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Synthesis, anticancer activity and apoptosis inducing ability of anthranilamide-PBD conjugates

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ABSTRACT

A series of novel anthranilamide linked pyrrolo[2,1-*c*][1,4]benzodiazepine conjugates were prepared and evaluated for their anticancer activity. The effects of three promising PBD conjugates on cell cycle of cancerous cell line A375 were investigated. These promising compounds showed the characteristic features of apoptosis like enhancement in the levels of p53 and activation of caspase-3.

Pyrrolo[2,1-*c*][1,4]benzodiazepines (PBD) are naturally occurring DNA interactive antitumour antibiotics isolated from various *Streptomyces* species.[1] The PBD cytotoxins exert a powerful antitumoural activity by binding to the minor groove of double stranded DNA by forming a covalent bond to the exocyclic amino group of a central guanine within a three base pair recognition site.[2,3] The natural occurring PBDs have potent anticancer activity, however they have been precluded from clinical studies due to problems relating to side effects.[4] Hence, there has been a considerable interest in the design and synthesis of conjugate agents with active moieties of known antitumour agents to enhance the sequence selectivity as well as antitumour activity. An anthranilamide moiety present in the structures of agents like AAL-993 (**2**) and PD 184352 (CI-1040, **3**)[5,6] is considered responsible for the potent antitumour properties.[7,8] Similarly, it is interesting to observe that in the last decade, a number of piperazine derivatives have been synthesized to exploit their chemotherapeutic potential.[9–11]

Michejda and co-workers[12] reported symmetrical bifunctional agents as promising antitumour class of compounds with remarkable selectivity against colon cancers that has a piperazine moiety in its linker spacer. Recently, transdiamine dichloroplatinum (II) complexes with piperazine ligands have also exhibited significant cytotoxicity.[13] Some representative members of the anthranilamides, DC-81 and its conjugates are illustrated in Figure 1.

In the past few years, several hybrid compounds, in which known antitumour agents tethered to PBD moiety, have been designed, synthesized and evaluated for their biological activity.[14–18] Recently, we have been not only involved in the development of new synthetic strategies[19,20] for the preparation of PBD ring system but also in the design of structurally modified PBDs and their hybrids.[21–23] In continuation of these efforts, it is considered of interest to design and synthesize hybrid molecules in which anthranilamide moiety is linked through 4-piperazinyl alkane spacer to the C8-position of the A ring of PBD ring system to explore their potential as a new class of anticancer agents.

The synthesis of the target conjugates was performed in a convenient manner as outlined in Schemes 1 and 2. Isatoic anhydride (**5a**) or 6-chloroisatoic anhydride (**5b**) was treated with *N*-boc piperazine in 1:1.2 M ratio, and refluxed in 1,4-dioxane to give the 4-(2-aminobenzoyl)*N*-boc piperazine (**6a**) and 4-(2-amino-5-chlorobenzoyl) *N*-boc piperazine (**6b**), respectively, in almost quantitative yield. These compounds **6a** and **6b** upon reductive amination with different aromatic aldehydes by using sodium cyano borohydride ($NaCNBH_3$) in methanol with catalytic amount of acetic acid afforded compounds **7a–f**. These on deprotection

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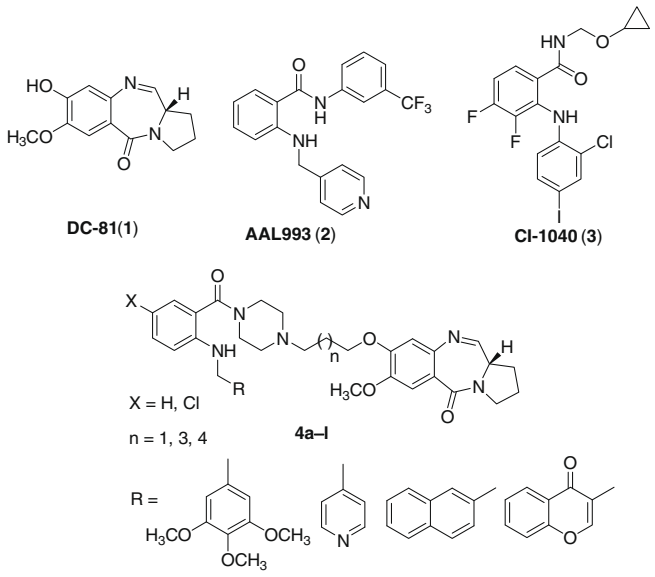
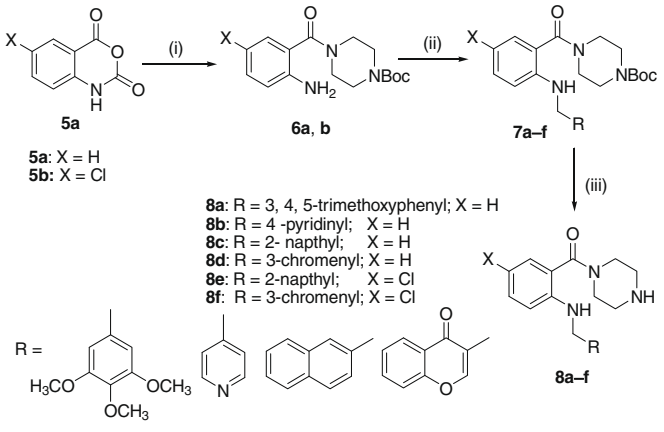


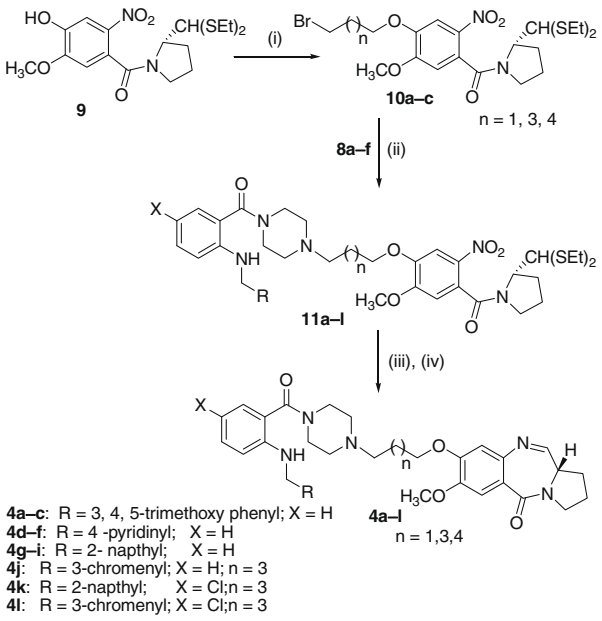
Figure 1. Chemical structures of DC-81 (**1**), anthranilamide AAL993 (**2**), CI-1040 (**3**), anthranilamide-PBD conjugates (**4al**).

with triflouroacetic acid gave precursors **8a–f** as shown in Scheme 1. The synthesis of C8-linked anthranilamide-PBD conjugates (**4a–l**) was carried out by coupling (2*S*)-*N*-{4-[*n*-bromoalkoxy-5-methoxy-2-nitrobenzoyl} pyrrolidine-2-carboxaldehyde diethyl thioacetal (**10a–c**) (prepared by the methods reported in our previous studies[21–23]) with anthranilamide precursors (**8a–f**) using K_2CO_3 in acetone to provide the nitro thioacetal intermediates **11a–l**. Finally, these upon reduction with $SnCl_2{\cdot}2H_2O$ in methanol followed by deprotection using $HgCl_2/CaCO_3$ provided the desired PBD conjugates (**4a–l**)[24] as shown in Scheme 2.

These conjugates **4a–l** were evaluated for their anticancer activity in selected human cancer cell lines of lung, breast, oral, colon, cervix, ovary and prostate by using sulforhodamine B (SRB) protein assay. This assay is used for cell density determination, based on the measurement of cellular protein content. The experimental protocol is given in Supplementary data. The compounds exhibiting $GI_{50} \leqslant 10^{-5}$ M (10 μM) are considered to be active on the respective cell lines. All these compounds **4a–l** exhibited potent anticancer activity with GI_{50} values ranging from 0.13 to 29 μM. The positive control compound adriamycin[25] demonstrated significant activity with the GI_{50} in the range from 0.10 to 7.25 μM and



Scheme 1. Reagents and conditions: (i) *N*-boc piperazine, 1,4-dioxane, reflux, 4 h; (ii) R-CHO, $NaCNBH_3$, MeOH, CH_3COOH, rt, 22 h; (iii) TFA, CH_2Cl_2, rt, 5 h.



Scheme 2. Reagents and conditions: (i) dibromoalkane, K_2CO_3, acetone, reflux, 24 h; (ii) K_2CO_3, acetone, reflux, 24 h; (iii) $SnCl_2{\cdot}2H_2O$, MeOH, reflux, 6 h; (iv) $HgCl_2$, $CaCO_3$, CH_3CN/H_2O (4:1), rt, o/n.

for DC-81 the GI_{50} ranged from 0.10 to 2.37 μM. As shown in Table 1, most of these conjugates exhibited higher anticancer activity than **1** (DC-81), particularly against Zr-75-1, MCF7, KB, and DWD cell lines. The significant anticancer activity showed by some of the compounds like **4g**, **4h** and **4j** prompted us to evaluate the cell viability of human melanoma cell line A375 cells, as this cell line is rapidly proliferating and malignant cell line resistant to radio and chemotherapy.[26] Further, to confirm the effectiveness of these conjugates, they were compared to both the precursors **1** (DC-81), **8c** and **8d** (anthranilamide precursors used to obtain compounds **4g**, **4h** and **4j**, respectively). As shown in Supplementary Figure 1, all these three conjugates (**4g**, **4h** and **4j**) exhibited higher anticancer activity relative to **1** (DC-81), **8c** and **8d** in A375 cells.

In order to study the effect of these conjugates on cell cycle progression, human melanoma cell lines (A375) were treated with compounds **1**, **8c**, **8d**, **4g**, **4h** and **4j** and analyzed using fluorescence activated cell sorter (FACS shown in Supplementary Figs. 2 and 3). Treatment of cells with these compounds at 1 μM concentration for 24 h induced apoptotic effects up to 2.63%, 1.23%, 1.15%, 5.48%, 7.34% and 3.48%, respectively, of sub G1 DNA peak (G0 phase) of A375 cells compared to 0.74% for the control cells. These results show that all these conjugates (**4g**, **4h** and **4j**) were more efficient in inducing apoptosis than **1** (DC-81) in A375 cells as shown in Figure 2.

Activation of tumour suppressor genes such as p53 is found to be important in the regulation of apoptotic pathway induced by various stimuli.[27] The p53 gene, is a tumour suppressor gene, that is, its activity stops the formation of tumours. To investigate whether these PBD conjugates induce apoptosis through p53-dependent pathway, the expression levels of p53 protein was checked after treating the A375 cells with compounds and western blot analysis was carried out using p53-specific antibody. As shown in Figure 3, it was observed that p53 protein expression levels were up-regulated when treated with compound **4h** indicating a clear-cut evidence of p53-dependent pathway. Further, all these conjugates increased the levels of p53 when compared to DC-81.

As we observed the increase in levels of p53, we tried to investigate the down stream signalling events in the process of apoptosis that is mainly mediated by caspases. Caspase-3 is

Table 1
GI_{50} values[a] (in μM) for compounds **4al** in selected human cancer cell lines

Compd	A549[b]	Hop62[b]	Zr-75-1[c]	MCF7[c]	DWD[d]	KB[d]	Gurav[d]	PC-3[e]	A2780[f]	Colo205[g]	SiHa[h]
4a	2.0	2.1	0.17	0.14	0.15	0.15	0.17	0.16	0.18	0.17	0.17
4b	1.9	1.9	0.16	0.14	0.14	0.14	0.16	0.14	0.16	2.2	0.17
4c	0.18	1.9	0.18	0.14	0.15	0.15	0.17	0.16	0.17	0.16	1.8
4d	2.0	2.2	0.18	0.13	0.17	0.15	0.18	1.8	0.19	0.19	1.8
4e	2.0	2.3	2.0	0.16	0.15	0.15	0.17	0.17	0.17	25	1.8
4f	2.2	25	2.0	0.15	0.17	0.15	0.18	1.8	0.19	2.6	1.9
4g	0.17	1.7	0.15	0.14	0.14	0.15	0.17	0.14	0.17	0.18	0.16
4h	0.17	1.8	0.15	0.13	0.13	0.13	0.14	0.14	0.14	0.15	0.15
4i	0.17	1.9	0.16	0.14	0.16	0.15	0.17	0.15	0.19	0.18	0.15
4j	0.16	1.8	0.16	0.14	0.16	0.15	0.17	0.14	0.18	2.4	0.13
4k	2.0	2.0	0.18	0.14	0.17	0.14	0.17	0.17	0.18	29	1.8
4l	0.17	1.8	0.16	0.15	0.17	0.16	0.17	0.16	0.18	2.3	0.16
ADR[i]	7.25	0.14	1.79	0.17	0.10	0.17	0.17	1.81	0.16	0.14	0.17
DC-81 (**1**)	0.16	0.15	2.37	0.17	1.49	0.17	0.16	0.20	0.14	0.11	0.17

[a] 50% growth inhibition and the values are mean of three determination.
[b] Lung cancer.
[c] Breast cancer.
[d] Oral cancer.
[e] Prostate cancer.
[f] Ovary cancer.
[g] Colon cancer.
[h] Cervix cancer.
[i] Adriamycin.

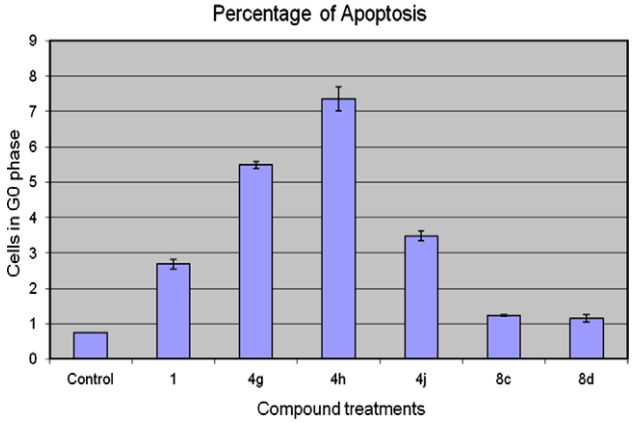


Figure 2. FACS analysis of cell cycle distribution of A375 cells after treatment with anthranilamide-PBD conjugates **1**, **4g**, **4h**, **4j**, **8c** and **8d** at 1 μM concentration for 24 h. The cells which fall in G0 phase of cell cycle indicates the percentage of apoptotic cells.

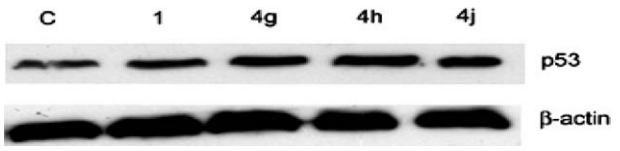


Figure 3. Effect on p53 protein expression. A375 cells were treated with 1 μM concentration of anthranilamide-PBD conjugates for 24 h. The cell lysates were used for western blot analysis using p53-specific antibody. C: cells without treatment. β-Actin was used as internal or loading control.

considered to be the effector caspase and is considered as the therapeutic target for the treatment of cancer.[28] Hence, we examined the involvement of caspases and their role in the process of apoptosis. From the results obtained in caspase-3 assay, an up regulation of caspase-3 (2.5-fold) was observed when treated with compound **4h** compared to untreated cells, while a slight increase in caspase-3 level was observed when treated with compounds **4g** and **4j** compared to DC-81 as shown in Figure 4. Similar type of observations were reported for the indole-PBD (IN6CPBD) conjugate.[29]

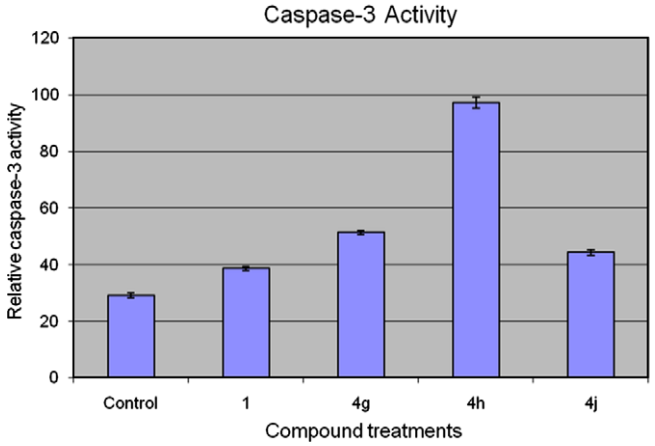


Figure 4. Effect of anthranilamide-PBD conjugates on caspase-3 activity in A375 cells determined by flourimetry.

Further, PBD compounds are effective as DNA binding agents particularly at G-rich sequences.[30] Therefore, in order to check the superior activity of these PBD conjugates over the naturally occurring PBD compounds such as DC-81, the restriction enzyme digestion assay (RED 100 assay) was carried out for the most active compound **4h** along with the naturally occurring PBD compound DC-81 (**1**). In this assay pBR 322 vector having *Bam* HI restriction site (5′-G′GATCC-3′) with the G-rich regions around the *Bam* HI site was used. The experiment is based on the principle that the drug binding at *Bam* HI site should inhibit the restriction digestion mediated by *Bam* HI enzyme. The experimental protocol is described in the previous study.[31] In this study, we used various concentration gradients ranging from 2 to 32 μM. It was observed from Figure 5 that, compound **4h**, the effective compound in our other studies was found to inhibit the restriction digestion even at 4 μM concentration, whereas DC-81 showed similar effects at much higher concentrations (i.e., 16 μM). This showed the improved selectivity of the compound **4h** than the naturally occurring DC-81(**1**).

In conclusion, in the present study, a series of new anthranilamide-PBD conjugates were synthesized. All these PBD conjugates

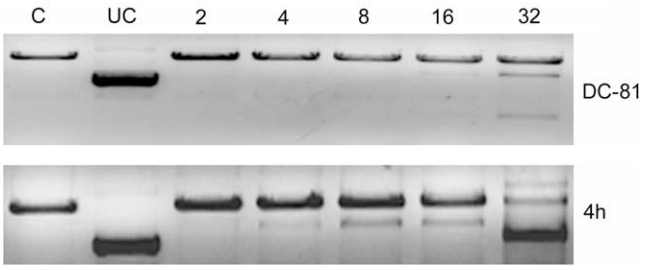


Figure 5. RED 100 assay: The figure in the top panel depicts the Red assay using DC-81(**1**) and figure in the bottom panel depicts the compound **4h**. C, the Cut vector DNA with *Bam* HI enzyme in the absence of compound and UC, the uncut vector DNA. The numbers 2, 4, 8, 16, 32 depicts the concentration in micro molar of the compound used in the RED assay.

(**4a–l**) showed significant anticancer activity in 11 human cancer cell lines. The FACS analysis clearly indicated that compound **4h** showed highest G0/G1 phase cells (G1 arrest). Then P53 which is considered as the master regulator of apoptosis was examined and the expression was found to be highest particularly in case of compound **4h**. Further, caspase-3 which is effector caspase was found to be highly up-regulated in case of compound **4h** treated cells in comparison to the other two compounds (**4g** and **4j**) and as well as DC-81 (**1**). Further, from the RED assay studies, it was observed that compound **4h** was found to be the most effective one in inhibiting the *Bam* H1 enzyme compared to DC-81 (**1**). Further, most of the PBD conjugates consisting of anthranilamide moiety are more effective as anticancer agents than **1** (DC-81). From these studies it may be concluded that compound **4h** which could be a promising compound can be taken up for further in vivo cancer studies that may be of interest in cancer chemoprevention.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.04.037.

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