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Potent antitumor bifunctional DNA alkylating agents, synthesis and biological activities of 3a-aza-cyclopenta[*a*]indenes

Rajesh Kakadiya [a,f], Huajin Dong [b], Pei-Chih Lee [a,e], Naval Kapuriya [a,f], Xiuguo Zhang [b], Ting-Chao Chou [b], Te-Chang Lee [a,d], Kalpana Kapuriya [a], Anamik Shah [f], Tsann-Long Su [a,c,*]

[a] *Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan*
[b] *Preclinical Pharmacology Core Laboratory, Molecular Pharmacology and Chemistry Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA*
[c] *Graduate Institute of Pharmaceutical Chemistry, China Medical University, Taichung, Taiwan*
[d] *National Research Institute of Chinese Medicine, Taipei 112, Taiwan*
[e] *Institute of Pharmacology, School of Medicine, National Yang-Ming University, Taipei, Taiwan*
[f] *Department of Chemistry, Saurashtra University, Rajkot, Gujarat, India*

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ABSTRACT

A series of bifunctional DNA interstrand cross-linking agents, bis(hydroxymethyl)- and bis(carbamates)-8*H*-3a-azacyclopenta[*a*]indene-1-yl derivatives were synthesized for antitumor evaluation. The preliminary antitumor studies revealed that these agents exhibited potent cytotoxicity in vitro and antitumor therapeutic efficacy against human tumor xenografts in vivo. Furthermore, these derivatives have little or no cross-resistance to either Taxol or Vinblastine. Remarkably, complete tumor remission in nude mice bearing human breast carcinoma MX-1 xenograft by **13a**,**b** and **14g**,**h** and significant suppression against prostate adenocarcinoma PC3 xenograft by **13b** were achieved at the maximum tolerable dose with relatively low toxicity. In addition, these agents induce DNA interstrand cross-linking and substantial G2/M phase arrest in human non-small lung carcinoma H1299 cells. The current studies suggested that these agents are promising candidates for preclinical studies.

1. Introduction

A large number of synthetic compounds or naturally occurring products contain two reactive nucleophilic centers in the molecule. These compounds often exhibit potent antitumor activity since they can covalently bind to DNA. For example, many synthetic *N*-mustards[1] bearing a *N*,*N*-bis(2-chloroethyl)amine active pharmacophore and *cis*-platinum complexes[2,3] are currently used clinically for cancer chemotherapy.[4] The antibiotic anticancer mitomycin C (MMC, **1**, Fig. 1)[5–7] and its analogue, indoloquinone EO9 (**2**),[8] have been demonstrated to be able to cross-link to DNA double strands after bioreductive activation.[9] Another class of compounds, such as thioimidazoles (i.e., carmethizole, **3**),[10,11] bis(hydroxymethyl)-pyrrolidines (i.e., **4**[12,13] and **5**[14]), and 2,3-dihydroxy-6,7-bis(hydroxymethyl)-1*H*-pyrrolizines [i.e., **6** (IPP)],[15,16] have been also demonstrated to have DNA interstrand cross-linking activity, that have certain similarities with MMCs.[14] The halo-substituted pyrrolizine bis(carbamate) **6** was shown to have significant reproducible antitumor activity against a broad range of experimental murine neoplasias and human tumor xenografts in nude athymic mice and was selected for more extensive preclinical

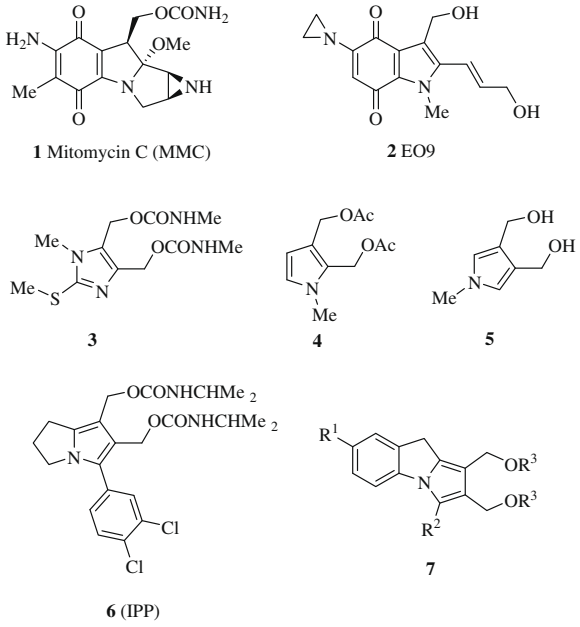

Figure 1. Chemical structure of some DNA bifunctional alkylating agents.

* Corresponding author. Tel.: +886 2 2789 9045; fax: +886 2 2782 5573.
E-mail address: tlsu@ibms.sinica.edu.tw (T.-L. Su).

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studies.[17] However, no further information has been published whether this agent was in clinical trials.

Studies on the mechanism of action showed that DNA interstrand cross-linking by bis(carbamate)pyrroles or pyrrolizines was probably via a S_N1 electrophilic reaction.[14,16] These derivatives are capable of forming an DNA interstrand cross-link with the short oligonucleotide 5′-ACGT at the 5′-CG residues at the minor groove region.[14] The potential electrophilic reactivity of these agents (the carbamates moieties are leaving group in an alkyl-oxygen cleavage mechanism) would be modulated by the degree of electronic perturbation in participating of the pyrrole. Thus, the electronic effects induced from the substituted phenyl groups at C2 of pyrroles or substituent at C5 of pyrrolizines would affect the cytotoxicity of these congeners. However, it was reported that the in vivo antileukemic activity and the host toxicity were not altered to considerable degree as a function of electronic properties of phenyl substituent probably due to the insignificance of the electronic effects.[18,19] In contrast with pyrrole derivatives, the C5-phenyl and the pyrrole ring in 5-phenylpyrrolizines are coplanar. It was suggested that the planarity of the molecule may be required for their antitumor activity. Studies on the structure–activity relationships (SAR) of 5-phenylpyrrolizines revealed that compounds having electron-donating substituent(s) (OMe) on the phenyl ring were generally more toxic than those compounds bearing electron-withdrawing substituent(s) (halogen). While, the in vivo antitumor activities were comparable or slightly less potent in compounds having an electron-donating substituent.[20,21] These studies also suggested that the lipophilicity of compound might affect its antitumor potency.

Based on the potent antitumor activities and mechanism of action of MMC and bis(carbamates)pyrrolizidines, we have synthesized a series of bis(hydroxymethyl) of 3a-azacyclopenta[*a*]indene-1-yl (**7**, R^3 = OH, Fig. 1) and their bis(methylcarbamate) derivatives (**7**, R^3 = OCONHMe) for antitumor studies. These agents can be considered as 'benzologues' of pyrrolizines. One can expect that the newly synthesized compounds might be able to cross-link to the macromolecular DNA via a similar mechanism of action as that of pyrroles or pyrrolizines (Scheme 1). We have introduced an alkyl or substituted phenyl moiety at C3 of the 3a-azacyclopenta[*a*]indene-1-yl to investigate their effects on antitumor activity. The results revealed that the newly synthesized compounds exhibited significant in vitro cytotoxicity and potent in vivo therapeutic efficacy. The synthesis of 3a-azacyclopenta[*a*]indene-1-yl derivatives and their structure–activity relationships for the growth inhibitory properties in a panel of tumor cell lines are included in this study. Furthermore, we also describe the capability of DNA interstrand cross-linking and influence on cell cycle of the newly synthesized derivatives.

2. Results and discussion

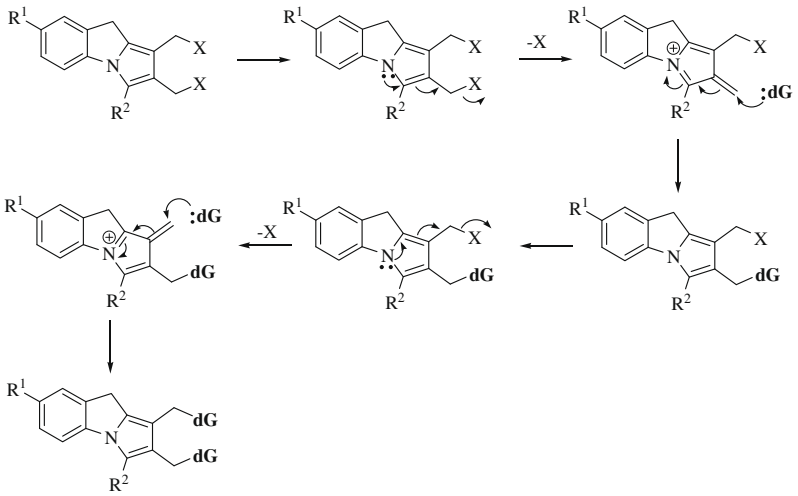
2.1. Chemistry

The (2-hydroxymethyl-3-phenyl-8*H*-3a-azacyclopenta[*a*]indene-1-yl derivatives (**13a–l**) and their bis(methylcarbamate) (**14a–l**) were synthesized by following the method developed by Anderson et al.[18] with modification (Scheme 2). Treatment of indoline-2-carboxylic acid (**8**) with appropriate acid chlorides afforded 1-acyl-indoline-2-carboxylic acids (**11b–k**), which were reacted with dimethyl acetylenedicarboxylate (DMAD) in acetic anhydride by heating at 120 °C to yield diester **12b–k**. The diester **12a** was prepared directly from **8** by reacting with acetic anhydride and DMAD. While, the diester **12l** was synthesized starting from 5-methoxyindole **9** via N-acylation by treating with 4-methoxybenzoyl chloride in the presence of triethylamine followed by catalytic hydrogenation using PtO_2/H_2 in EtOH to give intermediate **11l**. Reaction of **11l** with acetic anhydride and DMAD afforded compound **12l**. The diester functions of **12a–l** were reduced to bis-alcohol derivatives **13a–l** by treating with $LiAlH_4$ in a mixture of ether/CH_2Cl_2 in an ice bath. Treatment of **13a–l** with methyl isocyanate afforded the desired bis(carbamate) derivatives **14a–l** in good yield.

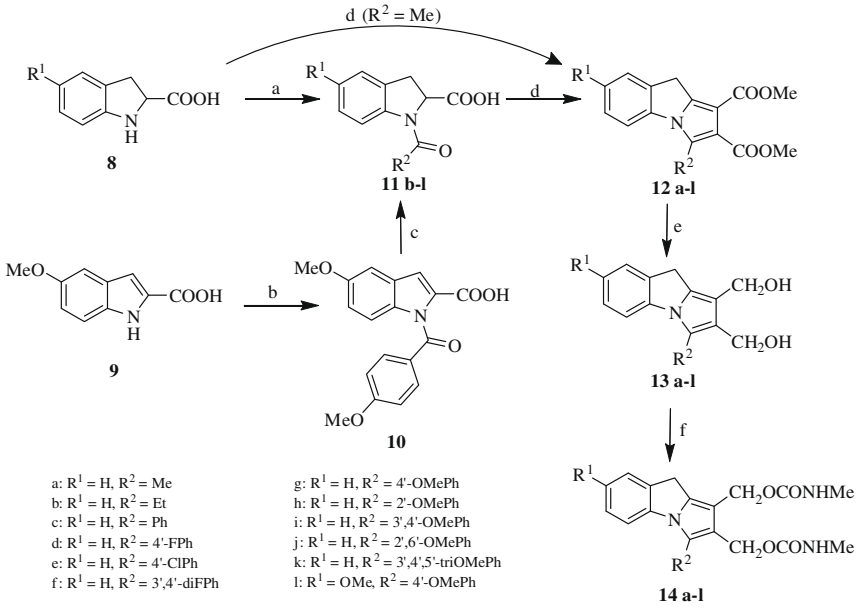
2.2. Biological results

2.2.1. In vitro cytotoxicity

Table 1 shows the cytotoxicity of 1,2-bis(hydroxylmethyl) of cyclopenta[*a*]indenes (**13a–l**) and their counterparts 1,2-bis(methylcarbamate) derivatives (**14a–l**) against human lymphoblastic leukemia CCRF/CEM cell line and its subcell lines resistant to Vinblastine and Taxol. It shows that the size and the electron property of the substituent at C3 altered the cytotoxicity of these agents. In the series of bis(hydroxymethyl) derivatives (**13a–l**), the C3 alkyl substituted derivatives (**13a**,**b**) are more cytotoxic than the phenyl substituted derivatives (**13c–l**). In the series of C3 alkyl substituted compounds, the C3-Me derivative (**13a**) is more potent than **13b** (C3-Et), whereas the order of cytotoxic effects for the series of C3 phenyl substituted derivatives is as follows: Ph > OMe-Ph > halo-Ph, with the exception of C2′,6′-diOMe derivative (**13j**; the least



Scheme 1. The proposed mechanism of DNA bis-alkylation by cyclopenta[*a*]indenes.



Scheme 2. Reagents and conditions: (a) acid chloride/Et_3N/dry CH_2Cl_2, 0–25 °C, 3 h; (b) 4-methoxybenzoyl chloride/Et_3N/dry CH_2Cl_2/DMAP, 0–25 °C, 5 h; (c) PtO_2/H_2/EtOH, 14 h; (d) dimethyl acetylenedicarboxylate/AC_2O, 120 °C; (e) $LiAlH_4/CH_2Cl_2/EtO_2$, 0 °C, 15 min; (f) MeNCO/CH_2Cl_2/Et_3N, rt, 4–5 h.

potent). It clearly demonstrated that the electron property of the substituent at the C3 phenyl ring affected the cytotoxic effects of these agents; compounds having an electron-donating substituent were generally more potent than compounds bearing electron-withdrawing functionality. But, this effect was not shown in bis(methylcarbamate) derivatives. Among the OMe substituted derivatives, **13g**, **13h**, and **13i**, bearing OMe function(s) at C4′, C2′, and C3′,4′, respectively, were more cytotoxic than those derivatives having the same substituent at C2′,6′ and C3′,4′,5′ (**13j** and **13k**, respectively), indicating that introduction of a bulky substituent at C3 decreased the cytotoxicity of cyclopent[*a*]indenes. Similar observations were found in the series of bis(methylcarbamate) derivatives.

We used CCRF-CEM/Taxol and CCRF-CEM/VBL, which are subcell lines of CCRF-CEM cells that are 330-fold resistant to Taxol, and 680-fold resistant to Vinblastine, respectively, when comparing with the IC_{50} of the parent cell line, to study whether the newly synthesized compounds process multi-drug resistance toward Taxol or Vinblastine. As shown in Table 1, the newly synthesized cyclopenta[*a*]indenes have little or no cross-resistance to either Taxol or Vinblastine. This suggests that all newly synthesized compounds are neither a good substrate of membrane multidrug resistance transporters (i.e., p-glycoprotein) nor mutated tubulin. Thus, the newly synthesized compounds are effective against multi-drug resistant tumors although both Taxol and Vinblastine are more cytotoxic than the newly synthesized derivatives.

The antiproliferative activity of cyclopenta[*a*]indenes in inhibiting human solid tumors such as breast carcinoma MX-1 and colon carcinoma HCT-116 (Table 1) cell growth in vitro were also investigated. To explore further, compounds **13a**, **13b**, **13g**, **13l**, **14a**, **14b**, **14g**, and **14l** were selected to study their cell growth inhibitory effect against other solid tumor such as lung carcinoma H1299, oral tumor OECM1, prostate tumor PC3, and brain glioma U87 (Table 2). Generally, the bis(methylcarbamate) compounds were more cytotoxic than the corresponding parent bis(hydroxymethyl) derivatives with the exception of compounds **13a**, **13c**, **13e**, and **13k**. These agents were more effective against CCRF/CEM, MX-1, and OECM1 cell growth among the tumor cell lines tested.

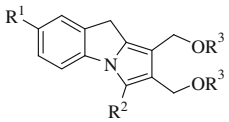
2.2.2. In vivo therapeutic efficacy

Based on the results of the in vitro cytotoxicity, solubility and toxicity to the host, we selected compound **13a**,**b** and **14g**,**h** for further antitumor evaluation in animal model. Figure 2A and B shows the therapeutic efficacy of **13a**,**b** and **14g**,**h** in nude mice bearing human breast carcinoma MX-1 xenograft. At the maximal tolerate doses [**13b**/50 mg/kg and **14g**/5 mg/kg, daily for 3 times (QD × 3); for **13a**/18 mg/kg (Q2D × 2) and **14h**/15 mg/kg (Q2D × 4), via intravenous (iv) injection], these agents induce total tumor remission (complete remission, CR) with relatively low toxicity. In human prostate adenocarcinoma PC3 xenograft in nude mice (Fig. 3A and B), compound **13b** produced complete tumor suppression at the dose of 50 mg/kg, QD × 4, iv inj. Although, 15% of body weight loss was observed during the treatment, it recovered soon after the cessation of drug administration. The antitumor efficacy of **13a** and **13b** in nude mice bearing human ovarian adenocarcinoma SK-OV-3 xenograft was also studied (Fig. 4A and B). We found that **13a** and **13b** yielded 53% and 78% tumor suppression, respectively, on day 22 at the dose of 20 mg/kg, QD × 4, and 50 mg/kg, QD × 3), respectively, via iv injection. These studies suggest that the newly synthesized cyclopent[*a*]indenes possess superior therapeutic efficacy against human breast MX-1 and human prostate adenocarcinoma PC3 over human ovarian adenocarcinoma SK-OV-3 xenograft.

2.2.3. DNA cross-linking study by alkaline agarose gel shift assay

Early works on the study of DNA interstrand cross-linking by IPP (**6**) and related compounds demonstrated that these agents preferentially cross-link deoxyguanosine residues at the duplex nucleotide (a short oligonucleotide 5′-ACGT) at the sequence 5′-CG residues, a common target site in duplex DNA.[14] To determine whether the newly synthesized derivatives are able to cross-link to DNA double-strands, we adopted alkaline agarose gel shift assay[25] to investigate the DNA cross-linking activity of several representative bis(hydroxymethyl) derivatives (**13a**, **13b**, and **13l**) and bis(carbamate) derivatives (**14g**, **14h**, and **14l**). We first mixed the pEGFP-N1 plasmid DNA with various concentrations of tested compounds (1, 10, and 20 μM) or melphalan

Table 1
The cytotoxicity of 1,2-bis(methanol) derivatives (**13a–l**)[a] and 1,2-bis(methylcarbamate) derivatives (**14a–l**)[b] of cyclopenta[*a*]indenes against human lymphoblastic leukemia (CCRF-CEM) and its drug-resistant sublines (CCRF-CEM/Taxol and CCRF-CEM/VBL) and human solid tumor (breast carcinoma MX-1 and colon carcinoma HCT-116) cell growth in vitro[c]



Compd	R^1	R^2	Cell growth inhibition (IC_{50}, μM)[c]				
			CCRF/CEM	CCRF-CEM/VBL[d]	CCRF-CEM/Taxol[d]	MX-1	HCT-116
13a	H	Me	0.010 ± 0.0004	0.067 ± 0.0002 [0.88×][e]	0.032 ± 0.002 [0.39×]	0.36 ± 0.03	0.34 ± 0.0008
14a	H	Me	0.12 ± 0.07	0.063 ± 0.005 [0.53×]	0.12 ± 0.004 [0.67×]	0.34 ± 0.01	0.3 ± 0.001
13b	H	Et	0.36 ± 0.1	0.23 ± 0.004 [0.64×]	0.14 ± 0.009 [0.39×]	1.1 ± 0.03	0.83 ± 0.002
14b	H	Et	0.07 ± 0.005	0.1 ± 0.0003 [1.5×]	0.08 ± 0.003 [1.2×]	0.66 ± 0.009	0.71 ± 0.001
13c	H	C_6H_5	0.25 ± 0.004	0.25 ± 0.03 [1.0×]	0.22 ± 0.02 [0.90×]	1.3 ± 0.011	1.9 ± 0.1
14c	H	C_6H_5	0.36 ± 0.01	0.55 ± 0.004 [1.6×]	1.7 ± 0.05 [4.7×]	5.9 ± 0.02	0.75 ± 0.01
13d	H	4′-FC_6H_4	4.5 ± 0.3	11 ± 0.1 [2.5×]	7.2 ± 0.2 [1.6×]	22 ± 0.1	8.7 ± 0.6
14d	H	4′-FC_6H_4	0.14 ± 0.004	0.28 ± 0.004 [2.0×]	0.30 ± 0.008 [2.2×]	0.74 ± 0.04	0.40 ± 0.007
13e	H	4′-ClC_6H_4	4.3 ± 0.004	4.5 ± 0.2 [1.0×]	2.4 ± 0.1 [0.55×]	6.1 ± 0.1	5.7 ± 0.09
14e	H	4′-ClC_6H_4	5.7 ± 0.6	2.3 ± 0.2 [0.40×]	3.3 ± 0.07 [0.58×]	9.9 ± 0.4	8.9 ± 0.4
13f	H	3′,4′-Di-FC_6H_3	5.2 ± 0.2	7.5 ± 0.3 [1.4×]	7.5 ± 0.2 [1.4×]	7.0 ± 0.02	11 ± 0.4
14f	H	3′,4′-Di-FC_6H_3	0.64 ± 0.007	0.65 ± 0.02 [1.0×]	0.61 ± 0.02 [0.97×]	8.2 ± 0.3	0.90 ± 0.02
13g	H	4′-$MeOC_6H_4$	1.2 ± 0.005	1.6 ± 0.02 [1.4×]	1.7 ± 0.03 [1.5×]	11 ± 0.005	6.1 ± 0.2
14g	H	4′-$MeOC_6H_4$	0.049 ± 0.003	0.063 ± 0.007 [1.3×]	0.070 ± 0.004 [1.4×]	1.9 ± 0.01	2.6 ± 0.1
13h	H	2′-$MeOC_6H_4$	1.0 ± 0.2	2.7 ± 0.03 [2.6×]	1.9 ± 0.007 [1.9×]	8.7 ± 0.04	12 ± 2
14h	H	2′-$MeOC_6H_4$	0.14 ± 0.029	0.36 ± 0.003 [2.7×]	0.25 ± 0.005 [1.8×]	1.3 ± 0.01	0.15 ± 0.004
13i	H	3′,4′-Di-$MeOC_6H_3$	1.0 ± 0.3	1.9 ± 0.07 [1.8×]	1.1 ± 0.02 [1.1×]	11 ± 0.05	4.3 ± 0.3
14i	H	3′,4′-Di-$MeOC_6H_3$	0.35 ± 0.07	0.50 ± 0.007 [1.4×]	0.39±0.02 [1.1×]	1.8 ± 0.001	0.66 ± 0.02
13j	H	2′,6′-Di-$MeOC_6H_3$	8.1 ± 0.2	7.5 ± 0.09 [0.92×]	6.2 ± 0.1 [0.76×]	26 ± 0.5	43 ± 0.3
14j	H	2′,6′-Di-$MeOC_6H_3$	0.64 ± 0.2	0.62 ± 0.01 [0.97×]	0.72 ± 0.05 [1.1×]	5.3 ± 0.3	1.1 ± 0.01
13k	H	3′,4′,5′-Tri-$MeOC_6H_2$	2.2 ± 0.1	0.99 ± 0.04 [0.45×]	1.1 ± 0.004 [0.52×]	17 ± 0.4	29 ± 2
14k	H	3′,4′,5′-Tri-$MeOC_6H_2$	12 ± 0.3	14 ± 0.04 [1.2 ×]	8.4 ± 0.003 [0.73×]	7.2 ± 0.07	1.9 ± 0.08
13l	OMe	4′-$MeOC_6H_4$	0.12 ± 0.005	0.14 ± 0.004 [1.2×]	0.062 ± 0.001 [0.53×]	1.5 ± 0.001	0.67 ± 0.03
14l	OMe	4′-$MeOC_6H_4$	0.062 ± 0.0001	0.098 ± 0.01 [1.6×]	0.062 ± 0.002 [1.0×]	0.87 ± 0.004	0.44 ± 0.02
		Taxol	0.0012 ± 0.0001	0.40 ± 0.04 [333×]	1.2 ± 0.05 [1000×]	0.046 ± 0.0007	0.0011 ± 0.0003
		VBT	0.00073 ± 0.0009	0.078 ± 0.01 [107×]	0.50 ± 0.1 [685×]	0.0022 ± 0.0002	0.0011 ± 0.0003

[a] R^3 = H.
[b] R^3 = CONHMe.
[c] Cell growth inhibition was measured by the XTT assay[28] for leukemic cells and the SRB assay[29] for solid tumor cells after 72-h incubation using a microplate spectrophotometer as described previously.[30] IC_{50} values were determined from dose–effect relationship at six or seven concentrations of each drug by using the CompuSyn software by Chou and Martin[32] based on the median-effect principle and plot.[33,34] Ranges given for Taxol and Vinblastine were mean ± SE (n = 4).
[d] CCRF-CEM/Taxol and CCRF-CEM/VBL are subcell lines of CCRF-CEM cells that are 333-fold resistant to Taxol, and 685-fold resistant to Vinblastine, respectively, when comparing with the IC_{50} of the parent cell line.
[e] Numbers in the brackets are fold of cross-resistant determined by comparison with the corresponding IC_{50} of the parent cell line.

Table 2
The cytotoxicity of 1,2-bis(methanol) derivatives (**13a–l**) and 1,2-bis(methylcarbamate) derivatives (**14a–l**) of cyclopenta[*a*]indenes against human solid tumor (lung carcinoma H1299, oral carcinoma OECM1, prostate carcinoma PC3, glioma U87) cell growth in vitro[a]

Compound	Cell growth inhibition (IC_{50}, μM)[b]			
	H1299	OECM1	PC3	U87
13a	15.16 ± 3.22	0.86 ± 1.21	19.68 ± 2.34	19.32 ± 3.56
14a	>50	1.45	27.18	>50
13b	14.14 ± 2.69	1.24 ± 2.51	16.56 ± 4.10	18.20 ± 1.33
14b	>50	11.8 ± 0.21	>50	>50
13g	50.3 ± 1.98	9.26 ± 2.85	55.5 ± 3.18	45.49 ± 3.11
14g	42.55 ± 2.47	4.63 ± 1.25	38.17 ± 0.93	34.35 ± 2.83
13l	19 ± 3.01	1.54 ± 0.72	23.2 ± 1.12	24.36 ± 1.83
14l	20.89 ± 2.45	1.27 ± 1.13	21.97 ± 3.25	38.28 ± 2.98

[a] Cell growth inhibition was measured by the WST-1 assay[31] after a 72 h-incubation using a microplate spectrophotometer as described in Section 4.
[b] The values are averages of at least two separate determinations.

(1 mM) in a tube and kept at 37 °C for 2 h. Afterward, the pEGFP-N1 plasmid DNA were linearized by digestion with *BamHI* and then subjected to electrophoresis on a 0.8% alkaline agarose gel. Ethidium bromide staining was used to visualize the DNA. As shown in Figure 5, all the tested compounds were able to interact with the plasmid DNA and resulted in interstrand cross-links, which could not separated into single strand under the alkaline condition. These results demonstrated that the newly synthesized 3a-azacyclopenta[*a*]indenes are capable of inducing DNA cross-linking formation.

2.2.4. DNA interstrand cross-linking in human non-small lung carcinoma H1299 cell lines

To determine whether the newly synthesized derivatives are able to form DNA cross-linking in cells, bis(hydroxymethyl) **13l** and its bis(methylcarbamate) derivative **14l** were selected as the representatives compounds for modified single cell gel electrophoresis assay (modified comet assay).[22,23] It is based on the property of negatively charged DNA fragments migration when electric field is applied to the gel after cell lysis.[24] DNA cross-linking agent mitomycin C (MMC) was chosen as a positive control although this agent was more effectively cross-link with DNA under anaerobic conditions.[5] Human non-small lung carcinoma H1299 cells were treated with **13l**, **14l**, and MMC for 1 h at various concentrations under aerobic conditions and then irradiated with a dose of 20 Gy to induce DNA strand breaks. Afterward, the irradiated cells were subjected for modified comet assay. Figure 6 shows the dis-

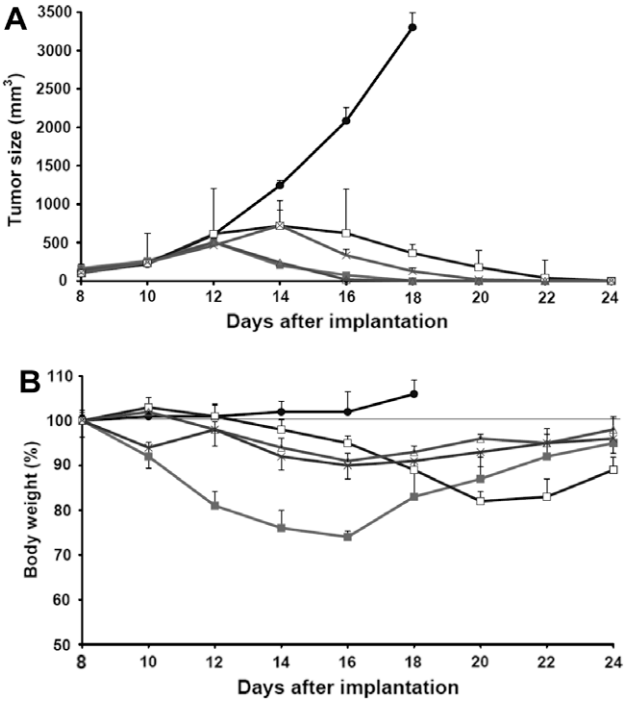


Figure 2. Therapeutic effect of **13a** (18 mg/kg, Q2D × 2, iv inj., $n = 5$, $p < 0.0001$ on day 14–24, no relapse on day 40), **13b** (50 mg/kg, QD × 3, iv inj., $n = 5$, $p < 0.0001$ on day 14–24, 3/5 CR on day 22; 5/5 CR on day 24, no relapse on day 40), **14g** (5 mg/kg, QD × 3, iv inj., $n = 5$, $p < 0.0001$, on day 16–24, 4/5 CR on day 20, 5/5 CR on day 22, 2/5 relapse on D28, 3/5 no relapse on day 40), and **14h** (15 mg/kg, Q2D × 4, iv inj., $n = 4$, $p < 0.0001$ on day 12–24, 3/4 CR on day 16, 4/4 CR on day 18, 1/4 relapse on D28, 3/4 no relapse on day 40) in nude mice bearing human mammary carcinoma MX-1 xenograft, control ($n = 5$); control (●), **13a** (■), **13b** (□), **14g** (×), **14h** (Δ); (A) average tumor size changes; (B) average body weight changes.

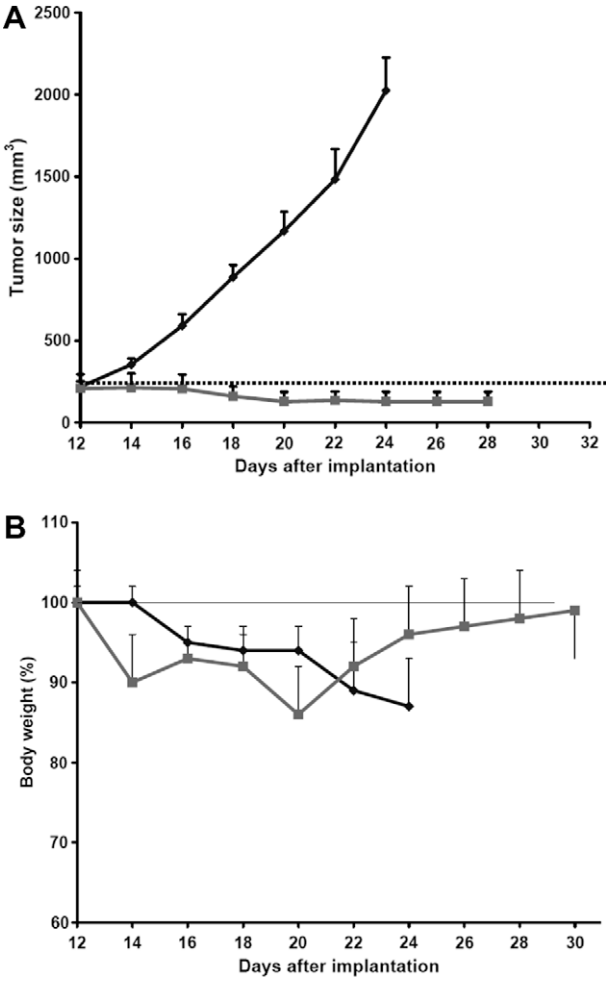


Figure 3. The therapeutic effects of **13b** (50 mg/kg, QD × 4, iv injection) in nude mice bearing human prostate adenocarcinoma PC3 xenograft; $n = 5$; control (♦), **13b** (■); (A) average tumor size changes; (B) average body weight changes; the p values for the treated versus the untreated group from day 20 to 28 is $p < 0.001$.

tribution of tail moment in H1299 cells. Since ionic radiation resulted DNA fragments in cells treated with DNA cross-linking agents would reduce their electrophoretic migration rate under alkaline condition, the length and fragment content of the tail is inversely proportional to the amount of DNA interstrand cross-linking. Since interstrand cross-link formation can be represented as percent decrease in tail moment, Figure 7 shows the levels of cross-linked DNA varied with compound concentrations. Under the same experimental conditions (100 μM), **14l**, MMC, and **13l** induce 97.9%, 76.5%, and 35.5% DNA interstrand cross-linking, respectively. The results showed that the order of potency for DNA cross-linking induction by these agents is **14l** > MMC > **13l**.

2.2.5. Cell cycle inhibition

DNA interacting/damaging agents are known to induce cell cycle perturbations and arrest the cell cycle progression predominantly at the G2/M boundary.[26] Compounds **13l**, a bis(hydroxymethyl) derivative, and **14l**, a bis(methylcarbamate) derivative, were selected as the representatives compounds to investigate their influence on cell cycle progression. We treated human non-small lung carcinoma H1299 cells with these compounds at the concentrations of 10 μM. At the time indicated (24, 48, and 72 h), the cells were harvested, stained with propidium iodide (PI) and analyzed with a flowcytometer. As shown in Figure 8, both **13l** and **14l** remarkably delayed the cell cycle progression. As compared to the control culture, we observed the accumulation of the S phase cells at 24 h after treatment with **13l** and **14l** and followed by the accumulation of the G2/M phase at 48 and 72 h. Furthermore, increased sub-G1 population were noticed in cells treated with these 2 compounds for 72 h (Fig. 8).

3. Conclusions

We have designed and synthesized a series of DNA bifunctional alkylating agents, bis(hydroxymethyl)-8*H*-3a-azacyclopenta[*a*]indene-1-yl derivatives (**13a–l**) and their bis(carbamate) derivatives (**14a–l**) for antitumor evaluation. These agents can be considered as 'benzologues' of pyrrolizines and may have a similar mechanism of action to bis-carbamoyloxymethyl derivatives of pyrroles and pyrrolizines or MMC. The present studies demonstrated that these newly synthesized compounds exhibited potent cytotoxicity against human leukemia and various solid tumor cell growths in vitro and potent antitumor efficacy in vivo with a relatively low toxicity. Detailed SAR studies demonstrated that the size and electron properties of the substituent at C3 affected the cytotoxicity of these agents. Compounds **13a**,**b** and **14g**,**h** were found to have potent therapeutic efficacy against human breast MX-1 xenograft in animal model. Complete tumor remission was achieved in nude mice bearing human breast carcinoma MX-1 xenograft by these derivatives. Interestingly, we also found that compound **13b** was able to significantly suppress human prostate adenocarcinoma PC3 xenograft in nude mice. Studies on the DNA interstrand cross-linking suggested that the newly synthesized derivatives are potent bifunctional DNA cross-linking agents. Furthermore, both derivatives induced substantial G2/M phase arrest. On the basis of the present studies, these agents should be considered as promising lead compounds for preclinical studies.

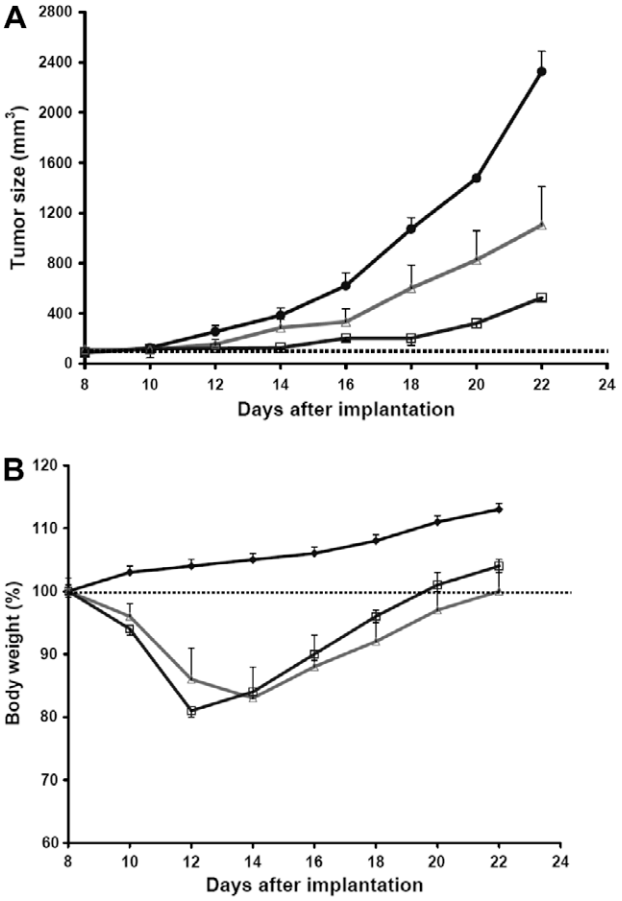


Figure 4. The therapeutic effects of **13a** (20 mg/kg, QD × 4, iv injection) and **13b** (50 mg/kg, QD × 3, iv injection) in nude mice bearing human ovarian adenocarcinoma SK-OV-3 xenograft; $n = 5$; control (●), **13a** (Δ), **13b** (□); (A) average tumor size changes; (B) average body weight changes; the values for the treated versus the untreated group from day 20 to 28 is $p < 0.001$.

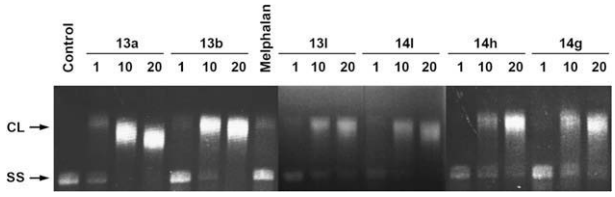


Figure 5. Representative DNA cross-linking gel shift assay for **13a**, **13b**, **13l**, **14h**, **14g**, and **14l** at the concentrations of 1, 10, and 20 μM. Control lane shows single-stranded DNA (SS), while CL shown in all tested lanes is DNA double-stranded cross-linking. Melphalan at 1 μM was used as a positive control.

4. Experimental

4.1. General methods and materials

Melting points were determined on a Fargo melting point apparatus and are uncorrected. Column chromatography was carried out over Silica Gel G60 (70–230 mesh). Thin-layer chromatography was performed on Silica Gel G60 F_{254} plates with short wavelength UV light for visualization. Elemental analyses were done on a Heraeus CHN–O Rapid instrument. ^{1}H NMR spectra were recorded on 400 MHz spectrometer with Me_4Si as an internal standard. The chemical shifts were reported in ppm (δ) relative to TMS and coupling constants (J) hertz (Hz).

4.2. 5-Methoxy-1-(4-methoxybenzoyl)-1*H*-indole-2-carboxylic acid (10)

To a solution of commercially available 5-methoxyindole-2-carboxylic acid **9** (6.69 g, 35 mmol) and triethylamine (10 mL, 71.74 mmol) in anhydrous CH_2Cl_2 (70 mL) containing catalytic amount of 4-dimethylaminopyridine (DMAP) was added dropwise a solution of 4-methoxybenzoyl chloride (6.20 g, 36.34 mmol) in CH_2Cl_2 (15 mL) at 0 °C. The reaction mixture was stirred at this temperature for 15 min and then at room temperature for 4 h. The reaction mixture was washed successively with 5% aqueous $KHSO_4$ solution (2 × 20 mL), brine (25 mL), dried over Na_2SO_4, and evaporated under reduced pressure to dryness. The solid residue was recrystallized from methanol to yield **10** as a white solid (7.4 g, 65%); mp 185–187 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 13.2 (br s, 1H), 7.59 (d, J = 8.9 Hz, 2H), 7.38 (d, J = 9.0 Hz, 1H), 7.33 (s, 1H), 7.28 (d, J = 2.5 Hz, 1H), 7.07 (d, J = 8.9 Hz, 2H), 7.02 (dd, J = 9.0, 2.5 Hz, 1H), 3.85 (s, 3H), 3.80 (s, 3H). Anal. Calcd for $C_{18}H_{15}NO_5 \cdot 0.1H_2O$: C, 65.73; H, 4.65; N, 4.25. Found: C, 65.77; H, 4.61; N, 4.24.

4.3. 1-Propionyl-2,3-dihydro-1*H*-indole-2-carboxylic acid (11b)

To a solution of indoline-2-carboxylic acid **8** (8.16 g, 50 mmol) and triethylamine (10 mL, 71.74 mmol) in anhydrous CH_2Cl_2 (60 mL) containing catalytic amount of DMAP was added dropwise a solution of propionyl chloride (5.55 g, 60 mmol) in CH_2Cl_2 (20 mL) at 0 °C. The reaction mixture was stirred at this temperature for 15 min and then at room temperature for 2.5 h. The reaction mixture was washed successively with 5% aqueous $KHSO_4$ solution (2 × 20 mL), brine (25 mL), dried over Na_2SO_4, and evaporated under reduced pressure to dryness. The residue was crystallized from methanol to yield **11b** (9.19 g, 83%); mp 173–175 °C; δ = 13.26 (br s, 1H), 8.09–8.07 (m, 1H), 7.23–7.15 (m, 2H), 7.01–6.98 (m, 1H), 5.15 (d, J = 16.0 Hz, 1H), 3.57 (dd, J = 16.0, 11.0 Hz, 1H), 3.17 (d, J = 16.0 Hz, 1H), 2.51 (q, J = 7.6 Hz, 2H), 1.05 (t, J = 7.6 Hz, 3H). Anal. Calcd for $C_{12}H_{13}NO_3$: C, 65.74; H, 5.98; N, 6.39. Found: C, 65.73; H, 6.07; N, 6.34.

By following the same procedure as describe for **11b**, the following compounds **11c–k** were synthesized.

4.3.1. 1-Benzoyl-2,3-dihydro-1*H*-indole-2-carboxylic acid (11c)

Compound **11c** was synthesized from indoline-2-carboxylic acid **8** (6.52 g, 40 mmol), triethylamine (11 mL, 78 mmol), benzoyl chloride (5.62 g, 40 mmol); yield (10.66 g, 81%); mp 209–210 °C (lit. mp 191–193 °C);[27] ^{1}H NMR (400 MHz, DMSO-d_6) δ = 13.01 (br s, 1H), 8.17–8.14 (m, 1H), 7.49 (m, 5H), 7.26–7.25 (m, 2H), 7.06 (m, 1H), 4.97 (s, 1H), 3.63–3.61 (m, 1H), 3.09 (d, J = 16.3 Hz, 1H); MS: m/z 268.0 $(M+H)^+$.

4.3.2. 1-(4-Fluorobenzoyl)-2,3-dihydro-1*H*-indole-2-carboxylic acid (11d)

Compound **11d** was synthesized from indoline-2-carboxylic acid **8** (6.52 g, 40 mmol), triethylamine (11 mL, 78 mmol), and 4-fluorobenzoyl chloride (6.34 g, 40 mmol); yield (9.28 g, 81%); mp 215–216 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 12.98 (br s, 1H), 8.16–7.03 (m, 8H), 5.01 (d, J = 9.3 Hz, 1H), 3.63 (dd, J = 16.3, 11.1 Hz, 1H), 3.10 (d, J = 16.5 Hz, 1H). Anal. Calcd for $C_{16}H_{12}FNO_3$: C, 67.36; H, 4.24; N, 4.91. Found: C, 67.22; H, 4.58; N, 4.72.

4.3.3. 1-(4-Chlorobenzoyl)-2,3-dihydro-1*H*-indole-2-carboxylic acid (11e)

Compound **11e** was synthesized from indoline-2-carboxylic acid (**8**) (6.52 g, 40 mmol), triethylamine (11 mL, 78 mmol), and 4-chlorobenzoyl chloride (5 g, 40 mmol); yield (9.35 g, 77%); mp 184–187 °C; ^{1}H NMR (MHz, DMSO-d_6) δ = 12.91 (br s, 1H), 7.81–7.53 (m, 4H), 7.27–7.25 (m, 2H), 7.18–7.03 (m, 2H), 5.02 (d,

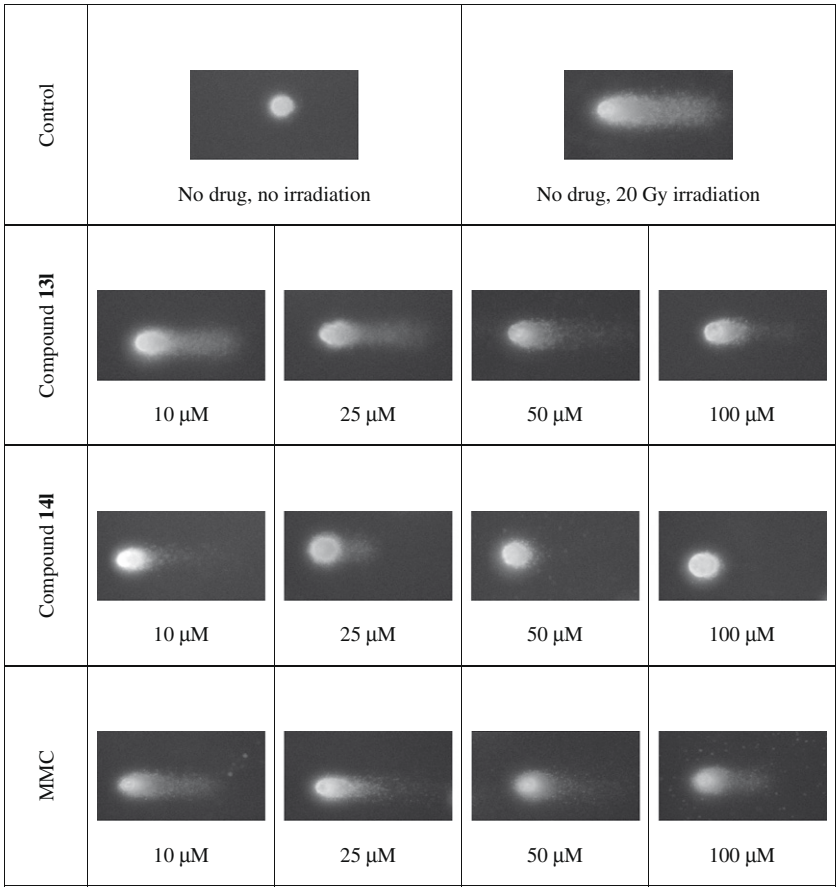


Figure 6. Typical comet images of DNA interstrand cross-linking study by modified comet assay under the aerobic conditions in H1299 cells. Cells were treated with various concentrations of **13l** and **14l** for 1 h prior to 20 Gy X-ray irradiation. Mitomycin C (MMC) was used as a positive control.

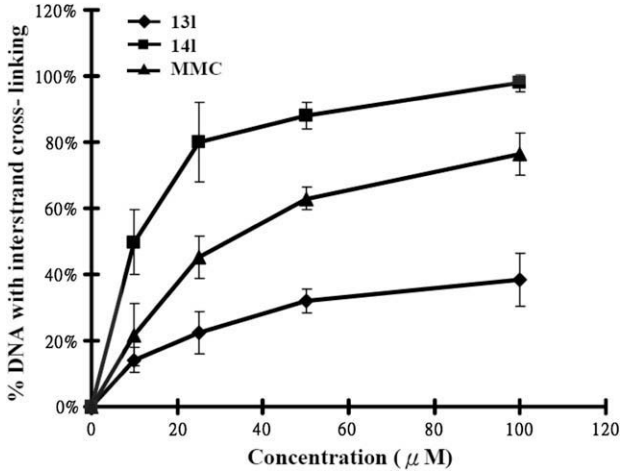


Figure 7. Percentages of DNA interstrand cross-linking in H1299 cells induced by **13l** and **14l** at various concentrations. Interstrand cross-link formation represented as percent of decreased tail moment. Mitomycin C (MMC) was served as a positive control. Data were presented as mean of 3 independent experiments. Bars, SD.

J = 9.8 Hz, 1H), 3.63 (dd, *J* = 16.6, 11.2 Hz, 1H), 3.13 (d, *J* = 16.6 Hz, 1H). Anal. Calcd for $C_{16}H_{12}ClNO_3$: C, 63.69; H, 4.01; N, 4.64. Found: C, 62.15; H, 3.96; N, 4.51.

4.3.4. 1-(3,4-Difluorobenzoyl)-2,3-dihydro-1*H*-indole-2-carboxylic acid (11f)

Compound **11f** was synthesized from indoline-2-carboxylic acid (**8**) (6.52 g, 40 mmol), triethylamine (11 mL, 78 mmol) and 3,4-difluorobenzoyl chloride (7.06 g, 40 mmol); yield (8.53 g, 70%); mp 248–250 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 12.99 (br s, 1H), 8.15 (m, 1H), 7.64 (t, *J* = 8.7 Hz, 1H), 7.60–7.55 (m, 1H), 7.41 (s, 1H), 7.28–7.06 (m, 3H), 5.02 (dd, *J* = 10.9, 2.9 Hz, 1H), 3.63 (dd, *J* = 16.4, 11.2 Hz, 1H), 3.12 (d, *J* = 16.2 Hz, 1H). Anal. Calcd for $C_{16}H_{11}F_2NO_3$: C, 63.37; H, 3.66; N, 4.62. Found: C, 63.12; H, 3.97; N, 4.83.

4.3.5. 1-(4-Methoxybenzoyl)-2,3-dihydro-1*H*-indole-2-carboxylic acid (11g)

Compound **11g** was synthesized from indoline-2-carboxylic acid (**8**) (6.52 g, 40 mmol), triethylamine (11 mL, 78 mmol), and 4-methoxybenzoyl chloride (6.82 g, 40 mmol); yield (10.88 g, 92%); mp 196–198 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 12.91 (br s, 1H), 7.50–7.46 (m, 2H), 7.24 (d, *J* = 7.3 Hz, 1H), 7.09–6.97 (m, 5H), 5.01 (dd, *J* = 11.0, 3.3 Hz, 1H), 3.82 (s, 3H), 3.61 (dd, *J* = 16.6, 11.0 Hz, 1H), 3.08 (dd, *J* = 16.6, 3.2 Hz, 1H). Anal. cacld. for $C_{17}H_{15}NO_4$: C, 68.68; H, 5.09; N, 4.71. Found: C, 68.10; H, 5.06; N, 4.62.

4.3.6. 1-(2-Methoxybenzoyl)-2,3-dihydro-1*H*-indole-2-carboxylic acid (11h)

Compound **11h** was synthesized from indoline-2-carboxylic acid (**8**) (8 g, 49 mmol) and triethylamine (14 mL, 100 mmol) and 2-methoxybenzoyl chloride (6.82 g, 49 mmol); yield (13.17 g, 91%); mp 189–192 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 12.91 (br s, 1H), 7.49–7.47 (m, 2H), 7.24–7.23 (m, 2H), 7.08 (m, 1H), 7.02–6.98 (m, 3H), 5.02 (dd, *J* = 7.3, 2.2 Hz, 1H), 3.82 (s, 3H), 3.61 (dd, *J* = 10.9, 7.5 Hz, 1H), 3.08 (dd, *J* = 11.0, 1.6 Hz, 1H). Anal. Calcd for $C_{17}H_{15}NO_4$: C, 68.68; H, 5.09; N, 4.71. Found: C, 68.25; H, 5.04; N, 4.61.

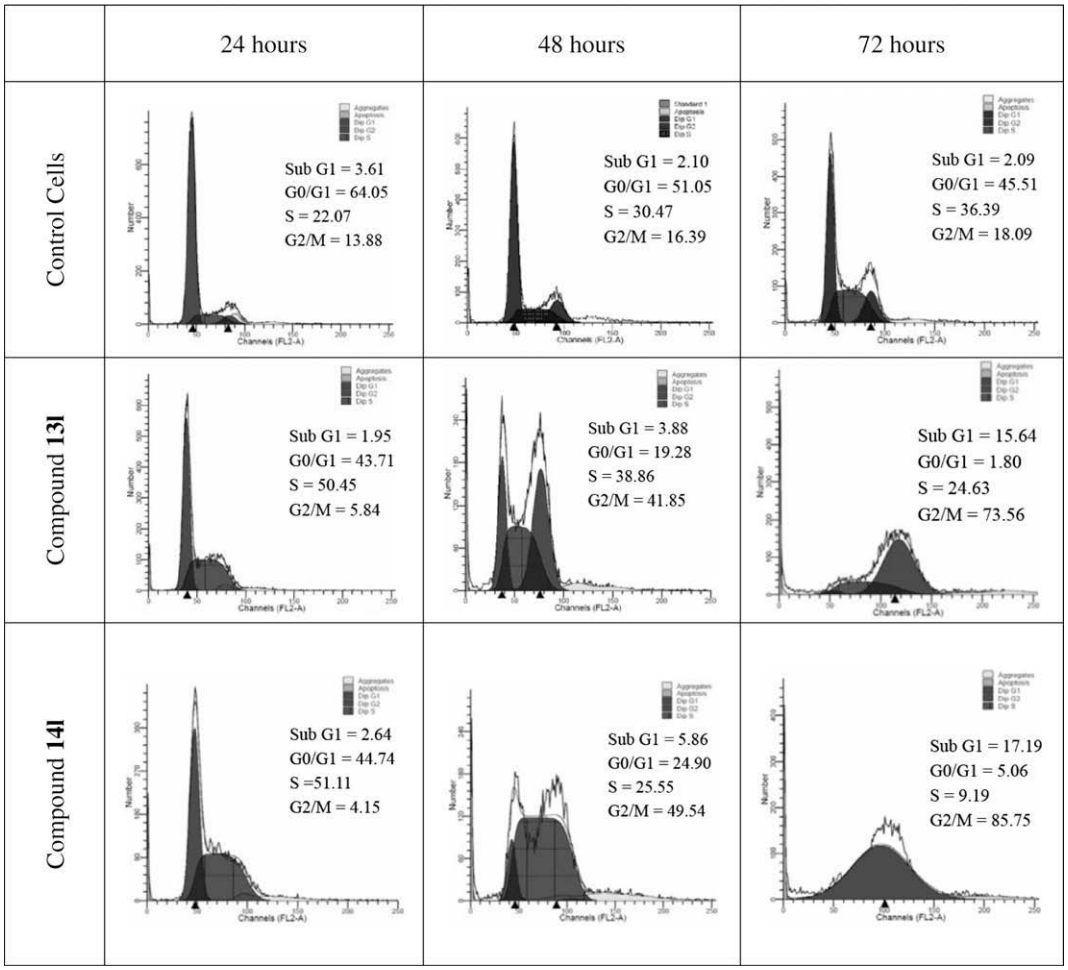


Figure 8. Effects of **13l** and **14l** on cell cycle progress in H1299 cells. Cells were treated with **13l** and **14l** for 24, 48, and 72 h and cell cycle phases were analyzed by flow cytometric technique. The distribution of cells at the sub-G1 (apoptotic cells), G0/G1, S, and G2/M phases was estimated with ModFit LT 2.0 software.

4.3.7. 1-(3,4-Dimethoxybenzoyl)-2,3-dihydro-1*H*-indole-2-carboxylic acid (11i)

Compound **11i** was synthesized from indoline-2-carboxylic acid (**8**) (5.03 g, 30.82 mmol), triethylamine (8.6 mL, 61.64 mmol), and 3,4-dimethoxybenzoyl chloride (6.18 g, 30.82 mmol); yield (8.20 g, 81%); mp 113–115 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 12.92 (br s, 1H), 7.26–7.24 (m, 1H), 7.10–6.98 (m, 6H), 5.00 (dd, J = 10.9, 3.1 Hz, 1H), 3.82 (s, 3H), 3.75 (s, 3H), 3.62 (dd, J = 16.6, 11.1 Hz, 1H), 3.07 (dd, J = 16.6, 2.9 Hz, 1H). Anal. Calcd for $C_{18}H_{17}NO_5$: C, 66.05; H, 5.23; N, 4.28. Found: C, 65.47; H, 5.74; N, 4.28.

4.3.8. 1-(2,6-Dimethoxybenzoyl)-2,3-dihydro-1*H*-indole-2-carboxylic acid (11j)

Compound **11j** was synthesized from indoline-2-carboxylic acid (**8**) (7.88 g, 48.28 mmol), triethylamine (8.6 mL, 61.64 mmol), and 2,6-dimethoxybenzoyl chloride (9.69 g, 48.28 mmol); yield (12.77 g, 81%); mp 84–85 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 12.88 (br s, 1H), 7.25–7.23 (m, 1H), 7.10–6.97 (m, 6H), 5.00 (dd, J = 10.9, 3.2 Hz, 1H), 3.82 (s, 3H), 3.75 (s, 3H), 3.61 (dd, J = 16.6, 11.1 Hz, 1H), 3.07 (dd, J = 16.6, 2.9 Hz, 1H). Anal. Calcd for $C_{18}H_{17}NO_5$?H_2O: C, 62.60; H, 5.54; N, 4.05. Found: C, 62.54; H, 5.25; N, 3.97.

4.3.9. 1-(3,4,5-Trimethoxybenzoyl)-2,3-dihydro-1*H*-indole-2-carboxylic acid (11k)

Compound **11k** was synthesized from indoline-2-carboxylic acid (**8**) (6.01 g, 36.81 mmol), triethylamine (10.4 mL, 74.61 mmol), and 3,4,5-trimethoxybenzoyl chloride (8.49 g, 36.81 mmol); yield (9.92 g, 75%); mp 224–227 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 13.01 (br s, 1H), 8.31 (s, 1H), 7.25 (d, J = 7.49 Hz, 1H), 7.15 (m, 1H), 7.03–7.02 (m, 1H), 6.77 (s, 2H), 4.97–4.95 (m, 1H), 3.77 (s, 6H), 3.71 (s, 3H), 3.65–3.59 (m, 1H), 3.10–3.05 (m, 1H). Anal. Calcd for $C_{19}H_{19}NO_6$: C, 63.86; H, 5.36; N, 3.92. Found: C, 62.27; H, 5.29; N, 3.76.

4.3.10. 5-Methoxy-1-(4-methoxybenzoyl)-2,3-dihydro-1*H*-indole-2-carboxylic acid (11l)

To a solution of **10** (7.02 g, 21.57 mmol) in ethanol (200 mL) was added platinum oxide (0.7 g). The mixture was hydrogenated for 6 h at 32 psi. The reaction mixture was filtered through a pad of Celite. The filter cake was washed with ethanol. The combined filtrate and washings was evaporated in vacuo to dryness. The solid residue was recrystallized from ethanol to yield **11l** (6.35 g, 90%); mp 151–152 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 12.92 (br s, 1H), 7.45 (d, J = 8.6 Hz, 2H), 7.00 (d, J = 8.6 Hz, 2H), 6.86 (m, 2H), 6.67 (s, 1H), 5.01–4.97 (m, 1H), 3.81 (s, 3H), 3.70 (s, 3H), 3.63–3.56 (m, 1H), 3.05 (m, 1H). Anal. Calcd for $C_{18}H_{17}NO_5$: C, 66.05; H, 5.23; N, 4.28. Found: C, 65.69; H, 5.47; N, 4.14.

4.4. 3-Methyl-8*H*-3a-aza-cyclopenta[*a*]indene-1,2-dicarboxylic acid dimethyl ester (12a)

A mixture of indoline-2-carboxylic acid **8** (10.60 g, 65 mmol) and dimethyl acetylenedicarboxylate (9.24 mL, 80 mmol) in acetic anhydride (110 mL) was stirred in a flask equipped with a reflux

condenser and a gas bubbler to monitor carbon dioxide evolution during the reaction. The mixture was heated to 120 °C where carbon dioxide evolution was most intense. The temperature was maintained until gas evolution had ceased. The dark solution was concentrated in vacuo and the solid residue was crystallized from methanol to yield **12a** (14.40 g, 78%); mp 162–164 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.53–7.46 (m, 2H), 7.37–7.33 (m, 1H), 7.27–7.20 (m, 1H), 4.01 (s, 2H), 3.89 (s, 3H), 3.85 (s, 3H), 2.74 (s, 3H). Anal. Calcd for $C_{16}H_{15}NO_4$: C, 67.36; H, 5.30; N, 4.91. Found: C, 67.30; H, 5.31; N, 4.85.

4.4.1. 3-Ethyl-8*H*-3a-aza-cyclopenta[*a*]indene-1,2-dicarboxylic acid dimethyl ester (12b)

A mixture of **11b** (4.49 g, 20.5 mmol) and dimethyl acetylenedicarboxylate (5.68 mL, 40 mmol) in acetic anhydride (40 mL) was stirred in a flask equipped with a reflux condenser and a gas bubbler to monitor carbon dioxide evolution during the reaction. The mixture was heated to 120 °C where carbon dioxide evolution was most intense. The temperature was maintained until gas evolution had ceased. The dark solution was concentrated in vacuo to dryness and the solid residue was recrystallized from methanol to yield **12b** (4.61 g, 75%); mp 86–88 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.64–7.59 (m, 2H), 7.47–7.43 (m, 1H), 7.31–7.27 (m, 1H), 4.07 (s, 2H), 3.76 (s, 3H), 3.75 (s, 3H), 3.08 (q, J = 7.4 Hz, 2H), 1.22 (t, J = 7.4 Hz, 3H). Anal. Calcd for $C_{17}H_{17}NO_4$: C, 68.21; H, 5.72; N, 4.68. Found: C, 68.22; H, 5.74; N, 4.65.

By following the same procedure as describe for **12b**, the following compounds **12c–l** were synthesized.

4.4.2. 3-Phenyl-8*H*-3a-aza-cyclopenta[*a*]indene-1,2-dicarboxylic acid dimethyl ester (12c)

Compound **12c** was synthesized from **11c** (7.48 g, 28 mmol) and dimethyl acetylenedicarboxylate (4 mL, 32 mmol) in acetic anhydride (40 mL); yield (7.5 g, 77%); mp 149–151 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.67–7.51 (m, 6H), 7.23–7.18 (m, 2H), 6.67–6.60 (m, 1H), 4.15 (s, 2H), 3.79 (s, 3H), 3.62 (s, 3H). Anal. Calcd for $C_{21}H_{17}NO_4{\cdot}0.9H_2O$: C, 69.40; H, 5.21; N, 3.85. Found: C, 69.42; H, 4.65; N, 3.83.

4.4.3. 3-(4-Fluorophenyl)-8*H*-3a-aza-cyclopenta[*a*]indene-1,2-dicarboxylic acid dimethyl ester (12d)

Compound **12d** was synthesized from **11d** (7.13 g, 25 mmol) and dimethyl acetylenedicarboxylate (4 mL, 32 mmol) in acetic anhydride (40 mL); yield (6.63 g, 72%); mp 152–155 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.54–7.51 (m, 2H), 7.47 (d, J = 7.4 Hz, 1H), 7.21–7.15 (m, 3H), 6.65 (d, J = 7.93 Hz, 1H), 7.12 (t, J = 7.4 Hz, 1H), 4.12 (s, 2H), 3.89 (s, 3H), 3.72 (s, 3H). Anal. Calcd for $C_{21}H_{16}FNO_3{\cdot}0.67H_2O$: C, 66.84; H, 4.63; N, 3.71. Found: C, 66.94; H, 4.46; N, 3.65.

4.4.4. 3-(4-Chlorophenyl)-8*H*-3a-aza-cyclopenta[*a*]indene-1,2-dicarboxylic acid dimethyl ester (12e)

Compound **12e** was synthesized from **11e** (7.54 g, 25 mmol) and dimethyl acetylenedicarboxylate (4 mL, 32 mmol) in acetic anhydride (40 mL); yield (7.32 g, 79%); mp 166–168 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.64–7.54 (m, 5H), 7.25–7.21 (m, 2H), 6.68–6.64 (m, 1H), 4.16 (s, 2H), 3.79 (s, 3H), 3.62 (s, 3H). Anal. Calcd for $C_{21}H_{16}ClNO_4$: C, 66.06; H, 4.22; N, 3.67. Found: C, 65.92; H, 4.22; N, 3.64.

4.4.5. 3-(3,4-Difluoro-phenyl)-8*H*-3a-aza-cyclopenta[*a*]indene-1,2-dicarboxylic acid dimethyl ester (12f)

Compound **12f** was synthesized from **11f** (8.14 g, 26.82 mmol) and dimethyl acetylenedicarboxylate (4.7 mL, 37.3 mmol) in acetic anhydride (40 mL); yield (8.37 g, 81%); mp 156–158 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.70–7.67 (m, 1H), 7.64–7.59 (m, 2H), 7.41–7.40 (m, 1H), 7.25–7.22 (m, 2H), 6.68–6.65 (m, 1H), 4.16 (s, 2H), 3.80 (s, 3H), 3.62 (s, 3H). Anal. Calcd for $C_{21}H_{15}F_2NO_4$: C, 65.80; H, 3.94; N, 3.65. Found: C, 65.83; H, 4.03; N, 3.62.

4.4.6. 3-(4-Methoxyphenyl)-8*H*-3a-aza-cyclopenta[*a*]indene-1,2-dicarboxylic acid dimethyl ester (12g)

Compound **12g** was synthesized from **11g** (7.42 g, 25 mmol) and dimethyl acetylenedicarboxylate (4.7 mL, 37.3 mmol) in acetic anhydride (40 mL); yield (8.42 g, 89%); mp 157–159 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.59–7.58 (m, 1H), 7.44 (d, J = 8.3 Hz, 2H), 7.21–7.20 (m, 2H), 7.09 (d, J = 8.3 Hz, 2H), 6.67–6.65 (m, 1H), 4.15 (s, 2H), 3.85 (s, 3H), 3.78 (s, 3H), 3.60 (s, 3H). Anal. Calcd for $C_{22}H_{19}NO_5$: C, 70.02; H, 5.07; N, 3.71. Found: C, 69.80; H, 6.08; N, 3.62.

4.4.7. 3-(2-Methoxy-phenyl)-8*H*-3a-aza-cyclopenta[*a*]indene-1,2-dicarboxylic acid dimethyl ester (12h)

Compound **12h** was synthesized from **11h** (9.07 g, 30.5 mmol) and dimethyl acetylenedicarboxylate (5 mL, 40.81 mmol) in acetic anhydride (40 mL); yield (8.90 g, 77%); mp 162–164 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.58–7.53 (m, 2H), 7.36–7.34 (m, 1H), 7.21–7.19 (m, 3H), 7.11–7.07 (m, 1H), 6.53–6.51 (m, 1H), 4.23–4.09 (m, 2H), 3.79 (s, 3H), 3.65 (s, 3H), 3.58 (s, 3H). Anal. Calcd for $C_{22}H_{19}NO_5$: C, 70.02; H, 5.07; N, 3.71. Found: C, 70.03; H, 5.06; N, 3.69.

4.4.8. 3-(3,4-Dimethoxyphenyl)-8*H*-3a-aza-cyclopenta[*a*]indene-1,2-dicarboxylic acid dimethyl ester (12i)

Compound **12i** was synthesized from **11i** (9.01 g, 27.53 mmol) and dimethyl acetylenedicarboxylate (4 mL, 32 mmol) in acetic anhydride (40 mL); yield (8.9 g, 79%); mp 186–187 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.60–7.58 (m, 1H), 7.24–7.22 (m, 2H), 7.12 (s, 1H), 7.10 (s, 1H), 7.08–7.05 (m, 1H), 6.77–6.75 (m, 1H), 4.16 (s, 2H), 3.85 (s, 3H), 3.79 (s, 3H), 3.75 (s, 3H), 3.63 (s, 3H). Anal. Calcd for $C_{23}H_{21}NO_6$: C, 67.80; H, 5.20; N, 3.44. Found: C, 67.57; H, 5.22; N, 3.39.

4.4.9. 3-(2,6-Dimethoxyphenyl)-8*H*-3a-aza-cyclopenta[*a*]indene-1,2-dicarboxylic acid dimethyl ester (12j)

Compound **12j** was synthesized from **11j** (12.11 g, 37 mmol) and dimethyl acetylenedicarboxylate (5.5 mL, 44.90 mmol) in acetic anhydride (55 mL); yield (10.53 g, 70%); mp 84–86 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.58–7.55 (m, 1H), 7.49 (t, J = 8.3 Hz, 1H), 7.22–7.18 (m, 2H), 6.82 (s, 1H), 6.80 (s, 1H), 6.50–6.55 (m, 1H), 4.15 (s, 2H), 3.79 (s, 3H), 3.63 (s, 6H), 3.54 (s, 3H). Anal. Calcd for $C_{23}H_{21}NO_6$: C, 67.80; H, 5.20; N, 3.44. Found: C, 67.12; H, 5.20; N, 3.41.

4.4.10. 3-(3,4,5-Trimethoxyphenyl)-8*H*-3a-aza-cyclopenta[*a*]indene-1,2-dicarboxylic acid dimethyl ester (12k)

Compound **12k** was synthesized from **11k** (9 g, 25.18 mmol) and dimethyl acetylenedicarboxylate (4.2 mL, 34.28 mmol) in acetic anhydride (45 mL); yield (9.62 g, 87%): mp 205–207 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.59 (d, J = 6.9 Hz, 1H), 7.29–7.21 (m, 2H), 6.87 (d, J = 7.6 Hz, 1H), 6.80 (s, 2H), 4.16 (s, 2H), 3.99 (s, 3H), 3.77 (s, 3H), 3.76 (s, 6H), 3.65 (s, 3H). Anal. Calcd for $C_{24}H_{23}NO_7$: C, 65.90; H, 5.30; N, 3.20. Found: C, 65.81; H, 5.35; N, 3.12.

4.4.11. 6-Methoxy-3-(4-methoxyphenyl)-8*H*-3a-aza-cyclopenta[*a*]indene-1,2-dicarboxylic acid dimethyl ester (12l)

Compound **12l** was synthesized from **11l** (4.24 g, 12.9 mmol) and dimethyl acetylenedicarboxylate (2.4 mL, 19.5 mmol) in acetic anhydride (24 mL); yield (3.75 g, 71%); mp 240–242 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.42 (d, J = 6.9 Hz, 2H), 7.19 (d, J = 2.4 Hz, 1H), 7.08 (d, J = 8.7 Hz, 2H), 6.77 (dd, J = 8.8, 2.6 Hz, 1H), 6.55 (d, J = 8.8 Hz, 1H), 4.09 (s, 2H), 3.85 (s, 3H), 3.77 (s, 3H),

3.73 (s, 3H), 3.60 (s, 3H). Anal. Calcd for $C_{23}H_{21}NO_5$: C, 67.80; H, 5.20; N, 3.44. Found: C, 67.83; H, 5.19; N, 3.39.

4.5. (3-Methyl-2-hydroxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl)methanol (13a)

A solution of **12a** (3.14 g, 11 mmol) in anhydrous dichloromethane (30 mL) was added dropwise to a stirred suspension of $LiAlH_4$ (1.04 g, 27.5 mmol) in anhydrous ether (45 mL) at 0 °C. The mixture was stirred for 15 min after the addition was complete. The excess $LiAlH_4$ was carefully decomposed by the slow, sequential addition of water (1.3 mL), 15% NaOH aqueous solution (1.3 mL), and water (3.6 mL). The precipitated solid inorganic salt was removed by filtration and washed with dichloromethane. The combined filtrate and washings was concentrated in vacuo to dryness. The residue was triturated with ether, the solid product was collected by filtration, and dried to give **13a** (1.95 g, 77%); mp 123–125 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.48–7.46 (m, 2H), 7.33–7.29 (m, 1H), 7.10–7.07 (m, 1H), 4.61 (t, J = 5.2 Hz, 1H), 4.47 (t, J = 5.2 Hz, 1H), 4.44 (d, J = 5.2 Hz, 2H), 4.35 (d, J = 5.2 Hz, 2H), 3.80 (s, 2H), 2.53 (s, 3H). Anal. Calcd for $C_{14}H_{15}NO_2$: C, 73.34; H, 6.59; N, 6.11. Found: C, 73.08; H, 6.59; N, 6.04.

By following the same procedure as describe for **13a**, the following compounds **13b–l** were synthesized.

4.5.1. (3-Ethyl-2-hydroxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl)methanol (13b)

Compound **13b** was synthesized from **12b** (2.5 g, 8.35 mmol) in anhydrous dichloromethane (30 mL) and $LiAlH_4$ (0.793 g, 20.87 mmol) in anhydrous ether (30 mL); yield (1.31 g, 64%); mp 131–133 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.46–7.45 (m, 1H), 7.39–7.37 (m, 1H), 7.34–7.30 (m, 1H), 7.09–7.06 (m, 1H), 4.63 (t, J = 5.2 Hz, 1H), 4.47 (t, J = 5.2 Hz, 1H), 4.45 (d, J = 5.2 Hz, 2H), 4.36 (d, J = 5.2 Hz, 2H), 3.81 (s, 2H), 2.89 (q, J = 7.3 Hz, 2H), 1.18 (t, J = 7.3 Hz, 3H). Anal. Calcd for $C_{15}H_{17}NO_2$: C, 74.05; H, 7.04; N, 5.76. Found: C, 73.22; H, 6.94; N, 5.71.

4.5.2. (2-Hydroxymethyl-3-phenyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl)methanol (13c)

Compound **13c** was synthesized from **12c** (2.08 g, 6 mmol) in anhydrous dichloromethane (35 mL) and $LiAlH_4$ (0.568 g, 15 mmol) in anhydrous ether (50 mL); yield (1.24 g, 71%); mp 169–171 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.55–7.44 (m, 6H), 7.12–7.09 (m, 1H), 7.05–7.03 (m, 1H), 6.74–6.73 (m, 1H), 4.80 (t, J = 4.8 Hz, 1H), 4.68 (t, J = 4.8 Hz, 1H), 4.55 (d, J = 4.8 Hz, 2H), 4.27 (d, J = 4.8 Hz, 2H), 3.93 (s, 2H). Anal. Calcd for $C_{19}H_{17}NO_2$: C, 78.33; H, 5.88; N, 4.81. Found: C, 78.50; H, 5.98; N, 4.72.

4.5.3. (3-(4-Fluorophenyl)-2-hydroxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl)methanol (13d)

Compound **13d** was synthesized from **12d** (2.78 g, 7.6 mmol) in anhydrous dichloromethane (26 mL) and $LiAlH_4$ (0.72 g, 19 mmol) in anhydrous ether (40 mL); yield (1.69 g, 72%); mp 164–166 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.56–7.54 (m, 1H), 7.46 (d, J = 7.31 Hz, 1H), 7.38–7.34 (m, 2H), 7.12 (t, J = 7.31 Hz, 1H), 7.04 (t, J = 7.31 Hz, 1H), 6.71 (d, J = 8.1 Hz, 1H), 4.79 (t, J = 3.9 Hz, 1H), 4.68 (t, J = 3.9 Hz, 1H), 4.57 (d, J = 4.5 Hz, 2H), 4.27 (d, J = 4.5 Hz, 2H), 3.92 (s, 2H). Anal. Calcd for $C_{19}H_{16}FNO_2$: C, 73.77; H, 5.21; N, 4.53. Found: C, 73.30; H, 5.48; N, 4.32.

4.5.4. (3-(4-Chlorophenyl)-2-hydroxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl)methanol (13e)

Compound **13e** was synthesized from **12e** (1.91 g, 5 mmol) in anhydrous dichloromethane (20 mL) and $LiAlH_4$ (0.47 g, 12.5 mmol) in anhydrous ether (35 mL); yield (1.16 g, 71%): mp 213–215 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.62–7.45 (m, 5H), 7.16–7.13 (m, 1H), 7.08–7.04 (m, 1H), 6.78 (d, J = 7.8 Hz, 1H), 4.80 (t, J = 5.3 Hz, 1H), 4.73 (t, J = 5.3 Hz, 1H), 4.55 (d, J = 5.3 Hz, 2H), 4.26 (d, J = 5.3 Hz, 2H), 3.93 (s, 2H). Anal. Calcd for $C_{19}H_{16}ClNO_2$: C, 70.05; H, 4.95; N, 4.30. Found: C, 69.58; H, 5.05; N, 4.20.

4.5.5. (3-(3,4-Difluoro-phenyl)-2-hydroxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl)methanol (13f)

Compound **13f** was synthesized from **12f** (3.83 g, 10 mmol) in anhydrous dichloromethane (30 mL) and $LiAlH_4$ (0.95 g, 25 mmol) in anhydrous ether (40 mL); yield 2.25 g (68%): mp 146–147 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.62–7.57 (m, 2H), 7.49 (d, J = 4.89 Hz, 1H), 7.36–7.34 (m, 1H), 7.16 (t, J = 5.23 Hz, 1H), 7.07 (t, J = 4.92 Hz, 1H), 6.78 (d, J = 5.27 Hz, 1H), 4.77 (t, J = 3.5 Hz, 1H), 4.73 (t, J = 3.5 Hz, 1H), 4.56 (d, J = 3.5 Hz, 2H), 4.26 (d, J = 3.5 Hz, 2H), 3.93 (s, 2H). Anal. Calcd for $C_{19}H_{15}F_2NO_2$: C, 69.72; H, 4.62; N, 4.28. Found: C, 69.39; H, 4.68; N, 4.13.

4.5.6. (3-(4-Methoxyphenyl)-2-hydroxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl)methanol (13g)

Compound **13g** was synthesized from **12g** (2 g, 8.35 mmol) in anhydrous dichloromethane (30 mL) and $LiAlH_4$ (0.76 g, 20 mmol) in anhydrous ether (30 mL); yield (1.33 g, 78%): mp 143–144 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.46 (d, J = 7.5 Hz, 1H), 7.49–7.42 (m, 2H), 7.11 (t, J = 7.5 Hz, 1H), 7.08 (d, J = 8.6 Hz, 2H), 7.04 (t, J = 7.4 Hz, 1H), 6.72 (d, J = 7.9 Hz, 1H), 4.74 (t, J = 5.2 Hz, 1H), 4.60 (t, J = 5.2 Hz, 1H), 4.55 (d, J = 5.2 Hz, 2H), 4.25 (d, J = 5.2 Hz, 2H), 3.90 (s, 2H), 3.84 (s, 3H). Anal. Calcd for $C_{20}H_{19}NO_3$: C, 74.75; H, 5.96; N, 4.36. Found: C, 74.53; H, 6.05; N, 4.18.

4.5.7. (3-(2-Methoxyphenyl)-2-hydroxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl)methanol (13h)

Compound **13h** was synthesized from **12h** (2.54 g, 6.73 mmol) in anhydrous dichloromethane (25 mL) and $LiAlH_4$ (0.8 g, 21 mmol) in anhydrous ether (40 mL); yield 1.65 g (76%): mp 132–134 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.50–7.42 (m, 2H), 7.34 (dd, J = 7.4, 1.68 Hz, 1H), 7.17–7.15 (d, J = 8.1 Hz, 1H), 7.11–7.05 (m, 2H), 6.99 (t, J = 7.4 Hz, 1H), 6.47 (d, J = 7.81 Hz, 1H), 4.77 (t, J = 5.3 Hz, 1H), 4.57–4.55 (m, 1H), 4.54 (t, J = 5.2 Hz, 1H), 4.33 (d, J = 5.2 Hz, 2H), 4.10 (d, J = 5.2 Hz, 2H), 3.96–3.81 (m, 1H), 3.63 (s, 3H). Anal. Calcd for $C_{20}H_{19}NO_3$: C, 74.75; H, 5.96; N, 4.36. Found: C, 74.45; H, 6.01; N, 4.34.

4.5.8. (3-(3,4-Dimethoxyphenyl)-2-hydroxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl)methanol (13i)

Compound **13i** was synthesized from **12i** (3.26 g, 8 mmol) in anhydrous dichloromethane (20 mL) and $LiAlH_4$ (1 g, 26.35 mmol) in anhydrous ether (40 mL); yield (2.17 g, 77%); mp 160–162 °C; H NMR (400 MHz, DMSO-d_6) δ = 7.28 (d, J = 7.2 Hz, 1H), 7.15–7.01 (m, 5H), 6.80 (d, J = 8.0 Hz, 1H), 4.73 (t, J = 5.2 Hz, 1H), 4.61 (t, J = 5.2 Hz, 1H), 4.55 (d, J = 5.2 Hz, 2H), 4.26 (d, J = 5.2 Hz, 2H), 3.91 (s, 2H), 3.84 (s, 3H), 3.75 (s, 3H). Anal. Calcd for $C_{21}H_{21}NO_4$: C, 71.78; H, 6.02; N, 3.99. Found: C, 70.65; H, 6.02; N, 3.82.

4.5.9. (3-(2,6-Dimethoxyphenyl)-2-hydroxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl)methanol (13j)

Compound **13j** was synthesized from **12j** (2.5 g, 6.13 mmol) in anhydrous dichloromethane (30 mL) and $LiAlH_4$ (0.83 g, 21.87 mmol) in anhydrous ether (50 mL); yield (1.72 g, 80%); mp 146–147 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.46–7.40 (m, 2H), 7.05 (t, J = 7.8 Hz, 1H), 6.97 (t, J = 7.7 Hz, 1H), 6.80 (s, 1H), 6.78 (s, 1H), 6.39–6.37 (m, 1H), 4.81 (t, J = 5.3 Hz, 1H), 4.55 (d, J = 5.3 Hz, 2H), 4.28 (t, J = 4.8 Hz, 1H), 4.15 (d, J = 5.0 Hz, 2H), 3.87 (s, 2H), 3.62 (s, 6H). Anal. Calcd for $C_{21}H_{21}NO_4$: C, 71.78; H, 6.02; N, 3.99. Found: C, 71.19; H, 6.01; N, 3.92.

4.5.10. (3-(3,4,5-Trimethoxyphenyl)-2-hydroxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl)methanol (13k)

Compound **13k** was synthesized from **12k** (2.62 g, 6 mmol) in anhydrous dichloromethane (35 mL) and $LiAlH_4$ (0.83 g, 21.87 mmol) in anhydrous ether (40 mL); yield (1.71 g, 75%); mp 132–134 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.47 (d, J = 7.3 Hz, 1H), 7.17 (t, J = 7.5 Hz, 1H), 7.05 (t, J = 7.2 Hz, 1H), 6.92 (d, J = 7.9 Hz, 1H), 6.79 (s, 2H), 4.73 (t, J = 4.7 Hz, 1H), 4.66 (t, J = 4.7 Hz, 1H), 4.55 (d, J = 4.4 Hz, 2H), 4.30 (d, J = 4.4 Hz, 2H), 3.91 (s, 2H), 3.78 (s, 6H), 3.76 (s, 3H). Anal. Calcd for $C_{22}H_{23}NO_5$: C, 69.28; H, 6.08; N, 3.67. Found: C, 68.33; H, 6.04; N, 3.60.

4.5.11. (2-Hydroxymethyl-6-methoxy-3-(4-methoxyphenyl)-8*H*-3a-aza-cyclopenta[*a*] inden-1-yl)methanol (13l)

Compound **13l** was synthesized from **12l** (1.73 g, 3.56 mmol) in anhydrous dichloromethane (20 mL) and $LiAlH_4$ (0.45 g, 11.85 mmol) in anhydrous ether (40 mL); yield (1.35 g, 91%); mp 186–187 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.40 (d, J = 8.7 Hz, 2H), 7.10 (d, J = 2.3 Hz, 1H), 7.07 (d, J = 8.7 Hz, 2H), 6.69 (dd, J = 8.7, 2.5 Hz, 1H), 6.64 (d, J = 8.7 Hz, 1H), 4.71 (t, J = 4.4 Hz, 1H), 4.56 (t, J = 4.4 Hz, 1H), 4.53 (d, J = 4.4 Hz, 2H), 4.24 (d, J = 4.4 Hz, 2H), 3.87 (s, 2H), 3.84 (s, 3H), 3.71 (s, 3H). Anal. Calcd for $C_{21}H_{21}NO_4$: C, 71.78; H, 6.02; N, 3.99. Found: C, 71.59; H, 5.90; N, 3.78.

4.6. Methylcarbamic acid 3-methyl-2-methylcarbamoyloxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl-methyl ester (14a)

To a solution of **13a** (0.80 g, 3.5 mmol) and triethylamine (2.5 mL) in anhydrous dichloromethane (10 mL) was added dropwise methyl isocyanate (2 mL, 35 mmol) in anhydrous dichloromethane (5 mL). The mixture was stirred at room temperature for additional 5 h. The mixture was concentrated under reduced pressure to dryness. The residue was triturated with a mixture of ether/methanol (15:1), the solid product was collected by filtration, washed with ether (10 mL), and dried in vacuo to yield **14a** (1.18 g, 98%); mp 86–88 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.52–7.47 (m, 2H), 7.34–7.30 (m, 1H), 7.14–7.10 (m, 1H), 6.86 (br s, 2H), 4.95 (s, 2H), 4.92 (s, 2H), 3.84 (s, 2H), 2.55 (d, J = 2.0 Hz, 3H), 2.54 (d, J = 2.0 Hz, 3H), 2.51 (s, 3H). Anal. Calcd for $C_{18}H_{21}N_3O_4$: C, 62.96; H, 6.16; N, 12.24. Found: C, 62.70; H, 6.35; N, 11.99.

By following the same procedure as describe for **14a**, the following compounds **14b–l** were synthesized.

4.6.1. Methylcarbamic acid 3-ethyl-2-methyl carbamoyloxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl-methyl ester (14b)

Compound **14b** was synthesized from **13b** (0.85 g, 3.5 mmol), triethylamine (2 mL), and methyl isocyanate (2 mL, 35 mmol); yield (1.24 g, 99%); mp 165–166 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.50–7.48 (m, 1H), 7.45–7.42 (m, 1H), 7.37–7.33 (m, 1H), 7.15–7.11 (m, 1H), 6.90 (br s, 1H), 6.84 (br s, 1H), 4.96 (s, 2H), 4.93 (s, 2H), 3.85 (s, 2H), 2.93 (q, J = 7.3 Hz, 2H), 2.55 (d, J = 2.8 Hz, 3H), 2.54 (d, J = 2.8 Hz, 3H), 1.18 (t, J = 7.3 Hz, 3H). Anal. Calcd for $C_{19}H_{23}N_3O_4$: C, 63.85; H, 6.49; N, 11.76. Found: C, 63.67; H, 6.71; N, 11.71.

4.6.2. Methylcarbamic acid 2-methylcarbamoyloxymethyl-3-phenyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl-methyl ester (14c)

Compound **14c** was synthesized from **13c** (0.73 g, 2.5 mmol), triethylamine (2.5 mL) and methyl isocyanate (1.47 mL, 25 mmol); yield (0.87 g, 86%); mp 196–198 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ = 7.48–7.42 (m, 5H), 7.38–7.368 (m, 1H), 7.05–7.02 (m, 2H), 6.77–6.76 (m, 1H), 5.20 (s, 2H), 5.01 (s, 2H), 4.73 (br s, 1H), 4.69 (br s, 1H), 3.94 (s, 2H), 2.80 (d, J = 4.7 Hz, 3H), 2.78 (t, J = 4.7 Hz, 3H). Anal. Calcd for $C_{23}H_{23}N_3O_4$: C, 68.13; H, 5.72; N, 10.36. Found: C, 67.69; H, 5.75; N, 10.31.

4.6.3. Methylcarbamic acid 3-(4-fluorophenyl)-2-methylcarbamoyloxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl-methyl ester (14d)

Compound **14d** was synthesized from **13d** (0.77 g, 2.5 mmol), triethylamine (2.5 mL), and methyl isocyanate (1.47 mL, 25 mmol); yield (0.92 g, 87%); mp 199–200 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.55–7.53 (m, 2H), 7.50 (d, J = 7.3 Hz, 1H), 7.38 (t, J = 8.7 Hz, 2H), 7.15 (t, J = 7.44 Hz, 1H), 7.09 (t, J = 7.4 Hz, 1H), 6.95 (br s, 1H), 6.92 (br s, 1H), 6.66 (d, J = 7.8 Hz, 1H), 5.05 (s, 2H), 4.79 (s, 2H), 3.96 (s, 2H), 2.58 (d, J = 4.5 Hz, 3H), 2.55 (t, J = 4.5 Hz, 3H). Anal. Calcd for $C_{23}H_{22}FN_3O_4$: C, 65.24; H, 5.24; N, 9.92. Found: C, 65.00; H, 5.35; N, 9.71.

4.6.4. Methylcarbamic acid 3-(4-chlorophenyl)-2-methylcarbamoyloxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl-methyl ester (14e)

Compound **14e** was synthesized from **13e** (0.81 g, 2.5 mmol), triethylamine (2.5 mL), and methyl isocyanate (1.47 mL, 25 mmol); yield (1.06 g, 96%); mp 204–206 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ = 7.51–7.38 (m, 5H), 7.08–7.04 (m, 2H), 6.77–6.76 (m, 1H), 5.19 (s, 2H), 4.98 (s, 2H), 4.71 (br s, 1H), 4.67 (br s, 1H), 3.94 (s, 2H), 2.73 (m, 6H). Anal. Calcd for $C_{23}H_{22}ClN_3O_4$: C, 62.80; H, 5.04; N, 9.55. Found: C, 62.72; H, 5.14; N, 9.41.

4.6.5. Methylcarbamic acid 3-(3,4-difluoro-phenyl)-2-methyl-carbamoyloxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl-methyl ester (14f)

Compound **14f** was synthesized from **13f** (0.81 g, 2.5 mmol), triethylamine (2.5 mL) and methyl isocyanate (1.47 mL, 25 mmol); yield (0.93 g, 84%); mp 151–152 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.63–7.57 (m, 2H), 7.52 (d, J = 4.5 Hz, 1H), 7.36 (s, 1H), 7.18 (d, J = 5.0 Hz, 1H), 7.12 (d, J = 5.1 Hz, 1H), 6.96 (br s, 1H), 6.95 (br s, 1H), 6.73 (d, J = 5.2 Hz, 1H), 5.05 (s, 2H), 4.82 (s, 2H), 3.97 (s, 2H), 2.58 (d, J = 4.4 Hz, 3H), 2.55 (t, J = 4.4 Hz, 3H). Anal. Calcd for $C_{23}H_{21}F_2N_3O_4$: C, 62.58; H, 4.80; N, 9.52. Found: C, 62.49; H, 4.97; N, 9.33.

4.6.6. Methylcarbamic acid 3-(4-methoxy-phenyl)-2-methyl-carbamoyloxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl-methyl ester (14g)

Compound **14g** was synthesized from **13g** (1 g, 3 mmol), triethylamine (3.5 mL), and methyl isocyanate (1.76 mL, 30 mmol); yield (0.73 g, 70%); mp 152–153 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.49 (d, J = 7.6 Hz, 1H), 7.40 (d, J = 8.5 Hz, 2H), 7.10–7.07 (m, 4H), 6.92 (br s, 1H), 6.90 (br s, 1H), 6.69 (d, J = 7.6 Hz, 1H), 5.04 (s, 2H), 4.78 (s, 2H), 3.95 (s, 2H), 3.85 (s, 3H), 2.58 (d, J = 4.8 Hz, 3H), 2.56 (t, J = 4.8 Hz, 3H). Anal. Calcd for $C_{24}H_{25}N_3O_5$: C, 66.19; H, 5.79; N, 9.65. found: C, 66.13; H, 5.61; N, 9.41.

4.6.7. Methylcarbamic acid 3-(2-methoxy-phenyl)-2-methyl-carbamoyloxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl-methyl ester (14h)

Compound **14h** was synthesized from **13h** (1.25 g, 3.89 mmol), triethylamine (2.5 mL) and methyl isocyanate (1 mL, 18.12 mmol); yield (1.47 g, 87%); mp 200–203 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.53–7.46 (m, 2H), 7.32–7.30 (m, 1H), 7.19 (d, J = 8.12 Hz, 1H), 7.14–7.04 (m, 3H), 6.95 (br s, 1H), 6.86 (br s, 1H), 6.47 (d, J = 7.7 Hz, 1H), 5.04 (s, 2H), 4.86 (d, J = 11.9 Hz, 1H), 4.60 (d, J = 11.9 Hz, 1H), 3.94 (s, 2H), 3.64 (s, 3H), 2.58 (d, J = 4.6 Hz, 3H), 2.54 (t, J = 4.6 Hz, 3H). Anal. Calcd for $C_{24}H_{25}N_3O_5$: C, 66.19; H, 5.79; N, 9.65. Found: C, 65.71; H, 5.87; N, 9.53.

4.6.8. Methylcarbamic acid 3-(3,4-dimethoxy-phenyl)-2-methyl-carbamoyloxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl-methyl ester (14i)

Compound **14i** was synthesized from **13i** (1.5 g, 4.27 mmol), triethylamine (2.5 mL) and methyl isocyanate (2.5 mL, 42.60 mmol); yield (1.80 g, 91%); mp 165–168 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.50–7.49 (m, 1H), 7.18–7.07 (m, 3H), 7.03–7.00 (m, 2H), 6.92 (br s, 2H), 6.80–6.79 (m, 1H), 5.04 (s, 2H), 4.81 (s, 2H), 3.95 (s, 2H), 3.85 (s, 3H), 3.75 (s, 3H), 2.58 (d, J = 4.6 Hz, 3H), 2.55 (t, J = 4.6 Hz, 3H). Anal. Calcd for $C_{25}H_{27}N_3O_6$: C, 64.50; H, 5.85; N, 9.03. Found: C, 64.28; H, 5.86; N, 8.95.

4.6.9. Methylcarbamic acid 3-(2,6-dimethoxyphenyl)-2-methyl-carbamoyloxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl-methyl ester (14j)

Compound **14j** was synthesized from **13j** (0.75 g, 2.13 mmol), triethylamine (1.5 mL), and methyl isocyanate (0.8 mL, 13.56 mmol); yield (0.89 g, 90%); mp 154–156 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.48–7.45 (m, 2H), 7.10–7.08 (m, 1H), 7.04–7.01 (m, 1H), 7.00–6.97 (m, 1H), 6.81 (s, 1H), 6.80 (br s, 1H), 6.79 (br s, 1H), 6.41–6.39 (m, 1H), 5.03 (s, 2H), 4.66 (s, 2H), 3.91 (s, 2H), 3.62 (s, 6H), 2.59 (d, J = 3.6 Hz, 3H), 2.52 (t, J = 3.6 Hz, 3H). Anal. Calcd for $C_{25}H_{27}N_3O_6$: C, 64.50; H, 5.85; N, 9.03. Found: C, 63.18; H, 5.74; N, 8.63.

4.6.10. Methylcarbamic acid 3-(3,4,5-trimethoxyphenyl)-2-methylcarbamoyloxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl-methyl ester (14k)

Compound **14k** was synthesized from **13k** (1.03 g, 2.7 mmol), triethylamine (1.5 mL) and methyl isocyanate (1 mL, 18.12 mmol); yield (1.27 g, 95%); mp 204–205 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.52–7.50 (d, J = 7.5 Hz, 2H), 7.22–7.18 (t, J = 7.2 Hz, 1H), 7.13–7.10 (t, J = 7.2 Hz, 1H), 6.94 (s, 1H), 6.93 (br s, 1H), 6.91 (br s, 1H), 6.77 (s, 2H), 5.05 (s, 2H), 4.83 (s, 2H), 3.96 (s, 2H), 3.79 (s, 6H), 3.78 (s, 3H), 2.57 (d, J = 4.6 Hz, 3H), 2.55 (t, J = 4.6 Hz, 3H). Anal. Calcd for $C_{26}H_{29}N_3O_7$: C, 63.02; H, 5.90; N, 8.48. Found: C, 62.74; H, 5.92; N, 8.42.

4.6.11. Methylcarbamic acid 6-methoxy-3-(4-methoxyphenyl)-2-methylcarbamoyl-oxymethyl-8*H*-3a-aza-cyclopenta[*a*]inden-1-yl-methyl ester (14l)

Compound **14l** was synthesized from **13l** (0.954 g, 2.71 mmol), triethylamine (1.5 mL), and methyl isocyanate (0.95 mL, 16.24 mmol); yield (1.17 g, 93%); mp 110–112 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.38 (d, J = 8.6 Hz, 2H), 7.13 (d, J = 2.5 Hz, 1H), 7.08 (d, J = 8.6 Hz, 2H), 6.92 (br s, 1H), 6.88 (br s, 1H), 6.71 (dd, J = 8.7, 2.5 Hz, 2H), 6.60 (d, J = 8.7 Hz, 1H), 5.02 (s, 2H), 4.80 (s, 2H), 3.92 (s, 2H), 3.84 (s, 3H), 3.71 (s, 3H), 2.58 (d, J = 4.5 Hz, 3H), 2.55 (t, J = 4.5 Hz, 3H). Anal. Calcd for $C_{25}H_{27}N_3O_6$: C, 64.50; H, 5.85; N, 9.03. Found: C, 63.41; H, 5.80; N, 8.84.

4.7. Biological experiments

4.7.1. Tumor and cell lines

Human colon carcinoma HCT-116 cells, human prostate adenocarcinoma PC3, ovarian adenocarcinoma SK-OV-3, human lung large cell carcinoma H1299, and human glioma cells U87 were obtained from American Type Culture Collection (ATCC, Rockville, MD). Human mammary carcinoma (MX-1) tumor cells were obtained from MSKCC cell bank. The CCRF-CEM human lymphoblastic leukemia cells and their Vinblastine resistant subline (CCRF-CEM/VBL, 680-fold resistance in vitro) were obtained from Dr. William Beck of the University of Illinois, Chicago, and CCRF-CEM/Taxol (330-fold resistance in vitro) resistant cells were produced by exposing the parent cells to increasing sublethal concentration (IC_{50}–IC_{90}) of paclitaxel for six months. OECM1 (human gingival squamous carcinoma cells) was obtained from Dr. T. Y. Liu of the National Yang-Ming University, Taiwan.

4.7.2. Cytotoxicity assays

The cytotoxic effects of the newly synthesized compounds were determined in T-cell acute lymphocytic leukemia (CCRF-CEM) and their resistant subcell lines (CCRF-CEM/Taxol and CCRF-CEM/VBL) by the XTT assay[28] and human solid tumor cells (i.e., breast carcinoma MX-1 and colon carcinoma HCT-116) the SRB assay[29] in a 72 h incubation using a microplate spectrophotometer as described previously.[30] After the addition of phenazine methosulfate-XTT solution, incubated at 37 °C for 6 h and absorbance at 450 and 630 nm was detected on a microplate reader (EL 340). The cytotoxicity of the newly synthesized compounds against non-small cell lung cancer (H1299), human prostate cancer cell line (PC3), human glioma (U87), and oral cancer cell line (OECM1) were determined by the WST-1 assay[31] in a 72 h incubation using a microplate spectrophotometer as described previously. After the addition of WST-1 solution, it was incubated at 37 °C for 4 h. Absorbance at 460 and 630 nm was detected on a microplate reader. IC_{50} values were determined from dose–effect relationship at six or seven concentrations of each drug using the CompuSyn software by Chou and Martin[32] based on the median-effect principle and plot.[33,34]

4.7.3. In vivo studies

Athymic nude mice bearing the nu/nu gene were obtained from NCI, Frederick, MD and used for all human tumor xenografts. Male nude mice 6 weeks or older weighing 20–24 g or more were used. Compounds were administered via the tail vein for iv injection as described previously.[30] Tumor volume was assessed by measuring length × width × height (or width) by using a caliper. Vehicle used was 50 μL DMSO and 40 μL Tween 80 in 160 μL saline. The maximal tolerate dose of the tested compound was determined and applied for the in vivo therapeutic efficacy assay. For tumor-bearing nude mice during the course of the experiment, the body weight refers to total weight minus the weight of the tumor. All animal studies were conducted in accordance with the guidelines for the National Institute of Health Guide for the Care and Use of Animals and the protocol approved by the Institutional Animal Care and Use Committee.

4.7.4. Alkaline agarose gel shift assay

Formation of DNA cross-linking was analyzed by alkaline agarose gel electrophoresis.[25] In brief, purified pEGFP-N1 plasmid DNA (1500 ng) was mixed with various concentrations (0.1–20 μM) of **13a**, **13b**, **13l**, **14h**, **14g**, and **14l** in 40 μL binding buffer (3 mM sodium chloride/1 mM sodium phosphate, pH 7.4, and 1 mM EDTA). The reaction mixture was incubated at 37 °C for 2 h. At the end of reaction, the plasmid DNA was linearized by digestion with BamHI and followed by precipitation with ethanol. The DNA pellets were dissolved and denatured in alkaline buffer (0.5 N NaOH–10 mM EDTA). An aliquot of 20 μL of DNA solution (1000 ng) was mixed with a 4 μL of 6 × alkaline loading dye and then electrophoretically resolved on a 0.8% alkaline agarose gel with NaOH–EDTA buffer at 4 °C. The electrophoresis was carried out at 18 V for 22 h. After staining the gels with an ethidium bromide solution, and the DNA was then visualized under UV light.

4.7.5. Modified single cell gel electrophoresis (comet) assay

Intracellular DNA interstrand cross-linking was analyzed using a modified comet assay.[22,23] All steps were carried out under subdued lighting. Briefly, H1299 cells (2×10^5 cells) were plated in a 60 mm dish and incubated at 37 °C with 5% CO_2 for 24 h. The growing cells were treated with tested compounds (**13l**, **14l**, and mito-

mycin C) for 1 h, and then irradiated with 20 Gy of X-ray to induce DNA strand breaks. Afterward, an aliquot of 5×10^5 cells were quickly embedded in 1.2% low melting point agarose that was layered on a 1% agarose-precoated microscope slide. The cells in agarose gel were treated with in lysis buffer [100 mmol/L sodium EDTA, 2.5 mol/L NaCl, 10 mmol/L Tris–HCl (pH 10)] containing 1% Triton X-100 and 10% dimethyl sulfoxide (immediately before use) for 1 h and followed with alkali buffer [300 mmol/L NaOH, 1 mmol/L sodium EDTA (pH 13.5)] for 20 min. Electrophoresis was then conducted at 25 V and 300 mA in the same buffer for 20 min. The slides were neutralized with neutralizing buffer [250 mmol/L Tris–HCl (pH 7.5)] for at least, and then stained with 20 μM YOYO-1 dye (Molecular Probes). The comet image was taken under fluorescence microscope (shortwave pass filter 450–490 nm, chromatic beam splitter 510 nm, and longwave pass filter 520 nm) with a digital camera (DCS-420; Kodak, Rochester, NY, USA). For each treatment, the tail moment of 100 cells was determined by aid of Comet Assay III software (available from www.perceptive.co.uk). The levels of interstrand cross-linking are proportional to the decrease in the tail moment in the irradiated drug treated sample compared to the irradiated untreated control. The decrease in tail moment is calculated by the following formula:

$$\% \text{ of DNA with interstrand cross-linking} = \left\{1 - \left(\frac{\text{TMdi} - \text{TMcu}}{\text{TMci} - \text{TMcu}}\right) \times 100\right\}$$

where TMdi = mean tail moment of drug treated, irradiated sample, TMcu = mean tail moment of unirradiated control sample, TMci = mean tail moment of irradiated control sample.

4.7.6. Flow cytometric analysis

The effects of compounds **13l** and **14l** on cell cycle progression were analyzed with a flow cytometer as described previously.[35] Briefly, human non-small cell lung carcinoma H1299 cells were treated with these compounds at 10 μM. At the time indicated, the attached cells were trypsinized, washed with phosphate buffer saline (PBS), fixed with ice-cold 70% ethanol for 30 min. The cells were stained with 4 μg/mL propidium iodide (PI) in PBS containing 1% Triton X-100 and 0.1 mg/mL RNase A. The stained cells were then analyzed using the FACS SCAN flow cytometer (Becton Dickinson, San Joes, CA, USA). The percentage of the cells in each cell cycle phase was determined using the ModFit LT 2.0 software based on the DNA histograms.

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Supplementary data

Supplementary data (elemental analysis data of compounds **11b–l**, **12a–l**, **13a–l**, and **14a–l**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.06.018.

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