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Mitotic Arrest Induced by a Novel Family of DNA Topoisomerase II Inhibitors

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A small structure-focused library of propargylic enol ethers was prepared by means of a modular and efficient chemodifferentiating organocatalyzed multicomponent reaction. The most active compound (GI_{50} 0.25 μM) against solid tumor cells was selected as lead. Cell cycle analysis and immunoblotting demonstrated arrest at the metaphase, pointing out human topoisomerase II as plausible molecular target. In vitro assays were carried out, showing that the lead behaves as a catalytic inhibitor of the enzyme.

Introduction

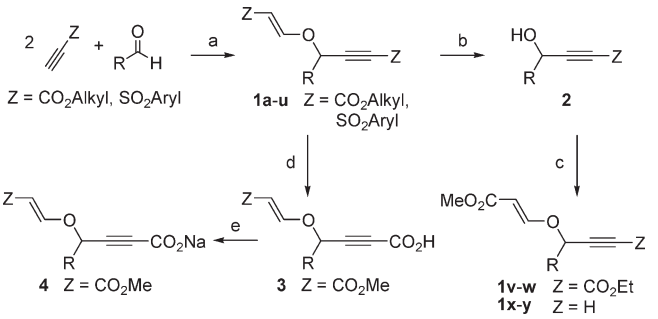
Cancer is a genetic disease characterized by uncontrolled accumulation of tumor cells caused by increased proliferation, decreased cell death, or both. Cell cycle is the major regulatory mechanism of cell proliferation, which is controlled by several proteins that represent attractive drug targets for therapeutic intervention.[1] Targeting one of these proteins, TOP2,[a] has demonstrated clinical benefit for patients.[2] TOP2 is a nuclear enzyme essential for cell division and possesses a wide array of biological functions mainly related with topological problems that arise in double-stranded DNA.[3] In particular, TOP2 is required for metaphase−anaphase transition,[4] and it has been proposed as a metaphase checkpoint.[5]

Within our program directed at the discovery of new antitumor agents, we have developed a fast and modular methodology for the synthesis of novel densely functionalized molecules incorporating three chemically differentiated constitutive blocks in an ordered and defined manner.[6] Hence, we prepared a small and structure-biased library of propargylic enol ethers to search for a lead. Herein we report on our preliminary results on this searching, which successfully afforded an unprecedented class of TOP2 inhibitors.

Results and Discussion

Chemistry. The library was built in parallel using an efficient manifold based on triethylamine catalyzed chemodifferentiating ABB′ 3CRs involving aldehydes and terminal conjugated alkynes (Scheme 1).[7] Compounds **1a**−**u** were obtained by the direct reaction of the appropriate aldehyde with the corresponding acetylide in average yield over 80%. In addition to propiolates, the method tolerates ethynyl *p*-tolyl sulfone. The enol ester group of **1** was exchanged in a two-step sequence to create a new functional-diversity point on the molecule (dissimilar ester groups). Derivatives **1v**−**y** were synthesized from the available propargylic alcohols **2** and were designed for structure−activity relationship studies. Product **1** was selectively hydrolyzed with LiOH to the propargylic acid **3**, which in turn was transformed into the sodium carboxylate **4**, providing an additional functional-diversity point on the molecule.

Scheme 1. Derivatization of Propargylic Enol Ether **1**[a]



[a] Reagents: (a) Et_3N (cat.), 80%; (b) TFA, 72%; (c) methyl propiolate, Et_3N, 98%; (d) (i) $LiOH \cdot H_2O$, THF:H_2O, 0 °C, 30 min; (ii) HCl (aq), 92%; (e) NaOH, H_2O, 88% (two steps). See Supporting Information for specific identity of compounds **1a**−**y**.

Cytotoxicity. In a preliminary screening, the antiproliferative activity of the library was determined in the human solid tumor cell lines SW1573 (nonsmall cell lung) and HBL-100 (breast). Analysis of the GI_{50} values allowed us to obtain some information on the lipophilic requirements involved in tumor cell growth inhibition providing the following structure−activity relationships (Figure 1). An ester or a

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[a] Abbreviations: TOP2, human DNA topoisomerase II; GI_{50}, 50% growth inhibition; SAR, structure−activity relationship; PARP, poly-(ADP-ribose) polymerase-1; Chk2, checkpoint kinase 2; CI, catalytic inhibitor; kDNA, intact kinetoplast DNA; Nck, nicked; Lin, linearized; SC, supercoiled; Rel, relaxed.

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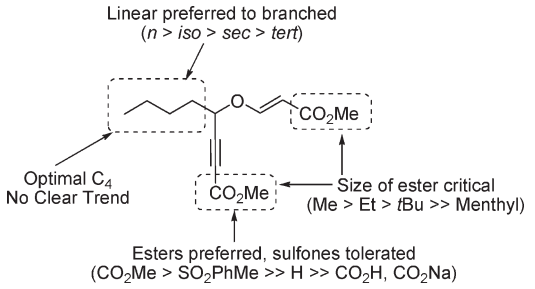

Figure 1. Summary of SAR.

Table 1. Antiproliferative Activity (GI_{50}) of Lead **1** (Z = CO_2Me, R = *n*Bu)[a]

		GI_{50}
solid tumor cell lines	HBL-100	0.25 ± 0.10
	HeLa	2.8 ± 0.24
	SW1573	0.24 ± 0.07
	T-47D	3.8 ± 1.3
	WiDr	3.1 ± 0.9
nontumor cell lines	Hs27	1.9 ± 0.6
	hTERT-HPNE	1.3 ± 0.3
	MCF-10A	34 ± 1.3

[a] Values in μM ± standard deviation. Means of three to five experiments. See Supporting Information for specific cytotoxicity data of compounds **1a**−**y**.

tosyl group at the alkyne end (Z) is essential for the activity. In this particular context, the bulkiness of the alkoxy group is inversely related to the biological effect. Thus, the antiproliferative activities are in the order Me > Et > *t*Bu > menthyl. Additional groups like hydrogen (**1v**−**w**), carboxylic acid (**3**), or carboxylate (**4**) produce a loss in either activity or potency. Regarding the alkyl side chain (R), compounds with linear substituents are more active than the corresponding acyclic branched analogues, and these are followed by the cyclic side chains, which are less active. Another important observation is obtained from the length of the alkyl side chain (R). There is no direct relationship between the number of carbon atoms on the side chain and the growth inhibition. The best result is obtained when R is *n*-butyl. On the basis of the in vitro profile of the synthesized compounds, derivative **1** (Z = CO_2Me, R = *n*Bu) was selected as a lead compound for further biological evaluation.

Lead **1** was evaluated against an extended panel of human solid tumor cell lines. Table 1 shows the GI_{50} values obtained in this study, which were in the range 0.24−3.8 μM. For comparative purposes, compound **1** was tested in a set of three immortalized nontumor cell lines. The GI_{50} values were in the range 1.3−34 μM and are listed in Table 1. Remarkably, lead **1** is more active against cells that divide faster, while it is less potent against cells with a much slower dividing profile such as MCF-10A.

Cell Cycle. To get insights into the mechanism of action, we analyzed whether **1** affected cell cycle progression by using flow cytometry. In all cell lines tested, **1** induced cell cycle arrest in G_2/M phase and in a dose dependent manner (Figure 2). This increase in G_2/M phase is concomitant with a decrease in the G_0/G_1 and the S stages. Incubation with **1** did not induce significant cell lysis, as there was no large increase of cells with a sub-G_1 DNA content, indicative of lack of apoptosis. Accordingly, as expected, Annexin V binding assays and PARP proteolysis experiments do not show any evidence of apoptosis.

G_2/M Transition. We next looked at protein expression at the G_2/M phase transition to discern between G_2 arrest and M arrest. The G_2 cell cycle arrest is thought to function as a defense mechanism (the so-called G_2 checkpoint) in response to DNA damage, which allows cells to repair DNA before progressing into mitosis.[8] To determine whether compound **1** was able to induce DNA damage to cells, we investigated two key proteins involved in the response to DNA damage: Chk2 and histone H2AX. Upon DNA damage, Chk2 and H2AX are phosphorylated. In cells treated with **1**, there was no evidence of phosphorylation of neither Chk2 nor H2AX (Figure 3a), denoting the absence of harm to DNA. These results show that the G_2 checkpoint is not activated by the exposure of cells to **1**, allowing cells to enter mitosis. This point was further confirmed with the expression of cyclin B1, which is required for mitotic initiation. Immunoblotting of HeLa cells treated with **1** showed that compound did not affect cyclin B1 levels (Figure 3b), thus indicating cell cycle progression into mitosis.

Time-dependent phosphorylation of the biological markers histone H3 and BubR1 also support that treated cells entered mitosis. In mammalian cells, phosphorylation of histone H3 on Ser^{10} (pS10-H3) starts in late G_2 phase, it is completed in late prophase, and it is maintained through metaphase. Dephosphorylation of pS10-H3 begins in anaphase and ends at early telophase.[9] Thus, high levels of pS10-H3 are only observed during mitosis.[10] Exposure of HeLa cells to **1** increased the levels of pS10-H3 in a time dependent manner (Figure 3b), as indicated by Western blotting analyses of cell extracts with an antibody that specifically recognizes the phosphorylated form. This result indicates that treated cells were not able to progress into anaphase. Further evidence of this result was derived from the study of BubR1 activation. The activation of BubR1 (pBubR1) by phosphorylation is crucial for mitotic timing. pBubR1 inhibits anaphase promoting complex/cyclosome[11] and is concomitant with a delayed progression into anaphase. Consistently with our previous findings for pS10-H3, we observed that treatment of HeLa cells with **1** increased the amount of pBubR1, as indicated by phosphorylation-induced retardation of the electrophoretic mobility of this protein. Altogether, immunobloting results show that **1** does not induce damage to DNA, allowing cells to enter mitosis until the cell cycle is arrested in metaphase.

A recent work using iRNA TOP2-depleted cells showed that the decatenation activity of TOP2 appeared to be essential for metaphase−anaphase transition.[12] Indeed, failure to initiate TOP2 decatenation resulted in metaphase arrest independent of DNA damage.[5] Taking into account all these considerations, the results obtained from the cell cycle and protein expression studies pointed out TOP2 as a plausible molecular target. Therefore, we next looked to the ability of **1** to inhibit TOP2.

TOP2 Assays. Drugs targeting TOP2 are divided into two broad classes: the so-called TOP2 poisons and the TOP2 CIs. Whereas TOP2 poisons produce DNA double-strand breaks through stabilization of the TOP2−DNA covalent complexes, TOP2 CIs are a heterogeneous group of compounds interfering with the catalytic cycle. To test whether compound **1** was active against TOP2, we performed three specific in vitro assays with commercially available purified enzyme and a set of small molecules of circular DNA as substrates. The use of these small DNA molecules greatly facilitates the visualization of the reactions by agarose gel electrophoresis.

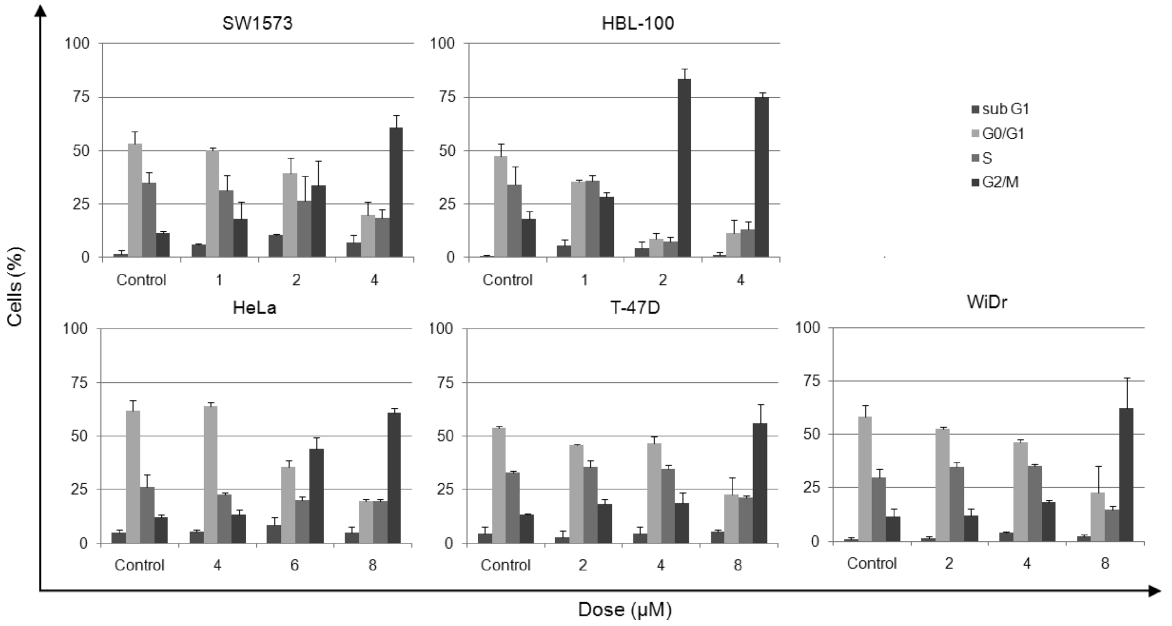

Figure 2. Cell cycle phase distribution of untreated cells (control) and cells treated with compound **1** for 24 h at three drug doses. Values are means of at least two experiments.

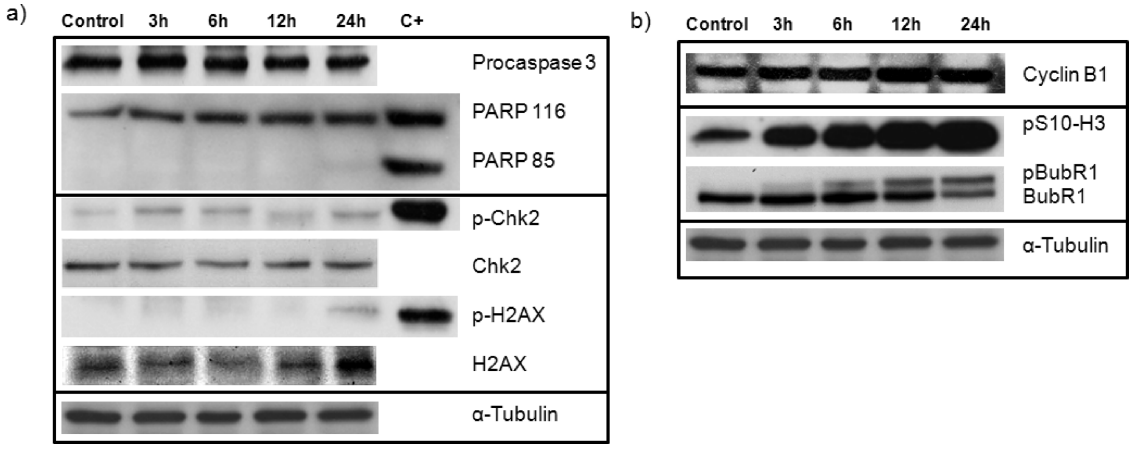

Figure 3. Immunobloting of protein extracts from HeLa cells exposed to **1** at 7.5 μM after 3, 6, 12, and 24 h of drug treatment. C+ (positive control).

We first tested the capacity of lead **1** to inhibit TOP2-mediated kDNA decatenation, the so-called decatenation assay. This assay is considered to be the most specific one to detect TOP2 activity.[13] kDNA cannot enter a typical agarose gel during electrophoresis unless DNA minicircles (Nck, SC and Rel) are previously released by the action of TOP2. In addition to **1**, the TOP2 CI ellipticine and the TOP2 poison VP-16 were also tested for comparison purposes. In Figure 4a, complete decatenation was seen in the positive control (TOP2+) and VP-16 lanes, while ellipticine and **1** inhibited TOP2 decatenation activity as seen by comparison with the sample that lacked the enzyme (TOP2).

In a second experiment, the SC DNA relaxation assay, we measured the TOP2-dependent relaxation of negatively SC plasmid pHOT1. Commercially available pHOT1 contains small amounts of two topoisomers: a relaxed covalently closed form (Rel) and a nicked single-strand break form (Nck). In an agarose gel electrophoresis under controlled conditions, the order of migration of these forms is, from fastest to slowest, SC, Nck, and Rel. As shown in Figure 4b, the effect of lead **1** showed a pattern that resembled that of the control without the enzyme (TOP2−). In contrast, VP-16 did not inhibit the enzyme, resembling the positive control (TOP2+) lane, appearing as a set of variably relaxed topoisomers that migrate more slowly than the SC form.

Both the decatenation and the relaxation assays show unequivocally that **1** is a TOP2 inhibitor. Although in both experiments there is a difference in activity profile between **1** and VP-16, it had to be established whether compound **1** could poison the TOP2 reaction by increasing the steady state of the cleavage complex, as it has been established for VP-16.[14] In fact, as shown previously, VP-16 has little effect on the overall TOP2 activity in spite of being a strong TOP2 poison. Therefore, we run the cleavage complex assay with pHOT1 as substrate. In this experiment, **1** was added once the relaxation reaction had already started and the electrophoresis was run under the presence of an excess of ethidium bromide to fully positively supercoil all the topoisomers (i.e., make them run as a sole band during the electrophoresis) to clearly unmask the linear form (Lin) of the DNA. This form

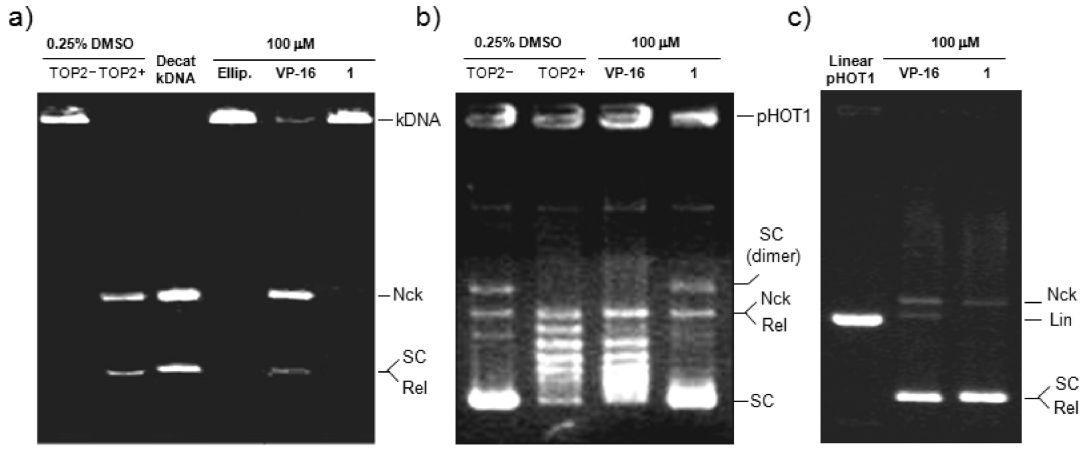

Figure 4. Agarose gel electrophoresis of TOP2 assays. (a) kDNA decatenation; (b) SC DNA relaxation; (c) cleavage complex.

is the result of the cut by the enzyme, and although it is normally rather transient and difficult to detect because the enzyme quickly reseals it, it can be seen with potent inhibitors of the ligation step of the catalytic cycle of TOP2, as is the case of VP-16. Figure 4c shows the presence of the Lin form in the VP-16 lane, while incubation with **1** did not lead to the Lin form.

The result of the cleavage complex assay confirmed that **1** is a TOP2 inhibitor that is not a poison, thus behaving as a true TOP2 CI. Additional support for this evidence was obtained from the immunoblotting experiments (Figure 3). Unlike TOP2 CIs, TOP2 poisons produce DNA damage and cell cycle arrest in G_2, which were not observed when cells were exposed to **1**. Like almost any drug, we cannot discard that compound **1** may have additional biological targets. However, all the experimental data presented herein suggests that the most relevant cellular target is TOP2.

Conclusion

In summary, we have identified a novel human TOP2 CI from a small and structure-focused library of propargylic enol ethers. These simple, linear, and densely functionalized fragments are assembled in a fast, modular, and efficient manner by a chemodifferentiating organocatalyzed ABB′ 3CR based manifold using commercially available aldehydes and alkyl propiolates. It is noteworthy that the synthesis of fragment lead **1** can be performed in a multigram scale without efficiency loss. Ongoing lead optimization studies will shed light on the mechanism of TOP2 inhibition and will be reported in due time.

Experimental Section

The purity of final compounds was assessed by elemental analyses and found to be >95% in all cases.

Methyl 4-((*E*)-2-(Methoxycarbonyl)vinyloxy)oct-2-ynoate (1). Methyl propiolate (2.00 mmol) and *n*-pentanal (1.1 mmol) were dissolved in 10 mL of CH_2Cl_2. After the mixture was cooled to 0 °C, triethylamine (1.0 mmol) was added and the reaction mixture was allowed to stir for 30 min. The solvent and excess reagents were then removed under reduced pressure. Product **1** was isolated by flash column chromatography (silica gel, *n*-hexane/EtOAc 90/10) as colorless oil, 83% yield. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.89 (t, 3H, J=7.4 Hz), 1.30−1.37 (m, 2H), 1.39−1.47 (m, 2H), 1.81−1.91 (m, 2H), 3.67 (s, 3H), 3.75 (s, 3H), 4.60 (t, 1H, J=6.4 Hz), 5.34 (d, 1H, J=12.5 Hz), 7.51 (d, 1H, J=12.5 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 13.7, 22.0, 26.8, 34.2, 51.1, 52.8, 70.5, 78.5, 83.0, 99.1, 153.1, 159.8, 167.5. IR ($CHCl_3$, cm^{-1}) 2957.1, 2870.8, 2241.4, 1721.4, 1628.5, 1436.8, 1260.4, 1134.5. MS, *m*/*z* (relative intensities) 254 (M+, 0.8), 153 (58), 121 (24), 93 (100), 91 (22), 79 (56), 77 (26), 59 (45), 55 (26). Anal. ($C_{13}H_{18}O_5$) C, H.

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Supporting Information Available: Experimental procedures for synthesis and biological tests. Characterization of compounds **1a**−**y**, **3**, and **4**. Antiproliferative activity of all compounds. Cell cycle histograms and Annexin V plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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