Alchemix: A Novel Alkylating Anthraquinone with Potent Activity against Anthracycline- and Cisplatin-resistant Ovarian Cancer

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Abstract

Chloroethylaminoanthraquinones are described with intercalating and alkylating capacity that potentially covalently cross-link topoisomerase II (topo II) to DNA. These compounds have potent cytotoxic activity (IC_{50} = 0.9–7.6 nM) against the A2780 human ovarian carcinoma cell line. Hydroxyethylaminoanthraquinones also reported in this paper have similar IC_{50} values (0.7–1.7 nM) in the same cell line. Alchemix (ZP281M, 1-{2-[*N,N*-bis(2-chloroethyl)amino]ethylamino}-4-{2-[*N,N*-(dimethyl)amino]ethylamino}-5,8-dihydroxy-9,10-anthracenedione), an alkylating anthraquinone, retains excellent antitumor activity in Adriamycin-resistant (2780AD) and cisplatin-resistant (2780/cp70) cell lines *in vitro* and *in vivo*. This indicates that Alchemix can evade both P-glycoprotein efflux pump and DNA mismatch repair-mediated resistance. In treated cells, Alchemix was shown to preferentially induce drug-stabilized covalent bound topo IIα-DNA complexes over topo IIβ-DNA complexes.

Introduction

A major problem with current cancer chemotherapy is the emergence of drug resistance. Therefore, it is of considerable therapeutic interest to develop compounds that are able to interact with their cellular targets in ways that will circumvent well-characterized resistant mechanisms. A number of clinically useful antitumor agents inhibit topo[3] II via drug-mediated stabilization of the topo II-DNA-cleavable complex and resulting in inhibition of poststrand passage DNA religation (1). This event is not lethal *per se* but initiates a cascade of events leading to cell death (2). Anthraquinones, as exemplified by mitoxantrone, are topo II inhibitors with proven success for the treatment of advanced breast cancer, non-Hodgkin's lymphoma, and acute leukemia (3). Intercalation is a crucial part of topo II inhibition by cytotoxic anthraquinones with high affinity for DNA (4). It is likely that the potent cytotoxicity of anthraquinones is related to their slow rate of dissociation from DNA, the kinetics of which favors long-term trapping of the topo-DNA complexes (5). However, currently available DNA intercalators at best promote a transient inhibition of topo II, because the topo-drug-DNA ternary complex is reversed by removal of the intracellular drug pool (6). Increased expression of efflux pumps and down-regulation or mutation of the target enzyme contribute to the mechanisms of resistance of available topo II poisons (6). It can be speculated that the reversible DNA binding of topo II poisons may contribute to these mechanisms of resistance.

We rationalize that DNA affinic anthraquinones and alkylating moieties with the potential to form covalent topo II-drug-DNA ternary complexes will be essentially irreversible, evade conventional resistance mechanisms, and lead to a more persistent inhibition of this enzyme. In recognition of this we have synthesized and evaluated a series of novel 1,4-disubstituted chloroethylaminoanthraquinones with the potential to bind covalently at the interface of DNA-topo II "cleavable complexes." We report here the *in vitro* cytotoxicity of several chloroethylaminoanthraquinones and their nonalkylating DNA-binding hydroxyethylaminoanthraquinone analogues. Furthermore, we also present the *in vivo* data of the most promising agent, Alchemix (ZP281M). In addition, we compare the interaction of Alchemix and its noncovalently binding analogue (ZP275) with topo IIα and IIβ.

Materials and Methods

***In Vitro* Studies.** Cytotoxicity (IC_{50}) was investigated with ovarian carcinoma cell lines A2780, 2780AD, 2780/cp70 (also known as A2780CisR or CP70), and B1 (CP70-ch3). Drug sensitivity was determined using a 96-well plate-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay with a 24-h drug exposure period and a 3-day growth period. The human ovarian carcinoma cell line A2780, a cisplatin-resistant subline A2780/cp70 and a doxorubicin-resistant subline 2780AD were obtained from Dr. Robert F. Ozols (Fox Chase Cancer Center, Philadelphia, Pennsylvania). A2780/cp70 is mismatch repair-deficient because it does not express *MLH1* protein because of hypermethylation of the *hMLH1* gene promoter (7). 2780AD is P-gp positive and does not express *MLH1* protein. B1 cells are a

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[3] The abbreviations used are: topo, topoisomerase; P-gp, P-glycoprotein; MDR, multidrug-resistance.

Table 1 Growth Inhibition (IC_{50})[a] of 1,4-disubstituted Aminoanthraquinones against wild-type *versus* resistant human ovarian carcinoma cell lines

Anthraquinone	A2780	2780AD		2780/cp70		B1	
	(nM)	(nM)	RF[b]	(nM)	RF	(nM)	RF
ZP257	1.69 ± 0.14[c]	40.4 ± 6.6	24	1.22 ± 0.45	1.0	0.93 ± 0.17	1.0
ZP265	0.71 ± 0.32	21.2 ± 1.5	30	1.55 ± 0.24	2.1	1.79 ± 0.24	2.5
ZP275	1.12 ± 0.14	144.5 ± 7.5	129	2.55 ± 0.44	2.2	2.19 ± 0.65	2.0
Alchemix	4.01 ± 0.57	16.0 ± 1.72	4	11.8 ± 1.4	2.9	7.02 ± 0.64	1.7
ZP282M	3.04 ± 0.57	245.8 ± 11.8	81	50.2 ± 3.9	16.5	19.6 ± 2.6	6.4
ZP285M	0.93 ± 0.31	573.3 ± 132.6	616	14.3 ± 1.0	15.3	5.81 ± 1.53	6.2
ZP289M	7.56 ± 0.88	2637 ± 154	348	125.9 ± 20.8	16.6	66.0 ± 4.5	8.7
Cisplatin	340.0 ± 60	3020 ± 220	9	2620 ± 730	7.7	1830 ± 230	5.3
Doxorubicin	4.98 ± 1.07	4860 ± 510	976	13.3 ± 0.9	2.6	11.7 ± 1.0	2.3

[a] IC_{50} is the concentration of drug (nM) required to inhibit cell growth by 50%.
[b] SE of the mean ($n = 3$).
[c] RF, resistance factor (IC_{50} in resistant cell line/IC_{50} in parent cell line).

variant of A2780/cp70 cells that have chromosome 3 introduced by microcell-mediated chromosome transfer and, as such, contain a wild-type copy of the *hMLH1* gene, which restores *MLH1* expression and mismatch repair proficiency (8). All of the cells were maintained in RPMI 1640 containing glutamine (2 mM) and FCS (10%). Additionally, the B1 cells were grown in the presence of hygromycin B (200 units/ml).

***In Vivo* Studies.** Monolayer cultures were harvested with trypsin/EDTA (0.25%/1 mM in PBS) and resuspended in PBS. Approximately 10^7 cells were injected s.c. into the right flank of athymic female nude mice (MF1 *nu/nu* mice; Harlan Olac). After 10–15 days, when the mean tumor diameter was ≥0.5 cm, animals were randomized into groups of 6 for experiments, and cytotoxic drugs were administered i.p. Alchemix was dissolved in DMSO and then diluted with sterile water to give a final concentration of 10%. Mice were weighed daily, and tumor volumes were estimated by caliper measurements assuming spherical geometry (volume = $d^3 \times \pi/6$). All of the animal procedures were carried out under project licenses issued by the Home Office, London, and United Kingdom Co-ordinating Committee on Cancer Research guidelines for the use of animals in experiments were adhered to throughout (9).

Trapped in Agarose DNA Immunostaining Assay. This assay has been described in detail previously (10). In brief, the assay used exponentially growing K562 cells on agarose-pretreated glass microscope slides treated with 0, 0.01, or 0.1 μM of agent for 2 h. Cells were then stained with antibodies raised to either recombinant human topo IIα or recombinant human topo IIβ. FITC-conjugated second antibody was used to visualize the topo IIα and IIβ. DNA was visualized with Hoechst 33258 using an epifluorescence microscope. Images were quantitated and statistically analyzed using Imager 2 software based on Visilog 4 and Graphpad Prism software.

Results and Discussion

Doxorubicin is routinely used in combination with cisplatin for the treatment of ovarian cancer (11), and although initially successful, therapy often ultimately fails because of acquired resistance to one or both of these agents. A series of compounds with both DNA intercalation and cross-linking potential was investigated against the A2780 human ovarian cancer cell line, the MDR1 overexpressing variant (2780AD), and the 2780/cp70 cell line, which is defective in the *hMLH1* subunit with consequent loss of DNA mismatch repair activity. The results from the cytotoxicity investigation highlighted mechanisms of resistance that distinguished the chloroethylaminoanthraquinones from the noncovalent binding hydroxyethylaminoanthraquinones. All of the compounds tested, with the exception of ZP289M, were found to be of the same order of potency as doxorubicin in the A2780 cell line, but showed a wide-ranging variation in the extent of cross-resistance in the doxorubicin-resistant cell line 2780AD (Table 1). 2780AD overexpresses the P-gp drug efflux pump, consistent with the MDR1 phenotype, which confers resistance to a variety of drugs including the anthracyclines, the *Vinca* alkaloids, and the epipodophyllotoxins (12). Three chloroethylaminoanthraquinones tested, ZP282M, ZP285M, and ZP289M, were shown to be poorly active in this P-gp-positive cell line, with resistance factors in the same order as that for doxorubicin. Resistance mechanisms other than MDR may contribute to the low cytotoxicity observed including elimination by conjugation with glutathione, often observed for classical nitrogen mustards (13). In contrast, Alchemix demonstrated very low resistance, which was 20–150-fold less than that observed with the other chloroethylaminoanthraquinones tested and 250-fold less resistant than doxorubicin.

In the cisplatin-resistant (2780/cp70) cell line ZP282M, ZP285M and ZP289M were shown to be 16-fold less cytotoxic than in the wild-type cell line. The 2780/cp70 cell line resistance is mainly because of the lack of expression of the *hMLH1* protein involved in mismatch repair (14). Increase in the cytotoxicity of the chloroethylaminoanthraquinones in the B1 cell line, which has the *hMLH1* gene restored, provides evidence that functional DNA mismatch repair is involved in the pathway that links recognition of the chloroethylaminoanthraquinone-induced DNA lesion and apoptosis. However, other resistance mechanisms have also been identified in the 2780CP cell line, including enhanced DNA repair through the induction of the *ERCC1* mRNA expression (15) and elevated levels of glutathione (16). This may also contribute to cellular resistance to these alkylating agents. The activity of the hydroxyethylaminoanthraquinones was

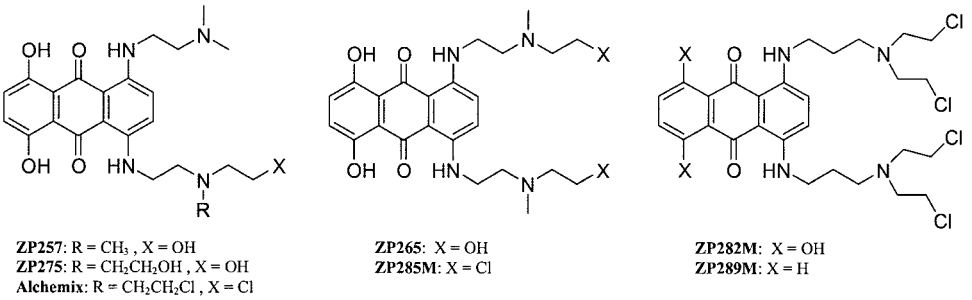

Fig. 1. Alchemix and other cytotoxic anthraquinones.

also measured in the cell lines of interest, because these compounds are close structural analogues of the alkylating aminoanthraquinones with hydroxylethyl groups in place of chloroethyl functionalities (see Fig. 1). The cytotoxicity of the hydroxyethylaminoanthraquinones was relatively unaffected by cisplatin resistance mechanisms, although some resistance was observed. Because these nonalkylating agents are unlikely to be influenced by DNA excision repair processes in cisplatin-resistant cells, the low-level resistance might be because of a decrease in drug accumulation. Previously, the 2780/cp70 subline was shown to be 2-fold more efficient than the A2780 wild-type at effluxing drugs (17).

These *in vitro* studies identified one agent, Alchemix, which was uniquely shown to maintain potent cytotoxicity against doxorubicin-resistant (2780AD) and cisplatin-resistant (2780/cp70) cell lines as shown in Table 1. To demonstrate that this effect was not restricted to the A2780 ovarian cell line, a separate study has shown that Alchemix retains cytotoxic activity (IC_{50} = 0.019 μM) against the CH1cisR human ovarian cancer cell line with acquired resistance to cisplatin compared with wild-type CH1 cells (IC_{50} = 0.018 μM). Alchemix was also 10-fold more active (IC_{50} = 0.49 μM) than cisplatin against intrinsically resistant SKOV-3 cells.[4] Alchemix differs structurally from the other chloroethylaminoanthraquinones in that it has a bis-alkylating functionality configured to only one side chain.

Two doses (8 and 10 mg/kg) of Alchemix were investigated in mice bearing sensitive (A2780) and resistant (2780AD and 2780/cp70) human xenograft tumors. Alchemix produced a significant antitumor effect at 8 mg/kg with a growth delay of 45–60% after 6 days of treatment (Fig. 2). A slightly better antitumor effect was observed at 10 mg/kg, but the higher dose resulted in >10% body weight loss (data not shown) and probably reflects the maximum tolerated dose.

The excellent *in vitro* and *in vivo* activity of Alchemix against anthracycline- and cisplatin-resistant tumors prompted additional investigation into the effect of this agent on topo II, its assumed target. A procedure was used that measures, in drug-treated cells, drug-stabilized covalent-bound topo II-DNA complexes that are detected using isoform-specific antibodies. Alchemix- or ZP275-treated cells were lysed, and nuclear proteins, including noncovalently bound topo II, were removed to leave the drug-stabilized covalent bound topo II-DNA complexes. In this

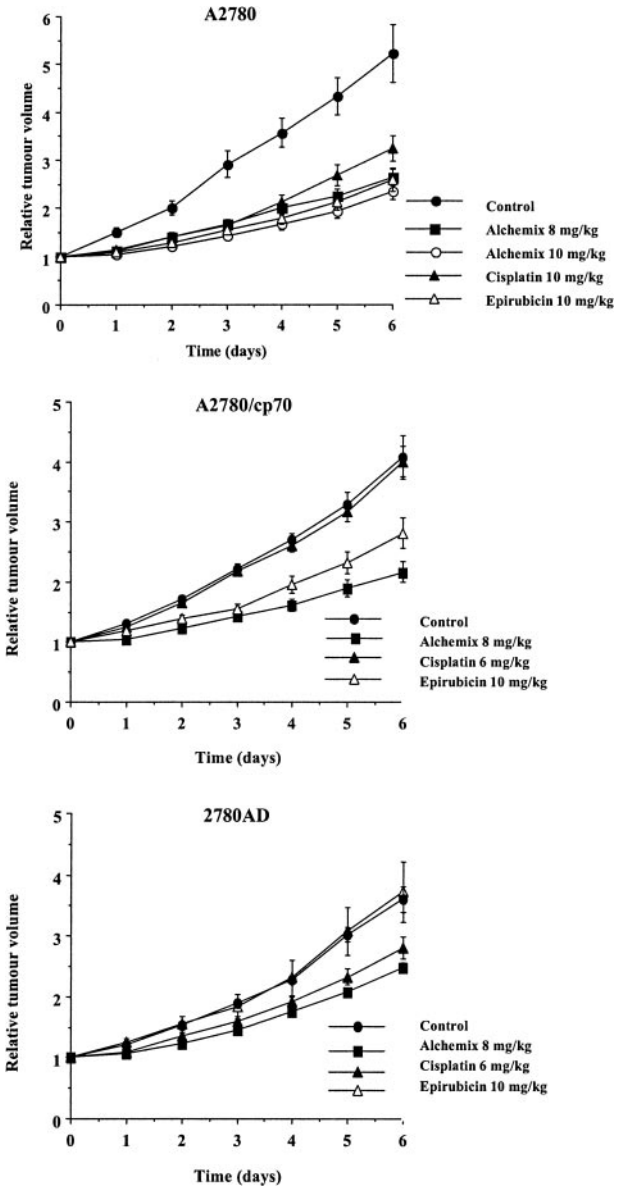

Fig. 2. Antitumor activity of Alchemix, cisplatin, and epirubicin i.p. dosing in mice bearing xenografted human tumors A2780, A2780/cp70, and 2780AD. The *in vivo* results are expressed as relative tumor volume, and the growth is measured over a period of 6 days; *bars,* ±SD.

[4] L. R. Kelland and L. H. Patterson, unpublished observations.

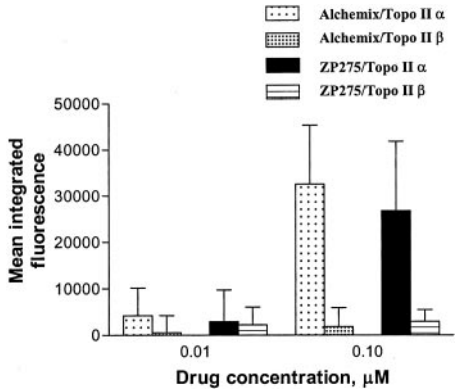

Fig. 3. Topo II-associated fluorescence in K562 cells after treatment with Alchemix or ZP275. Exponentially growing cells were treated with 0, 0.01, or 0.1 μM drug for 2 h, and then slides were prepared and stained with either 18511(α) or 18513(β)topo II antibodies. Plots show mean immunofluorescence values ± SE from four independent experiments; *bars,* ±SE.

assay, Alchemix and ZP275 were found to preferentially inhibit topo IIα, whereas leaving no significant effect on topo IIβ (see Fig. 3). In proliferating cells, topo IIα-isoform expression increases to a maximum during G_2/M transition, whereas topo II β-isoform expression remains constant throughout the cell cycle (18). Interestingly, there was no difference in the amount of drug-stabilized cleavable complexes between Alchemix and its nonalkylating analogue (ZP275). We have reported previously that Alchemix does possess DNA alkylating activity (19), and by analogy with other nitrogen mustards will be subject to inactivation by hydrolysis and binding by cell nucleophiles other than DNA. Inhibition of topo IIα by Alchemix after administration to live cells demonstrates that the alkylating functionality of this intercalating chloroethylaminoanthraquinone does not prevent its targeting of topo IIα. It has been shown previously that loss of either *hMSH2* or *hMLH1* is associated with resistance to doxorubicin, epirubicin, and mitoxantrone, suggesting the DNA mismatch repair proteins are involved in topo II-mediated cytotoxicity (20). The occurrence of these potent and potentially overlapping resistance mechanisms often confounds treatment with available antitumor agents. Alchemix represents a novel hybrid topo II/alkylating agent that retains potent activity in resistant ovarian cancer models. Alchemix warrants additional investigation to evaluate its activity against other resistant tumor types and to explore the mechanism by which it evades these resistance mechanisms.

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