KEYWORDS: Topoisomerase I; pyrazoloquinazoline derivatives; structure–activity relationships; medicinal chemistry.

Supporting Information Placeholder

ABSTRACT: In search for a novel chemotype to develop Topoisomerase I (Top1) inhibitors, the pyrazolo[1,5-*a*]quinazoline nucleus, structurally related to the indenoisoquinoline system precursor of well-known Top1 poisons, was variously decorated (i.e. a substituted phenyl ring at 2- or 3-position, a protonable side chain at 4- or 5-position) affording a number of Top1 inhibitors with cleavage patterns common to CPT and MJ-III-65. SARs data were rationalized by means of an advanced docking protocol.

Introduction

DNA topoisomerases (Top) are essential enzymes inducing DNA modification required during cellular processes such as replication, transcription, repair, etc.[1] There are two major families of Top: Type I (Top1) and Type II (Top2) depending on whether they cleave only one or two DNA strands.[2] Top1 relaxes supercoiled DNA by forming DNA single-strand breaks, and religates the broken strand, to rapidly restore intact duplex DNA.[2] At this stage, the enzyme is particularly vulnerable to a group of anticancer agents, the Top1 poisons, that reversibly trap the Top1-mediated cleavage complex, leading to irreversible DNA strand breaks, activation of apoptosis and cell cycle arrest.[2]

Top1 inhibitors are a relatively new group of anticancer agents with a wide range of activity in hematological and solid tumors. Camptothecin (CPT **I**, Chart I),[3] was the first small molecule identified as a Top1 inhibitor. Efforts to improve its toxicity profile and pharmacokinetics led to the development of two clinical water-soluble CPT derivatives, topotecan **II** (Chart I) and irinotecan,[4] as well as novel compounds currently under clinical evaluation.[5] However, CPTs are not ideal drugs as they display a number of limitations, including chemical instability,[6] and potential induction of cellular resistance.[7] To overcome the main drawbacks of CPTs, several chemical classes of non-CPT Top1 poisons were developed as promising antitumor drugs, including the phenanthridines **III**, and the indenoisoquinolines **IV** (Chart I).[8,9]

As part of our program in search for new antiproliferative agents, in the last decades we extensively studied several polyheterocyclic systems.[10-12] In the present study, we have directed our attention to the pyrazolo[1,5-*a*]quinazoline system **V** (Chart I),[13,14] as a novel scaffold to develop non-CPT agents acting against Top1. Actually, the core of **V** would mimic the A, B and C rings of **IV**, while the phenyl hanging from the pyrazole portion of **V** could mimic the substituted D ring of **IV** (Chart I). Compounds bearing a phenyl alternatively at 2- or 3-position of pyrazolo[1,5-*a*]quinazoline system were designed (**V**, Chart I). Further, as a protonable chain linked at the 5- or 6-position of the indenoisoquinoline ring or at the 5-position of the benzophenanthridine system is a common feature of the most active derivatives,[9] we decorated the pyrazoloquinazoline ring at the 4- or 5-position with aminoalkyl chains. In particular, we studied the influence on the Top1 inhibitory activity of: (i) the length of the chain, (ii) the nature of the linker between the ring and the chain; (iii) the nature of the terminal basic site. Interestingly, these compounds featuring a basic nitrogen in the side chain could be converted in the corresponding salts, thus increasing aqueous solubility that might facilitate their formulation.

In this study, derivatives **1**–**34** were prepared and evaluated for their ability to inhibit Top1 (Table 1), and an advanced docking protocol was employed to rationalize the biological results.

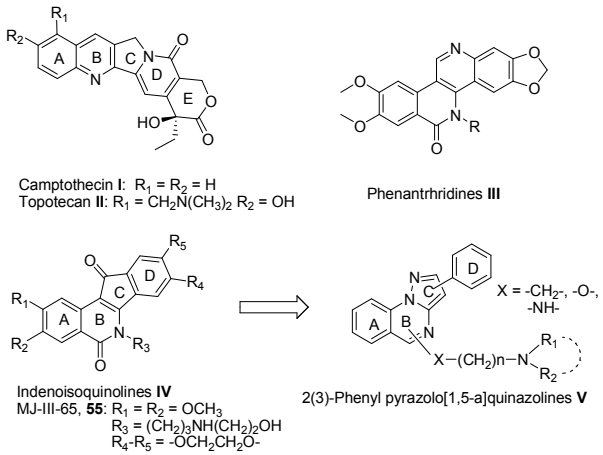
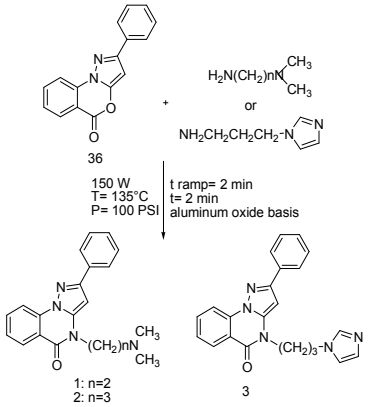


Chart I. Structures of known Top1 inhibitors; design of pyrazoloquinazolines **V**.

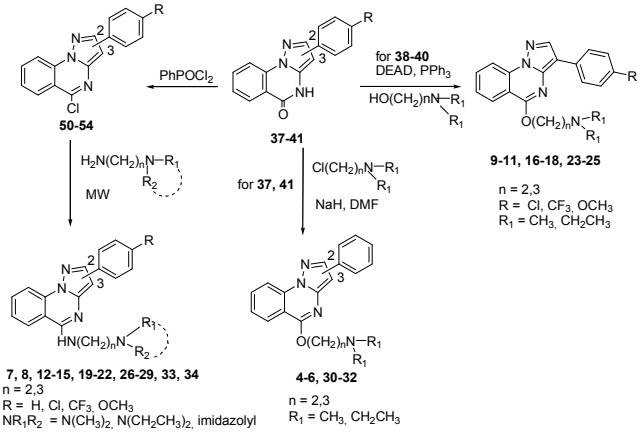
Chemistry

The 2-phenyl-5*H*-pyrazolo[1,5-*a*][3,1]benzoxazin-5-one **36** was obtained by an improved microwaves (MW)-assisted reported procedure[15,16] [experimental details, Scheme 1S, Supporting Information (SI)] and then reacted with the appropriate dialkylaminoalkylamine to obtain compounds **1**–**3** through the use of MW (Scheme 1).



Scheme 1. Synthesis of pyrazoloquinazolines **1**–**3.**

The phenylpyrazolo[1,5-*a*]quinazolin-5(4*H*)-ones **37**–**41** (Scheme 2) were obtained by improving known synthetic procedures (experimental details, Schemes 2S and 3S, SI).[13,14] Treatment of **37**, **41** with sodium hydride and addition of the appropriate dialkylaminoalkyl chloride gave derivatives **4**–**6, 30**–**34** (Scheme **2**). The best yields in the preparation of **9**–**11**, **16**–**18**, **23**–**25**, were obtained by a Mitsunobu reaction between **38**–**40** and the appropriate aminoalcohol (Scheme **2**). The synthesis of compounds **7**, **8**, **12**–**15**, **19**–**22**, **26**–**29**, **33**, **34** includes the transformation of **37**–**41** in the corresponding 5-chloro derivatives **50**–**54** (Scheme **2**, see SI for details), that were then reacted with the proper dialkylaminoalkylamines through the use of MW (Scheme **2**). All the products (**4**–**34**) were purified by conversion into the corresponding hydrochlorides by treatment with ethanol hydrochloride in absolute ethanol.



Scheme 2. Synthesis of pyrazoloquinazolines **4**–**34.**

Biological Results and Discussion

Table 1 lists the relative potencies of the pyrazoloquinazolines **1**–**34** toward the production of Top1-mediated DNA cleavage, ranked by a systematic visual analysis of the number of cleavage sites and their respective intensity in each lane as compared to the positive control lanes containing **I** or **55** (MJ-III-65, Chart I)[17,18] at 1 μM. A semi-quantitative ranking system is then used to rank the compounds from 0, not active; 0/+, trace of activity; +, weak activity; ++, moderate activity; +++, strong activity; to ++++, activity equivalent to 1 μM **I** or **55**.

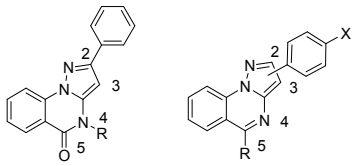
Compounds **1**–**3**, that were synthesized due to their structural similarities with the indenoisoquinolines **IV**, showed null Top1 inhibitory activity (Table 1). So we turned out our attention to 2-phenylpyrazoloquinazolines bearing different protonable chains at the 5-position (**4**–**8**). Also these substitution patterns did not produce favorable effects on biological activity. Only derivatives **4** and **5** showed a scarce activity as Top1 inhibitors.

Compounds **9**–**34** were then designed by shifting the pendant phenyl from the 2- to the 3-position, and slightly expanding the variability at the 5-chain with respect to **1**–**8**. Initially, an electron-donating OCH_3 was inserted at *p*-position of the 3-phenyl (**9**–**15**), due to the well-known beneficial effect of this substituent on the potency of Top1 inhibitors.[9] Then, variously (CF_3, Cl, H) 4'-substituted compounds **16**–**34** were developed to expand the SAR. The presence of the 3-phenyl produced a general improvement in the biological activity (**9**–**34**, Table 1), suggesting a specific arrangement of the molecule that is favorable for Top1 inhibition. The presence of a 4'-OCH_3 does not particularly favor the activity, yielding poorly active derivatives (**9**–**15**), whatever the nature of the chain at 5-position. An analogous effect is produced by a highly electron-withdrawing CF_3 substituent (**16**–**22**). Conversely, the presence of the lipophilic and electron-withdrawing 4'-Cl (**23**–**29**) determined an enhancement in the biological activity. In this subclass, the

nature of the 5-chain in terms of type (–O- or -NH-) and length of the linker, and terminal nitrogen containing group moderately influence Top1 inhibitory activity.

The imidazole-containing chain (**29**) scored the worst results, while the best activity was obtained with the dimethylaminoethylamino, dimethylaminopropylamino, and diethylaminoethylamino chains (**26**-**28**).

Table 1. Topoisomerase I Inhibitory Activities of **1**-**34**.



1-3 4-34

N	Phenyl position	R[a]	X	Top1 Inhibition
1	-	A	-	0/+
2	-	B	-	0
3	-	C	-	0
4	2	D	H	0/+
5	2	E	H	0/+
6	2	F	H	0
7	2	H	H	0
8	2	J	H	0
9	3	D	OCH_3	+
10	3	E	OCH_3	0/+
11	3	F	OCH_3	0/+
12	3	G	OCH_3	+
13	3	H	OCH_3	0/+
14	3	I	OCH_3	0/+
15	3	J	OCH_3	0
16	3	D	CF_3	0/+
17	3	E	CF_3	0
18	3	F	CF_3	0
19	3	G	CF_3	+
20	3	H	CF_3	0/+
21	3	I	CF_3	0/+
22	3	J	CF_3	0
23	3	D	Cl	+
24	3	E	Cl	0/+
25	3	F	Cl	+
26	3	G	Cl	++
27	3	H	Cl	++
28	3	I	Cl	++
29	3	J	Cl	0
30	3	D	H	++
31	3	E	H	+
32	3	F	H	+
33	3	H	H	+
34	3	J	H	0/+

[a]A: $(CH_2)_2N(CH_3)_2$; B: $(CH_2)_3N(CH_3)_2$; C: $(CH_2)_3$-1--imidazolyl; D: $O(CH_2)_2N(CH_3)_2$; E: $O(CH_2)_2N(C_2H_5)_2$; F: $O(CH_2)_3N(CH_3)_2$; G: $NH(CH_2)_2N(CH_3)_2$; H: $NH(CH_2)_2N(C_2H_5)_2$; I: $NH(CH_2)_3N(CH_3)_2$; J: $NH(CH_2)_3$-1-imidazolyl. The activity of the compounds to produce Top1-mediated DNA cleavage was expressed semi-quantitatively as follows: 0, not active; 0/+, trace of activity; +, weak activity; ++, moderate activity; +++, strong activity; ++++, activity equivalent to 1 μM **I** or **55**.

An analogous trend was observed when the 4'-position is unsubstituted (**30**-**34**), with the presence of a terminal imidazole nucleus that again results to be detrimental for the activity, whereas a 5-dimethylaminoethoxy moiety confers to **30** an increase in Top1 inhibition.

Figure 1 displays the Top1-mediated DNA cleavage patterns for compounds **2**, **18**, **24**, **26**-**29**, selected as representative of the whole series, along with those resulting from **I** and the indenoisoquinoline **55**.[17,18] It should be observed that compounds **26**-**28**, differently from the poorly active **2**, **18**, **24,** and **29** showed cleavage sites that are common to **I** and **55**.

Docking studies were performed to rationalize the Top1 inhibitory activity and the relative potencies of our pyrazoloquinazolines.

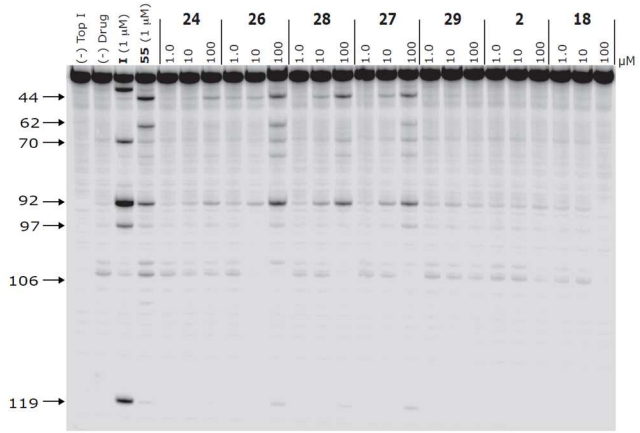


Figure 1. Top1-mediated DNA cleavage induced by compounds **24**, **26**, **28**, **27**, **29**, **2** and **18**. Lane 1: DNA alone; lane 2: Top1 alone; lane 3: **I**, 1 μM:; lane 4: Indenoisoquinoline **55**, 1 μM; lane 5-25: **24**, **26**, **28**, **27**, **29**, **2** and **18** at 1, 10 and 100 μM respectively from left to right. Numbers and arrows on the left indicate arbitrary cleavage site positions.

We selected the high resolution (2.10 Å) crystal structure of the human Top1 in complex with the poison Topotecan (**II**, Chart 1) and covalent complex with a 22 bp DNA duplex (PDB code: 1K4T).[19] In this structure **II** establishes direct H-bonds with E356, R364, K532 and D533, and additional water-mediated interactions with N722 and the phosphotyrosine 723 (P-Y723) (Figure S1a, SI). Thus, we decided to explicitly consider the waters molecules in docking calculations using the software Autodock4.2 (AD4).[20] In this respect, Forli *et al.* have recently developed a new AD4 force field and hydration docking method that allows for the automated prediction of waters mediating ligand binding.[21] To evaluate the predictive power of this approach in our system, we first ran self-docking calculations on the Top1-**II**-DNA complex. AD4 reproduced the experimental binding conformation as the lowest energy solution, with a ligand RMSD equal to 1.08 Å, also recapitulating the water-mediated interactions between **II** and the enzyme (Figure S1b, SI).

These results encouraged us to apply this protocol on our pyrazoloquinazolines. Among these, we selected compound **26** within the subclass displaying the most interesting pharmacological profile (**26**-**28**). According to docking results, **26** intercalates at the DNA cleavage site (Figure 2) stacking with its polyaromatic system between the downstream (-1) T-A and upstream (+1) G-C base pairs like other CPT-Top1 inhibitors, including **II (**Figure S2, SI**)**.[19]

In particular, the pyrazoloquinazoline scaffold stacks between the -1T and the +1G of the scissile strand, with the N^4 atom establishing water-mediated H-bonds with

R364 and the ribose endocyclic oxygen (O5) of the -1 adenosine on the non-scissile strand. The 3-phenyl establishes well-oriented parallel-displaced interactions with the -1A and the +1C which should be lost when the phenyl is moved to position 2, thus explaining the lower activity of compounds **1**-**8**.

As expected, in the 3-phenyl compounds enhancement of ligand-target charge-transfer interactions, through the introduction of an electron-withdrawing 4'-Cl (**23**-**29**), resulted in higher inhibitory potencies if compared to the unsubstitued **30**-**34**. Also, the Cl seems to perfectly fit in the crevice formed by the -1A and +1C residues of the non-scissile strand (Figure S3, SI); this is further confirmed by the lower potency displayed by analogues featuring bulkier substituents in the same position such as the *p*-OCH_3 and *p*-CF_3 substituted **9**-**22**.

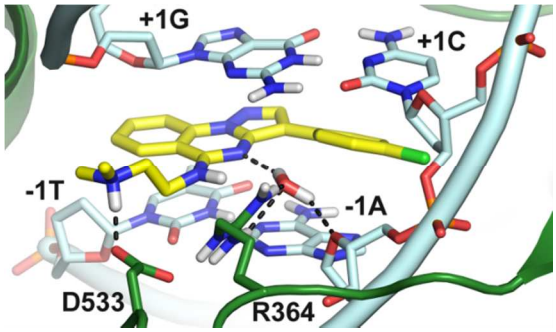


Figure 2. Binding mode of **26** (yellow sticks) at the Top1 (green cartoons) DNA (cyan cartoons) cleavage site. Residues important for ligand binding are highlighted as sticks. H-bonds are dashed black lines.

In the docking pose predicted for **26**, the 5-dimethylaminoethylamino branch extends outside the double-helix DNA towards a rather shallow protein pocket where the exocyclic NH group can donate a H-bond to the adjacent water molecule. Thus, it can be predicted that the lower Top1 inhibition rate displayed by compounds bearing exocyclic ether oxygen (**23**-**25**) might in part be ascribed to the loss of this water-mediated interaction. In this position, the terminal dimethylamino moiety of **26** establishes a tight salt bridge with the carboxylate group of D533. In this regard, the length of the aminoalkyl chain (**12** *vs* **14**, **19** *vs* **21**, and **26** *vs* **28**) or the alkyl on the terminal amine group (**12** *vs* **13**, **19** *vs* **20**, and **26** *vs* **27**) poorly influence the Top1 inhibitory potency. In this respect, longer but still flexible chains [the dimethylaminopropylamino (i.e. **14**, **21**, **28**), the diethylaminoethylamino (i.e. **13**, **20**, **27**) branches] can rearrange without steric restrictions to preserve the salt bridge with D533. However, the introduction of a more rigid and less basic substituent such as the imidazole (**8**, **15**, **22**, **29** and **34**) should result in low or null Top1 inhibitory activity due to the loss of the ionic interaction described above.

In conclusion, we designed and synthesized a series of novel non-CPT Top1 inhibitors based on the phenylpyrazolo[1,5-*a*]quinazolin-5(4*H*)-one scaffold, structurally related to the indenoisoquinoline nucleus. SARs emerging from this series, together with the theoretical model for the **26**/Top1/DNA ternary complex provided by hydrated docking calculations, allowed to identify the following structural requirements to gain Top1 inhibitory activity: (i) a properly-substituted 3-phenyl ring; (ii) a protonable dialkylaminoalkylamino chain at 5-position. Compounds **26**-**28** are among the most active Top1 inhibitors developed in this study showing cleavage patterns that are common to **I** and **55**.

Taken together, all these findings highlight the pyrazoloquinazoline nucleus as a suitable scaffold to further expand the chemical diversity in Top1 inhibitors, and provided SAR data for the optimized design of new derivatives with improved biological activity.

Experimental Section

Chemistry. General directions are in the SI. Purity of tested compounds is ≥95% (combustion analysis).

General procedure for the synthesis of 2-phenylpyrazolo[1,5-*a*]quinazolin-5(4*H*)-ones 1-3. A mixture of 2-phenyl-5*H*-pyrazolo[1,5-*a*][3,1]benzoxazin-5-one **36** (0.262 g, 1 mmol) and the proper alkylamine (0.9 mmol) in DMF (2 ml) was irradiated at a T=135 °C, P=100 PSI, power=150 W for 2 min. Products **1**-**3** (SI) crystallized from the reaction mixture by dilution with ice/water in the desired purity degree (≥95%).

General procedure for the synthesis of 5-(*N*,*N*-dialkylaminoalkoxy)-2(3)-phenylpyrazolo[1,5-*a*]quinazolines 4-6, and 30-32. Sodium hydride (1.1 mmol, 0.044 g, 60% dispersion in mineral oil) was added portion-wise to an ice-cooled solution of **37**, **41** (1 mmol) in 10 ml of DMF, and the mixture was stirred at rt for 1 h. The appropriate dialkylaminoalkyl chloride (1.1 mmol) was added dropwise, and stirring was continued for 24 h at rt (TLC analysis). The reaction mixture was evaporated to dryness, and the residue triturated with ice/water and extracted with $CHCl_3$. Evaporation of the organic phase yielded products **4**-**6**, **30**-**32** in the desired purity degree (≥95%). Samples of **4**-**6**, **30**-**32** were characterized as hydrochloride salts, obtained by treatment with ethanol hydrochloride in absolute ethanol (SI).

General procedure for the synthesis of 5-(substitutedalkylamino)-2(3)-phenylpyrazolo[1,5-*a*]-quinazolines 7, 8, 12-15, 19-22, 26-29, 33, and 34. A mixture of the appropriate 5-chloro derivatives **50**-**54** (1.2 mmol) and the proper alkylamine (2.4 mmol) were irradiated at a T=80 °C, P=100 PSI, power=100 W for 2 min, using aluminium oxide basis as solid support. Then, the mixture was dissolved in ethanol, the aluminium oxide basis was filtered off and the organic solvent was evaporated yielding compounds **7**, **8**, **12**-**15**, **19**-**22**, **26**-**29**, **33**, and **34** in the desired purity degree (≥95%). Samples of **7**, **8**, **12**-**15**, **19**-**22**, **26**-**29**, **33**, and **34** were characterized as hydrochloride salts, obtained by treatment with ethanol hydrochloride in absolute ethanol (SI).

General procedure for the synthesis of 5-(*N*,*N*-dialkylaminoalkoxy)-3-(4-substitutedphenyl)pyrazolo[1,5-*a*]quinazolines 9-11, 16-18, 23-25. The opportune pyrazolo[1,5-*a*]quinazolin-5(4*H*)-ones **38**-**40** (0.62 mmol) was stirred with PPh_3

(0.340g, 1.3 mmol) in dry THF under nitrogen atmosphere for 5 min. DEAD (0.21 ml, 1.2 mmol) was then added dropwise. After 15 min, the appropriate aminoalcohol (0.63 mmol) was added and the mixture was stirred for 16 h at rt (TLC analysis). The reaction mixture was evaporated to dryness, and the residue was treated with water and extracted with $CHCl_3$. Evaporation of the organic phase yielded products **9**–**11**, **16**–**18** and **23**–**25** in the desired purity degree (≥ 95%). Samples of **9**–**11**, **16**–**18** and **23**–**25** were characterized as hydrochloride salts, obtained by treatment with ethanol hydrochloride in absolute ethanol (SI).

ASSOCIATED CONTENT

Supporting Information. General chemistry directions; synthesis of compounds **35**–**41**, and **46**–**54**; yields, physical, and spectral data of compounds **1**–**34**; biological tests; computational studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

CPT, camptothecin; MW, microwaves; SAR, structure-activity relationship; Top1, Topoisomerase I.

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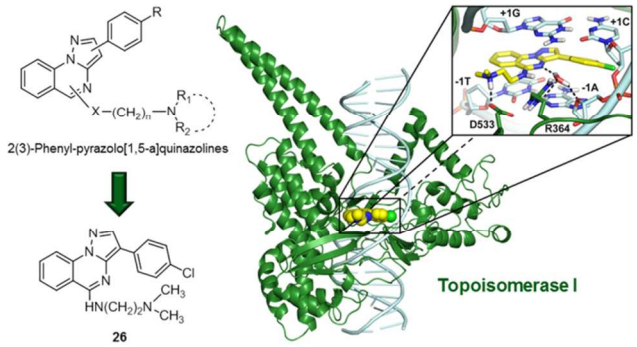

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