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INDUCTION OF ENDONUCLEOLYTIC DNA FRAGMENTATION AND APOPTOSIS BY THE DUOCARMYCINS

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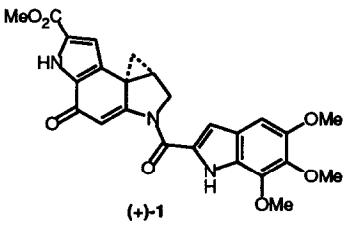
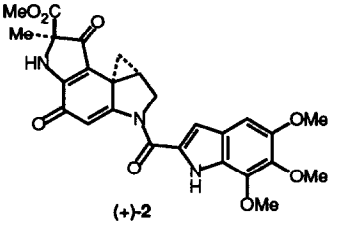
Abstract. The duocarmycins are exceptionally potent, naturally occurring antitumor antibiotics related to (+)-CC-1065. Short term exposure (1-4 h) of Molt-4 or L-1210 cells to the agents produced internucleosomal DNA fragmentation in approximately 200 base-pair multiples and at dose levels as low as 100 pM produced morphological changes characteristic of programmed cell death. These results were associated with strong inhibition of suspension culture growth and clonogenic survival suggesting that at physiologically relevant concentrations the agents induced cell death by a target cell activated pathway. Presumably, this is the consequence of the sequence selective alkylation of DNA by the duocarmycins potentially at internucleosomal sites, followed by intracellular signalling with activation of endonucleases, to trigger the apoptotic cell death mechanism.

Apoptosis represents an active process whereby selected cells undergo irreversible morphological changes including a characteristic heterochromatin condensation and degradation of genomic DNA into nucleosomal fragments prior to disintegration into characteristic structures (apoptotic bodies) suitable for phagocytosis.[1-4] It is an alternative form of cell death to necrosis and plays a major role in important developmental and tissue specific processes including tumor regression.[5-8] Recently, a number of useful antitumor agents[9] including Ara-C,[10] cisplatin,[11] doxorubicin,[12] and actinomycin D[13] which interact with DNA and the calicheamicins,[16] bleomycin[15] and synthetic enediynes[14,16] which cleave DNA have shown to induce cell death by apoptosis.

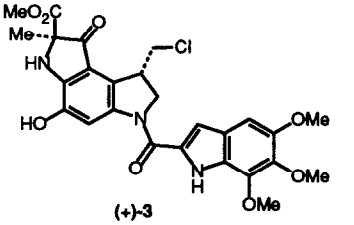
The duocarmycins[17,18] are exceptionally potent, naturally occurring antitumor antibiotics related to (+)-CC-1065[19-22] which derive their biological properties through the sequence selective alkylation of duplex DNA.[23-25] The characteristic alkylation occurs by 3′ adenine N3 addition to the least substituted carbon of the activated cyclopropane within selected minor groove AT-rich sites.[23-26] While studies have shown the intracellular target to be DNA,[27,28] the link between DNA alkylation and the ensuing biological properties remains to be established. During the course of the *in vitro* cytotoxic screening of the duocarmycins, (+)-CC-1065 and synthetic analogs, we have observed behavior characteristic of apoptosis and now wish to report our preliminary results.



(+)-1
(+)-duocarmycin SA



(+)-2
(+)-duocarmycin A



(+)-3
(+)-duocarmycin C_2

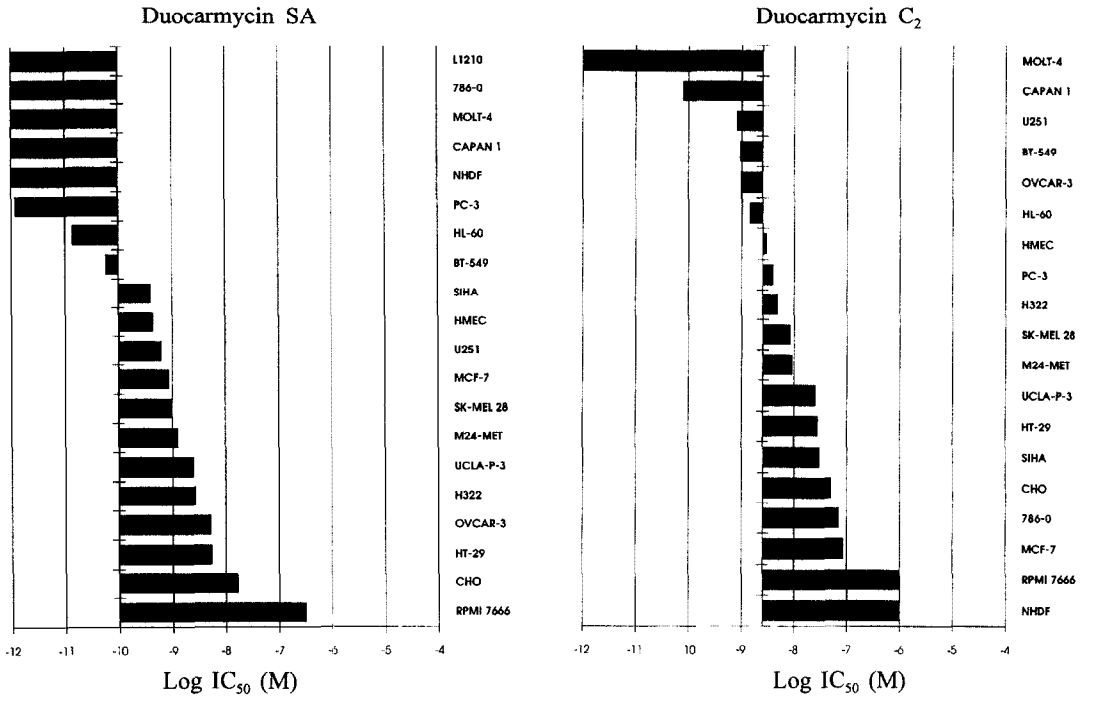


Figure 1. The relative cytotoxicities of duocarmycin SA and C_2 against a variety of cell lines.

Differential cytotoxicity profiles for duocarmycins C_2 and SA are shown in Figure 1. The mean IC_{50} values for duocarmycin C_2 and duocarmycin SA in the 20 cell line panel investigated were 2×10^{-8} and 1×10^{-10} M, respectively, with duocarmycin SA showing exceptional potencies against leukemias, pancreatic, renal and prostate tumor cell lines. Consistent with past observations, duocarmaycin SA proved to be substantially more potent than C_2 which correlates well with the observation that duocarmycin SA is substantially more stable than A.[29] Microscopic examination of Molt-4 leukemia cells several hours after incubation with duocarmycin SA showed shrinkage and blebbing of these cells without lysis, a phenomena characteristic of apoptosis. These observations prompted us to investigate the time course of cell death as a function of drug concentration after short drug exposure times. A 20 min exposure of Molt-4 cells to duocarmycin SA (0.1, 1, and 10 nM) followed by removal of the drug by low speed centrifugation and resuspension of the collected cells in medium without drug was sufficient to observe cell death. This suggested that duocarmycin SA was taken up rapidly by Molt-4 cells and that a program of cell death was being initiated by the pulsed drug treatment. While little effect on cell viability was observed in the subsequent incubation periods up to 3 h, a significant drop in the percentage of live cells was observed between 5

and 8 h particularly at dose levels above 1 nM. After 20 h, substantial growth inhibition was observed even with the 20 min exposure to 0.1 nM (100 pM) agent with nearly no cell survival above this dose level, Table 1. Examination of the cell morphology after a 4 h exposure of Molt-4 (Figure 2) and L-1210 (data not shown) cells to 10 nM duocarmycin C_2 or SA revealed the typical characteristics of apoptotic cell death when compared to untreated cells. Chromatin condensation and fragmentation of the nucleus occurred as well as disintegration of the affected cells (50-60%) into apoptotic bodies. Exposure of the less sensitive SK-Mel-28, Ovcar 3, or BT-549 cell lines to duocarmycin SA under similar conditions did not induce any of the morphological abnormalities characteristic of apoptosis indicating tumor cell line specificity for this programmed cell death that correlates well with the selective toxicity of the agent. However, these latter cell lines did show characteristic necrotic cell death morphologies in a standard 72 h tissue culture assay.

Table 1. Duocarmycin SA Induced Cell Death (Molt-4)

	Percent Viability			
Time of Incubation (h)	Control	10^{-10}	10^{-9}	10^{-8} [M]
1	95	95	95	95
3	95	95	95	92
5	90	90	85	80
8	84	65	52	42
20	92	58	15	10
28	88	58	12	10

5 x 10^6 Molt-4 cells were incubated for 20 min at 37 °C/5% CO_2 with the indicated concentration of duocarmycin SA and washed with PBS to remove the agent. Fresh media was added and cell viability as a function of time was measured microscopically using a hemacytometer and trypan blue stain.

DNA isolated from Molt-4 cells after a 4 h exposure to various levels of duocarmycin C_2 and SA were analyzed for nuclear endonuclease mediated internucleosomal DNA fragmentation. Figure 3 shows that exposure to these agents results in the characteristic ladder of DNA fragments of approximately 200 base-pair multiples. Intense fragmentation patterns were observed for duocarmycin SA in the concentration range between 10^{-7} to 10^{-10} M; Figure 3, lanes 3-6. Furthermore, Molt-4 cells which were incubated with duocarmycin SA (10 nM) in the presence of 500 μM $ZnCl_2$ (lane 8), an agent known to inhibit endonuclease activity,[30] did not show the characteristic nuclear ladder formation. Similar results were obtained for the L1210 cell line with both duocarmycin

Figure 2. Molt-4 leukemia cell morphology after exposure of cells to (I) 10 nM duocarmycin SA and (II) 10 nM duocarmycin C_2 for 4 h after which they were visualized by staining (Quik Diff). A = apoptotic, N = normal. The arrow shows apoptotic bodies attached to cell membrane.

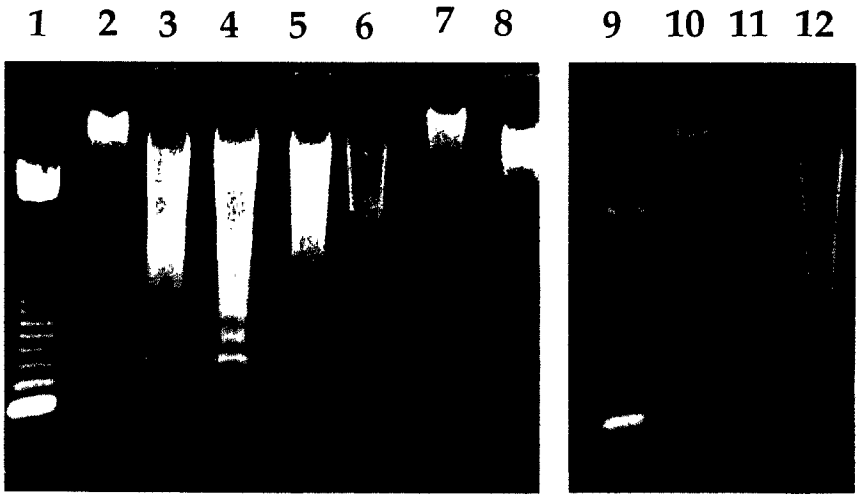


Figure 3. Agarose gel electrophoresis of ethidium bromide stained DNA fragments after 4 h drug exposure. Lane 1: molecular weight standard; 2: control without drug; 3-7: 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11} M duocarmycin SA; 8: 10^{-7} M duocarmycin SA with 500 μM $ZnCl_2$ added; 9: molecular weight standard; 10: control; 11: 10^{-7} M duocarmycin SA; 12: 10^{-7} M duocarmycin C_2.

SA (lane 11) and duocarmycin C_2 (lane 12). Quantitative analysis of DNA fragments derived from the lysed Molt-4 supernatant of 10^6 cells each treated with 100, 10 and 1 nM of duocarmycin SA for 4 h gave 33.5, 29.4 and 19.1% (average of triplicate runs) fragmented DNA compared to 2.5% fragmentation of untreated control Molt-4 cells. Similar treatment of Molt-4 cells with duocarmycin C_2 (100 nM, 2 h) produced 22.7% fragmented DNA which was compared alongside cyclohexamide (2.1%), actinomycin D (6.7%), dynemycin A (12.3%), and

calicheamicin (89%). Trypan blue staining following exposure to the drug gave cell viabilities of treater than 95% indicating that the action of these agents at physiologically relevant dose levels causing DNA degradation preceded any loss of cell viability (*i.e.*, cell membrane integrity). These results agree with previous investigations of anticancer drugs causing apoptosis where double-stranded DNA breaks occur following G-2 arrest prior to loss of membrane integrity.[11,31]

Thus, in sensitive cell lines including Molt-4 and L-1210, the duocarmycins and (+)-CC-1065 induce cell death through apoptosis. The agents induce apoptosis at concentrations in the same range as calicheamicin[16] and at significantly lower concentrations than cisplatin[11] (10^{-4} M), bleomycin[15] (10^{-5} M), doxorubicin[12] (10^{-5} M), synthetic enediynes[14] (10^{-7} M), and actinomycin D[13,14] (10^{-7} M). Presumably, sequence selective alkylation of duplex DNA potentially at internucleosomal sites initiates intracellular signalling of the apoptotic cell death mechanism with activation of endonucleases responsible for the heterochromatin condensation and degradation in the sensitive cell lines.

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