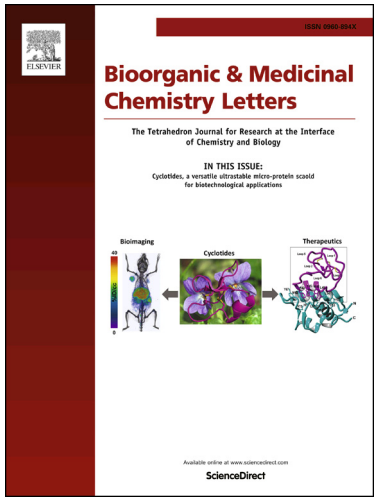

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Title Page

Synthesis and biological evaluation of novel carbazole-rhodanine conjugates as topoisomerease II inhibitors

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Abstract

In this study, a series of carbazole-rhodanine conjugates was synthesized and evaluated for their Topoisomerase II inhibition potency as well as cytotoxicity against a panel of four human cancer cell lines. Among these thirteen compounds, **3a**, **3b**, **3g**, and **3h** possessed Topoisomerase II inhibition potency at 20 μM. Mechanism study revealed that these compounds may function as Topo II catalytic inhibitors. It was found that the electron-withdrawing groups on the phenyl ring of compounds played an important role on enhancing both enzyme inhibition and cytotoxicity.

Keyword
Carbazole
Rhodanine
Topoisomerase II

Cytotoxic
Hybrid molecule

Human topoisomerase has been recognized as an important target in anticancer drugs discovery. Two types of topoisomerase exist in humans, namely, type I topoisomerase (Topo I) and type II topoisomerase (Topo II). Both isomers are nuclear enzymes essential to resolve topological problems that occur during DNA transcription, replication, and chromosome segregation[1-3].Topo II is the target for several commonly prescribed anticancer drugs, including etoposide, doxorubicin, and mitoxantrone[4]. Since the identification of amsacrine as a Topo II-targeted anticancer drug in 1984, about 50% of current treatment protocols still employ at least one drug directed against topoisomerases[5]. However, drug resistance and the severe side effects of Topo II-targeted drugs are an issue[6-8]. Novel and safer Topo II inhibitors for better anticancer therapeutics are highly active research field[9-14].

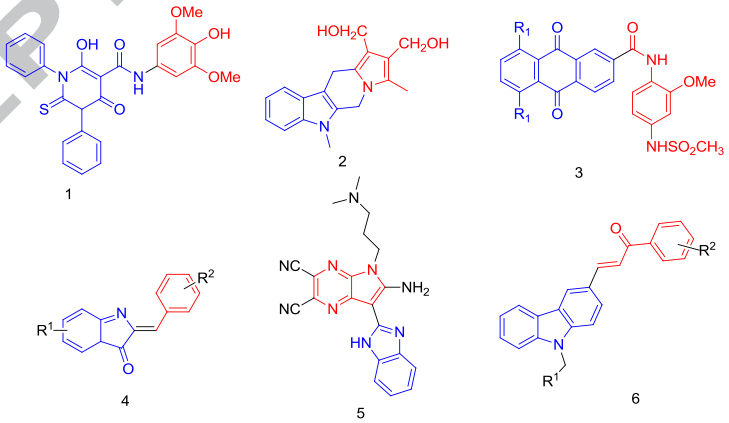


Fig. 1. Hybrid molecule as novel Topo II inhibitors

A hybrid molecule comprising two pharmacophoric groups is often used for the design of new drugs[15]. The hybrid may have increased potencies and/or modified

selectivity profiles compared to the corresponding single molecule. Accordingly, several hybrid compounds have been designed, synthesized and identified as novel Topo II inhibitors (Compounds **1**, **2**, **3**, and **4**; Fig. 1)[16-19]. Our previous studies demonstrated that these conjugates were generally more potent activity than the individual molecule alone (Compounds **5** and **6**; Fig. 1)[20,21]. Continuing with our interest in searching novel anticancer agents that targeting Topo II with high potency, we designed and synthesized novel carbazole-rhodanine conjugates, which contain biologically active carbazole and rhodanine moiety, as shown in Fig. 2.

Carbazole are of great interest due to their broad spectrum of biochemical effects and pharmaceutical functions, including antimalarial, antibacterial and antitumor activity[22]. In particular, there have been intense research efforts in recent years in the design and development of carbazole derivatives as a new class of Topo II inhibitors[21,23,24]. For example, compounds **7**, **8**, and **9** (Fig. 2) are function as potential Topo II inhibitors and display high cytotoxicity against human cancer cell lines[25-27].

Another pharmacophoric group in the target compounds is rhodanine moiety, which has been reported to present a variety of pharmacological activities, including antibacterial, antiparasitic, anti-microbial, and anticancer[28]. In recent years, a lot of efforts have focused on the anticancer activity of rhodanine compounds. For example (Fig. 2), GSK1059615 (compound **10**) is a reversible inhibitor of PI3Kα, which shows potential anticancer activity[29]. Compound **11** displays significant anti-proliferation activity against several cancer cell lines[30].

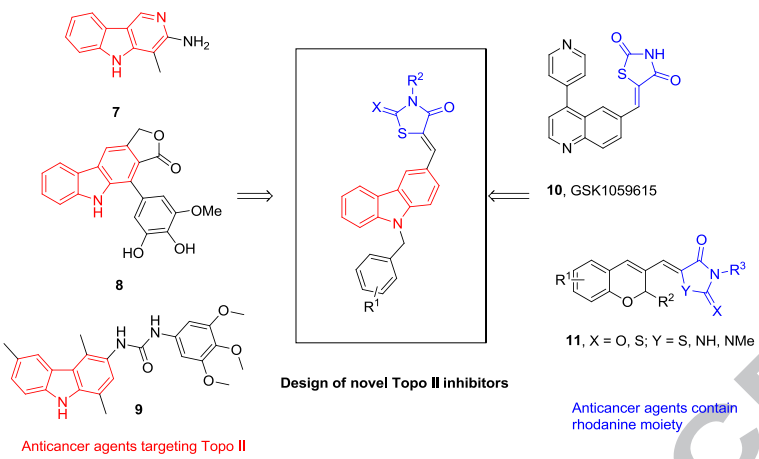
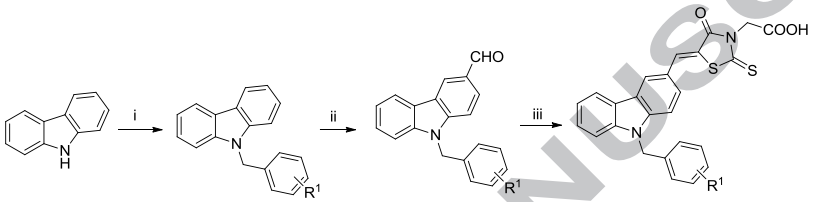


Fig. 2. Design of carbazole-rhodanine conjugates as novel Topo II inhibitors

In the previous study, we reported a series novel Topo II inhibitors via a pharmacophore hybridization strategy[20,21,31,32]. We found that carbazole derivatives containing chalcone analogs displayed strong Topo II inhibition potency and antiproliferation activity. The SAR study revealed that the benzyl group with different substituent linked to carbazole play an important role on the activity[21]. To pursue our mission to promote novel anticancer agents that target Topo II with high potency, we introduced carbazole and rhodanine moiety into a new hybrid scaffold and linked the benzyl groups with different substituent to carbazole[Fig. 2]. We found that these newly synthesized compounds displayed potent Topo II inhibitory activity and cytotoxic activity against four human cancer cell lines.

The synthetic route of carbazole-rhodanine conjugates is shown in scheme 1. *N*-alkylation of carbazole was carried out with appropriate benzyl bromides in the presence of KOH to form **1a**–**1m** in 53–84% yields. The *N*-subsititued-9*H*-3-carbaldehydes (**2a**-**2m**) were synthesized from **1a**–**1m** through

Vilsmeier-Haack reaction in 63–87% yields. The target compounds **3a**–**3m** were synthesized from **2a-2m** through the Hornor-Wadsworth-Emmons reaction of rhodanine moiety in 66–91% yield. The synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR, and HRMS (ESI), which was in full accordance with their depicted structures and the structure of these compounds are shown in Table. 1.

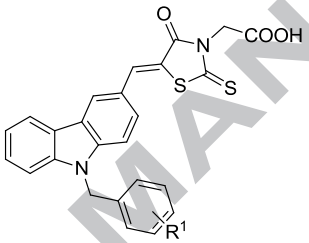


Scheme 1. Synthesis route of carbazole-rhodanine conjugates. Reagents and conditions: (i) DMF, appropriate benzyl bromide, K_2CO_3, rt, 24 h; (ii) $POCl_3$, DMF, DCM, 0 – 90 °C, 8 h; (iii) Rhodanine-3-acetic acid, NaOAc, acetic acid, 110 °C, 4 h.

The resulting synthetic **3a**–**3m** were evaluated for their cytotoxic activities against human lung adenocarcinoma A549, human cervical cancer Hela cells, human acute leukemia HL-60 cells, and human chronic myeloid leukemia K562 cells using MTT assay as described by Mosmann with modification[31]. Etoposide was taken as a positive control. As shown in Table 1, most of compounds exhibited significant cytotoxicities toward four cancer cell lines with low to moderate micromolar values of IC_{50}. Almost all the compounds showed a cytotoxic selectivity for the HL-60 and K562 cell lines, and several compounds such as **3b**, **3g**, and **3h** displayed a nanomolar level IC_{50} toward HL-60, which was consistent with etoposide. It was found that the substituent on the benzyl group had a significant effect on cytotoxic activity.

Compounds **3b**, **3g**, and **3h** with a strong electron-withdrawing group (-NO_2, -CN, and –CF_3, respectively) on the benzyl ring exhibited the most potent cytotoxic activity against all the tested cancer cell lines with an IC_{50} ranging between 0.96–7.51 μM, 0.71–8.20 μM, and 0.43–5.06 μM, respectively. In the contrast, compounds **3e** with an electron-donating group (-CH_3) on the benzyl ring exhibited poor cytotoxic activity.

Table 1. Cytotoxic and Topo II inhibition activity of carbazole-rhoadnine conjugates.



Cpd.	R^1	IC_{50} (μM)				Topo II Inhibition[a]
		Hela	A549	HL-60	K562	
3a	2-F,4-Br	11.72	9.44	3.51	6.92	++
3b	4-NO_2	7.51	3.94	0.96	2.70	++
3c	4-Br	>50	>50	12.67	27.99	-
3d	4-F	>50	>50	8.57	19.13	+
3e	4-CH_3	>50	>50	17.81	29.60	-
3f	4-Cl	28.30	16.18	4.86	13.36	-
3g	4-CN	5.06	2.18	0.43	1.85	++
3h	4-CF_3	8.20	3.40	0.71	2.16	++
3i	3-F,4-F	15.14	12.36	4.17	7.31	+
3j	H	>50	32.20	14.56	21.38	-
3k	2-F,4-F	>50	22.55	3.84	12.57	-
3l	3-F	>50	19.80	8.29	12.84	-
3m	2-F	21.26	13.59	1.82	11.75	-
Etoposide	-	32.05	13.71	0.33	8.45	++

[a]The relative potency Topo II inhibition of compounds are present as follows: -, no detectable activity at 20 μM; +, weak activity at 20 μM; ++, strong activity at 20 μM.

Next, these compounds were tested *in vitro* against Topo II using etoposide as positive control and pBR322 DNA as plasmid[10-12]. The results are present in Fig. 3 and Table 1 showed that most of tested compounds inhibited the Topo II-mediated relaxation of pBR322 DNA at 50 μM (Fig. 3A). Among these compounds, **3a**, **3b**, **3g**, and **3h** displayed strong Topo II inhibition potency at 20 μM (Fig. 3B). Specially, compound **3b** exhibited the most potent Topo II inhibition at 10 μM (Fig. 3C). It was observed that compounds **3b**, **3g**, and **3h** with a strong electron-withdrawing group (-NO_2, CF_3, and –CN, respectively) on the benzyl ring, exhibited better activities than those with a weak electron-withdrawing group (**3c**, **3d**, **3f**, **3i**, **3k**, **3l**, and **3m**). The 4-methyl substitution of the benzyl group (**3e**) did not improve the inhibition activity at all. These results indicated that carbazole-rhodanine conjugates possessed inhibitory activity on Topo II and the substitutions on the benzyl ring with an electron-withdrawing group played an important role on the activity. Topo I-mediated DNA relaxation assay was also carried out to test whether these compounds also target Topo I[11,12]. Results presented in Fig. 3D showed that camptothecin, a well-known Topo I inhibitor, strongly inhibited Topo I activity, while none of the tested compounds possessed inhibitory activity on Topo I in 100 μM. The result revealed that these compounds selectively inhibited the activity of Topo II.

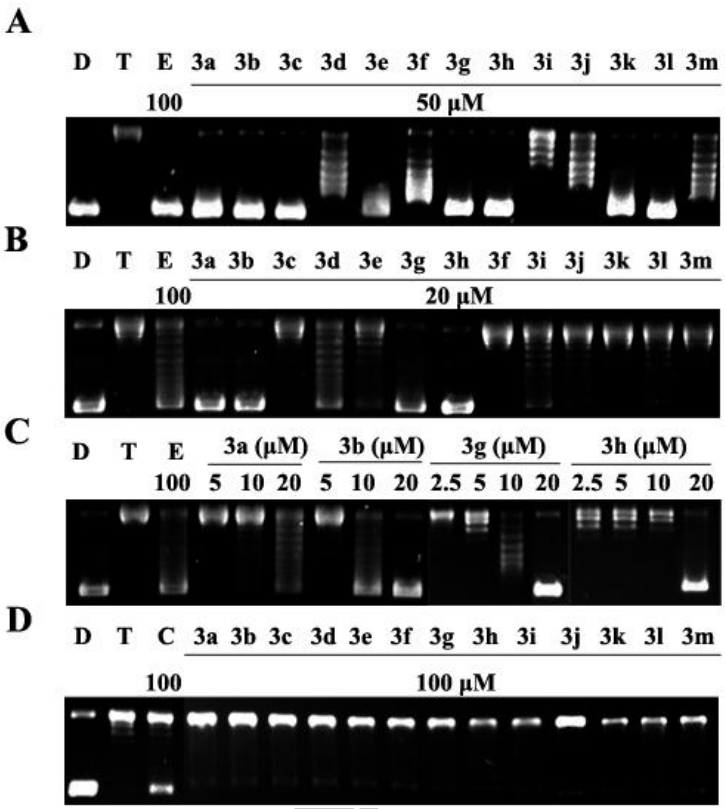


Fig.3. (A, B, and C) Topo II inhibition potency of compounds with various concentrations. Lane D, pBR322 DNA; lane T, pBR322 DNA + Topo II; lane E, pBR322 DNA+ Topo II + etoposide (100 μM); other lanes, pBR322 DNA + Topo II + compounds. (D) Topo I inhibition potency of compounds. Lane D: pBR322 DNA; lane T: pBR322 DNA + Topo I; lane C: pBR322 DNA + Topo I + camptothecin (100 μM); other lanes: pBR322 DNA + Topo I + compounds (100 μM).

Topo II inhibitors are classified according to their ability to induce DNA double-strand breaks with (Topo II poisons) or without (Topo II catalytic inhibitors) the formation of a cleaved complex, reflecting different inhibition mechanisms[1-3]. To gain insight into the mode of action, Topo II-mediated DNA cleavage assay was carried out to verify whether these compounds function as Topo II poisons or Topo II catalytic inhibitors. As shown in Fig. 4A, the well known Topo II poison etoposide produces discrete bands of the linear DNA, corresponding to its poison behavior. In

contrast to etoposide, the linear form of the DNA is not visible in **3g** and **3h** even at 50 μM. These observations indicated that the tested compounds function as Topo II catalytic inhibitor.

Topo II catalytic inhibitors have two types, namely, DNA intercalators and nonintercalators. In order to determine the intercalating or non-intercalating ability of the leading compounds, a DNA unwinding assay was carried out using supercoiled pBR322 DNA as substrate and ethidium bromide (EB; a classic DNA intercalator) as control. As shown in Fig. 4B, EB is able to transform the relaxed DNA into supercoiled DNA in the presence of excess Topo I. Compounds **3g** and **3h** displayed the similar effect of EB in a dose-dependent manner, suggesting that these compounds are intercalative Topo II catalytic inhibitors.

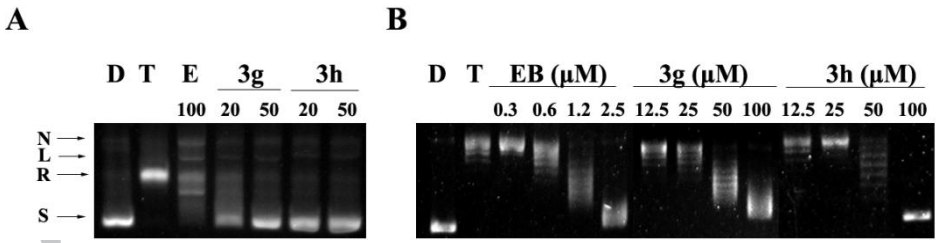


Fig. 4. (A) Topo II mediated cleavage of plasmid pBR322 DNA. Lane D: pBR322 DNA; lane T: pBR322 DNA + Topo II; lane E: 100 μM etoposide + Topo II + pBR322 DNA; other lanes: **3g** or **3h** + Topo II + pBR322 DNA. The positions of supercoiled DNA (S), relaxed DNA (R), linear DNA (L), and nicked DNA (N) are indicated. (B) The unwinding assay of **3g** and **3h**. Lane D, pBR322 DNA; lane T, pBR322 DNA + Topo I; other lanes, pBR322 DNA + Topo I + **3g**, **3h**, or EB with different concentrations.

In conclusion, we have synthesized a series of carbazoles-rhodanine conjugates and evaluated their pharmacological activity. Of the compounds **3a**, **3b**, **3g**, and **3h** could selectively impede Topo II function at 20 μM without affecting Topo I catalytic activity. Mechanism studies revealed that these compounds might function as intercalative Topo II catalytic inhibitors. These compounds displayed potential cytotoxic activity against four human cancer cell lines. The electron-withdrawing group on the benzyl ring enhancing both enzyme inhibition and cytotoxicity of these compounds. This study may provide valuable information to researchers working on the development of novel antitumor agents targeting Topo II.

Acknowledgements

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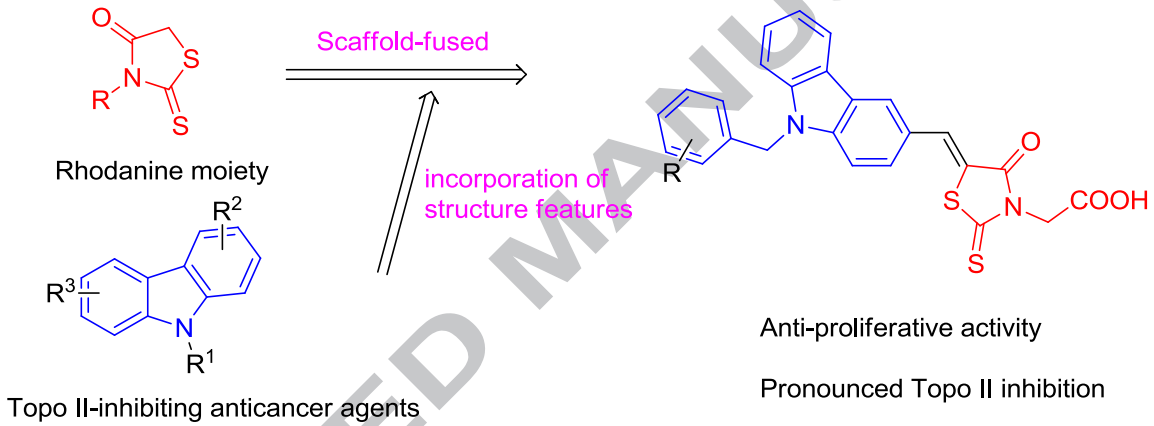

Highlight

1. Synthesis of 13 novel carbazole-rhodanine conjugates.

2. These compounds as potent intercalative Topo II catalytic inhibitors.

3. These compounds displayed potential anti-proliferation activity.