Structure-activity studies with cytotoxic anthrapyrazoles

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Received December 23, 2005; Accepted February 24, 2006

Abstract. Anthrapyrazoles have been investigated as cancer chemotherapeutic agents. The mechanism of action of these compounds is thought to involve inhibition of DNA topoisomerase II. A structure-activity study was carried out to determine the *in vitro* cytotoxic activity of nine novel anthrapyrazoles against human breast carcinoma, head and neck squamous cell carcinoma and leukemia cells, and against Chinese hamster ovary cells. The activity of these anthrapyrazole analogues was compared with that of two clinically tested anthrapyrazoles, losoxantrone and piroxantrone. Inhibition of topoisomerase II as a mechanism of action for the analogues was also investigated. The cytotoxic activity of the analogues was determined *in vitro* by MTT cell growth inhibition assay and inhibition of catalytic topoisomerase II activity by each compound was measured using a fluorometric DNA decatenation assay. All of the anthrapyrazole analogues inhibited the growth of the four cell lines with IC_{50} values that ranged from 0.1 to 45.2 μM. Losoxantrone was the most potent of the anthrapyrazole analogues studied. A tertiary amine in the basic side chain at N-2 increased the cytotoxic activity compared with a secondary amine in this side chain for many of the analogues, but not if there was a basic side chain at the C-5 position. A chlorine substituent on the basic side chain at N-2 did not have a consistent effect on activity. Moving the position of a chlorine substituent from C-5 to C-7 or introducing a basic side chain at C-5 did not have a consistent effect on cytotoxic activity. Anthrapyrazole analogues showed a broad range of activity for inhibiting topoisomerase II decatenation activity. Losoxantrone and piroxantrone were the most potent inhibitors of topoisomerase II activity. There was no significant correlation between the cytotoxic activity of the anthrapyrazole analogues and their ability to inhibit decatenation by topoisomerase II.

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Key words: anthrapyrazoles, structure-activity, cytotoxic activity, topoisomerase II inhibition

Introduction

Anthrapyrazoles are a class of antitumor agents that were developed as non-cardiotoxic analogues of anthracyclines (1). Because of their planar structure, these agents intercalate into DNA (2,3) and inhibit topoisomerase II (4), inhibit DNA synthesis (1) and cause DNA strand breaks (5). Anthrapyrazoles demonstrated a broad range of antitumor activity in model tumors (4,6) and showed variable levels of activity in doxorubicin-resistant cells (7). The dose-limiting toxicity of anthrapyrazoles in animals was myelosuppression, particularly leukopenia (1); however, there was little evidence of cardiotoxicity at doses that produced good antitumor activity.

Three anthrapyrazoles, losoxantrone (CI 941, DuP 941, biantrozole), piroxantrone (CI 942, DuP 942, oxantrazole) and teloxantrone (CI 937, DuP 937) have been tested in the clinical setting. The dose-limiting toxicity for all three drugs was myelosuppression with leukopenia predominating for losoxantrone and piroxantrone (8-11), and neutropenia being most important for teloxantrone (12,13). In general, there was no evidence of cardiac toxicity; however, a high incidence of cardiotoxicity was observed in patients receiving high cumulative doses of piroxantrone (1). In phase II trials all three anthrapyrazoles showed significant response rates in women with metastatic breast cancer (14-16), with a response rate of 63% in one study with losoxantrone (16). In addition, losoxantrone produced responses in 22% of patients with measurable hormone-refractory metastatic prostate cancer (17). In contrast, anthrapyrazoles showed little or no activity in non-small cell lung cancer, gastric cancer, soft tissue sarcoma, colorectal cancer, pancreatic cancer or malignant melanoma (1), and piroxantrone had limited activity in endometrial (18) and ovarian cancer (19).

Several previous studies have examined structure-activity relationships for anthrapyrazole cytotoxicity and DNA binding. Showalter *et al* (3) found that basic side chains with two to three carbon spacers between the nitrogens, at positions N-2 and C-5 of the anthrapyrazole ring structure, enhanced *in vivo* antitumor activity against P388 murine leukemia. Hydroxylation on the A-ring also enhanced cytotoxicity, with hydroxylation at C-7 appearing to be most important. Hartley *et al* (2) found that DNA binding and intercalation were also influenced by the side chains at N-2 and C-5 and by hydroxylation of the A-ring; however, the effects were not always consistent. For example, the side chains had a greater effect on DNA binding, while intercalation was affected more by hydroxylation of the A-ring. Hydroxylation at C-7 increased DNA binding, but hydroxyl groups at any position on the A-ring decreased intercalation. The potency of losoxantrone and teloxantrone inhibition of topoisomerase II was consistent with their cytotoxicity, and analysis of NCI screen data using the COMPARE analysis showed that the mechanism of cytotoxicity for these two agents correlated well with other topoisomerase II inhibitors (4). Thus, structural changes that alter intercalation and DNA binding may have an important effect on the ability of anthrapyrazoles to inhibit topoisomerase II and on their antitumor activity.

In this study, we examined a series of anthrapyrazole analogues with altered substituents at the C-5 and C-7 positions and also studied analogues having a tertiary amine at the distal nitrogen on the N-2 side chain or an alkylating moiety on this side chain. We determined the *in vitro* cytotoxic activity of these agents in human breast cancer, head and neck cancer and leukemia cell lines and in Chinese hamster ovary cells, and correlated these with their ability to inhibit the catalytic activity of topoisomerase II.

Materials and methods

Materials. RPMI-1640, DMEM:Hams F12 (1:1) and MEM media were obtained from Invitrogen (Burlington, ON, Canada). DMEM and 2 mM L-glutamine were obtained from Invitrogen (Grand Island, NY, USA). Hepes was from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Cansera (Etobicoke, ON, Canada). MCF-7, human breast carcinoma cells were obtained from Dr Robert Shiu (Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, MB, Canada) and were grown in DMEM/HamsF12(1:1) and 10% FBS. FaDu, human pharynx squamous carcinoma cells, were from the American Type Culture Collection (Rockville, MD, USA) and were grown in DMEM/HamsF12(1:1) and 10% FBS. Human leukemia K562 cells were obtained from the American Type Culture Collection and were maintained as suspension cultures in DMEM containing 10% fetal calf serum and 2 mM L-glutamine. Chinese hamster ovary (CHO) cells (type AA8; ATCC CRL-1859), were obtained from the American Type Culture Collection and were grown in MEM containing 20 mM Hepes and 10% fetal bovine serum. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), was from Sigma. kDNA was obtained from Topogen (Columbus, OH, USA). Losoxantrone and piroxantrone were obtained from the National Cancer Institute (Bethesda, MD, USA).

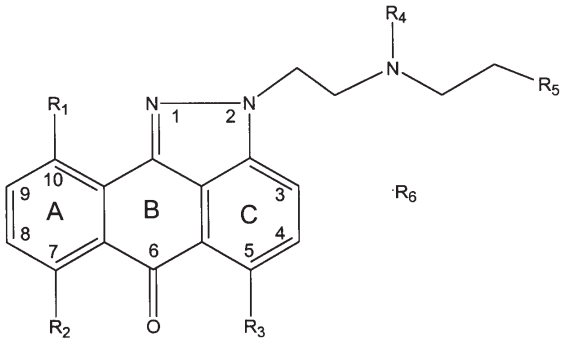
Preparation of anthrapyrazoles. The chloroanthrapyrazole derivative, AP-3, was prepared from commercially available 1,4-dichloroanthroquinone by reaction with 2-[(hydrazinoethyl) amino]ethanol in refluxing acetonitrile (20). The N-methylated derivative, AP-1, was prepared by reaction of unmethylated AP-3 with formaldehyde in formic acid at 110˚C. The N,N'-disubstituted anthrapyrazoles, AP-10 and AP-11, were prepared from the corresponding chloroanthrapyrazoles (AP-3 and AP-1, respectively) by reaction with excess N,N-dimethylethylenediamine at reflux. The chlorinated derivatives, AP-2 and AP-4, were prepared from the corresponding hydroxy compounds (AP-1 and AP-3, respectively) by reaction with excess thionyl chloride at room temperature. AP-8 was prepared by reaction of 1,5-dichloroanthroquinone with 2-[(hydrazinoethyl)amino] ethanol in refluxing acetonitrile (20) followed by chlorination of the basic side chain by reaction with excess thionyl chloride at room temperature. AP-9 was prepared by reaction of 1,5-dichloroanthroquinone with 2-[(hydrazinoethyl)amino]ethanol in refluxing acetonitrile (20) followed by methylation with formaldehyde in formic acid at 110˚C. AP-6 was prepared from AP-9 by chlorination of the basic side chain by reaction with excess thionyl chloride at room temperature. Anthrapyrazole hydrochlorides and dihydrochlorides were prepared by addition of excess methanolic hydrogen chloride followed by crystallization of the resulting solids from methanol-ether. All structures were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy.

Cytotoxicity assays. For the MCF-7 and FaDu cell lines, cells were plated from 100 to 1000 cells per well in 96-well plates in DMEM/HamsF12 (1:1) + FBS media for 24 h to allow for attachment, and then were treated with the test compounds. Drugs were dissolved in DMSO and diluted in DMEM/HamsF12 (1:1) + FBS media to desired concentrations. The final DMSO concentration was 1% and did not affect the growth or viability of the cells. Drug containing medium was added to the cells for a 1-h incubation. Cells were then washed with PBS and incubated with serum-supplemented DMEM/HamsF12 (1:1) media for 5-6 days. Cell viability was measured by the MTT assay as described previously (21). Briefly, MTT stock of 5 mg/ml was diluted to a final concentration of 0.2 mg/ml in RPMI-1640 with no HEPES. The medium was removed from cells and replaced with 125 μl of diluted MTT. Cells were incubated for approximately 4 h and then were spun at 480 x g for 10 min. MTT was removed and 200 μl DMSO added to each well. Plates were read using a plate reading spectrophotometer at 540 nm. IC_{50} values were defined as the drug concentration that resulted in 50% reduction of cell number as compared with untreated controls.

For K562 and CHO cells, cells in exponential growth were seeded at either 6000 cells/well (K562) or 2000 cells/well (CHO) in 96-well plates and allowed to attach for 24 h prior to treatment with drug for a further 72 h. The inhibition of cell growth was determined by the MTT assay as previously described (22,23).

kDNA decatenation. A modified and improved spectrofluorometric decatenation assay was used to determine the inhibition of topoisomerase IIα by anthrapyrazole analogues (24,25). The nuclear extract used in the kDNA decatenation assay was

Table I. Structures of anthrapyrazole analogues.



	R_1	R_2	R_3	R_4	R_5	R_6
AP-1	H	H	Cl	CH_3	OH	HCl
AP-2	H	H	Cl	CH_3	Cl	HCl
AP-3	H	H	Cl	H	OH	HCl
AP-4	H	H	Cl	H	Cl	HCl
AP-6	H	Cl	H	CH_3	Cl	HCl
AP-8	H	Cl	H	H	Cl	HCl
AP-9	H	Cl	H	CH_3	OH	HCl
AP-10	H	H	$NH(CH_2)_2N(CH_3)_2$	H	OH	2HCl
AP-11	H	H	$NH(CH_2)_2N(CH_3)_2$	CH_3	OH	2HCl
Losoxantrone	H	OH	$NH(CH_2)_3NH(CH_2)_2OH$	H	OH	-
Piroxantrone	OH	OH	$NH(CH_2)_3NH_2$	H	OH	-

prepared from CHO cells as described (25). kDNA consists of highly catenated networks of circular DNA. Topoisomerase II decatenates kDNA in an ATP-dependent reaction to yield individual minicircles of DNA. The 20-μl reaction mixture contained 0.5 mM ATP, 50 mM Tris-HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 30 μg/ml BSA, 40 ng kDNA, various concentrations of anthrapyrazole analogues and 10 ng of CHO nuclear extract (the amount that gave approximately 80% decatenation) (24,25). The assay incubation was carried out at 37˚C for 20 min and was terminated by the addition of 12 μl of 250 mM Na_2EDTA. Samples were centrifuged at 8000 x g at 25˚C for 15 min and 20 μl of the supernatant was added to 180 μl of 600-fold diluted PicoGreen® dye (Molecular Probes, Eugene, OR, USA) in a 96-well plate. The fluorescence, which was proportional to the amount of kDNA, was measured in a BMG Fluostar Galaxy (Durham, NC, USA) fluorescence plate reader using an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

Results

Structures of anthrapyrazole analogues. A series of anthrapyrazole analogues having various substituents on the ring system and on the N-2 side chain were prepared and structure-activity studies of these agents were carried out (Table I). The activity of these agents was also compared to that of losoxantrone and piroxantrone. AP-1 and AP-3, AP-2 and AP-4, AP-6 and AP-8, and AP-10 and AP-11 were compared to determine the effect of a methyl group at R_4. AP-1 and AP-2, AP-3 and AP-4, and AP-9 and AP-6 were compared to determine the effect of incorporating an alkylating group into the N-2 side chain by replacing the hydroxyl group at R_5 with a chlorine. AP-1 and AP-11, and AP-3 and AP-10 were compared to examine the effect of an amino side chain at R_3. AP-1 and AP-9, AP-2 and AP-6, and AP-4 and AP-8 were studied to investigate the effect of changing positions of chlorine substituents on the ring system.

Cytotoxic activity of anthrapyrazole analogues in vitro. MCF-7 human breast cancer cells or FaDu human pharynx squamous carcinoma cells were treated for 1 h with the anthrapyrazole analogues, while K562 human leukemia cells or CHO cells were treated continuously with the analogues, and the cytotoxic activity was determined by MTT assay. The IC_{50} for cytotoxic activity of the analogues ranged from 1.0±0.1 μM for AP-10 to 32.8±8.4 μM for AP-4 in MCF-7 cells (Table II). This compared to 0.3±0.1 μM for losoxantrone and 9.9±1.7 μM for piroxantrone. Similar results were obtained in FaDu cells. In general, lower IC_{50} values were obtained in the K562 human leukemia and CHO Chinese hamster ovary cells ranging from 0.1±0.1 μM for AP-10 and AP-11 to 3.6±0.3 μM for AP-4 in K562, with similar results in CHO cells (Table II). For AP-1, AP-2, AP-3, AP-4, AP-6 and AP-8 it appeared that a methyl group at R_4 increased cytotoxic activity in MCF-7 and FaDu cells but not in K562 or CHO cells; this effect appeared to be reversed with AP-10 and AP-11. A chlorine at R_5 did not have

Table II. Cytotoxic activity and inhibition of topoisomerase II decatenation by anthrapyrazole analogues.

	Cytotoxic activity IC_{50} (μM)				Inhibition of Topo II decatenation IC_{50} (μM)
	MCF-7 cells	FaDu cells	CHO cells	K562 cells	
AP-1	4.3±0.7	3.1±0.1	0.8±0.4	2.1±0.2	17.6±4.5
AP-2	1.8±0.3	1.1±0.1	0.7±0.1	1.4±0.1	12.5±2.3
AP-3	18.6±0.9	17.3±0.5	1.4±0.3	1.5±0.1	24.9±8.7
AP-4	32.8±8.4	31.8±2.9	2.7±0.7	3.6±0.3	48.4±7.3
AP-6	2.6±0.2	2.2±0.1	3.8±0.3	1.8±0.2	4.1±0.7
AP-8	29.1±5.3	45.2±4.3	3.6±0.2	2.2±0.2	10.2±1.4
AP-9	28.2±2.3	18.3±1.6	6.9±0.4	3.3±0.3	8.9±4.4
AP-10	1.0±0.1	0.5±0.1	0.8±0.3	0.1±0.1	ND
AP-11	4.8±0.6	11.6±1.2	0.3±0.1	0.1±0.1	ND
Losoxantrone	0.3±0.1	0.4±0.1	ND	ND	0.3±1.2
Piroxantrone	9.9±1.7	4.7±0.7	ND	ND	0.2±0.1

ND, not determined.

a consistent effect on cytotoxic activity. A chlorine at the R_2 position appeared to generally decrease activity compared with a chlorine at the R_3 position. An amino side chain at R_3 did not consistently alter cytotoxic activity compared with a chlorine at this position. All the cell lines were more sensitive to AP-10 compared with AP-3, but a comparison of AP-11 and AP-1 gave different results in different cell lines.

Inhibition of topoisomerase II activity by the anthrapyrazole analogues in vitro. The IC_{50} for inhibition of topoisomerase II decatenation activity for the anthrapyrazole analogues ranged from 4.1±0.7 to 48.4±7.3 μM. In contrast, losoxantrone and piroxantrone were much more potent inhibitors of decatenation activity with IC_{50} values of 0.3±1.2 and 0.2±0.1 μM, respectively.

QSAR analysis on the anthrapyrazole analogues. The data of Table II were subjected to linear correlation analysis to determine whether the growth inhibitory effects of the anthrapyrazole compounds were due to inhibition of topoisomerase II. Linear correlation analysis of the cell growth inhibitory log IC_{50} vs topoisomerase II inhibition log IC_{50} was poorly correlated for all cell lines (MCF-7: r^2=0.28, p=0.14; FaDu: r^2=0.31, p=0.12; CHO: r^2=0.13, p=0.43; K562: r^2=0.08, p=0.54). When losoxantrone and piroxantrone were excluded from the data set the correlations were not improved (MCF-7: r^2=0.20, p=0.31; FaDu: r^2=0.17, p=0.36). Thus, based on the QSAR analysis, it can be concluded that the growth inhibitory effects of the anthrapyrazole compounds was not due to the catalytic inhibition of topoisomerase II.

Discussion

Anthrapyrazoles have received extensive study as anticancer agents (1,6). Several agents, including losoxantrone and piroxantrone, have been tested in clinical trials as non-cardiotoxic analogues of anthracyclines (1). While these anthrapyrazole analogues generally showed low cardiotoxicity, piroxantrone did produce cardiotoxicity at high cumulative doses, and all the analogues caused myelosuppression (1). Losoxantrone and piroxantrone produced good responses in metastatic breast and prostate cancer (1,15-17), but showed little activity in other forms of cancer (1,18,19). Several previous structure-activity studies identified basic side chains at the N-2 and C-5 positions of the anthrapyrazole ring structure as important enhancers of antitumor activity, and suggested that the antitumor activity of the anthrapyrazoles was due to DNA intercalation and binding and inhibition of topoisomerase II activity (2-4).

In the current study, we examined the effect of changes to the basic side chain at N-2 on cytotoxic activity. We examined the effect of a tertiary amine compared with a secondary amine and the effect of introducing an alkylating group as a possible way of increasing DNA binding by forming a covalent adduct with DNA. We also investigated the effect of moving a chlorine substituent from the C-5 to the C-7 position. Finally, we correlated the cytotoxicity of the anthrapyrazole analogues with their ability to inhibit the DNA decatenation activity of topoisomerase II.

The cytotoxicity of the anthrapyrazole analogues was determined in human MCF-7 breast cancer, FaDu head and neck cancer, and K562 leukemia cell lines, and also in Chinese hamster ovary cells to determine if the results obtained were general for various cell types. In general, the IC_{50} values for cytotoxic activity obtained in the K562 and CHO cells were considerably lower than those obtained in MCF-7 and FaDu cells. However, this probably resulted from the longer exposure of the former cells to the analogues. All of the anthrapyrazole analogues studied were less potent than losoxantrone. A comparison of the IC_{50} values for AP-1 and AP-3, AP-2 and AP-4, and AP-6 and AP-8 suggested that a tertiary amine in the basic side chain at N-2 increased

cytotoxic activity compared with a secondary amine in this side chain. However, this did not appear to be the case for AP-11 and AP-10, particularly in MCF-7 and FaDu cells. While the reason for this difference is unknown, it is possible that the methyl group may enhance the ability of the analogues to interact with DNA resulting in increased cytotoxic activity. The ability of AP-10 to interact with DNA may already be enhanced by the basic side chain at C-5 (3); thus, a further increase in DNA interaction due to the methyl group may have little or no effect.

A comparison of the cytotoxic activity of AP-1 and AP-2, AP-3 and AP-4, and AP-6 and AP-9 showed that a chlorine at the R_5 position, which introduces an alkylating group on the N-2 basic side chain, did not have a consistent effect on activity. AP-2 and AP-6, which have the side chain chlorine, were more potent than AP-1 and AP-9 respectively, which do not have the chlorine, while the reverse effect was seen with AP-4 and AP-3. This suggests that the ability of the analogues to bind to DNA by alkylation does not contribute significantly to the antitumor activity of the anthrapyrazoles.

Comparing the cytotoxic activity of AP-1 and AP-9, AP-2 and AP-6, and AP-4 and AP-8 suggested that moving the chlorine from position C-5 to C-7 did not have a consistent effect on the activity of the analogues. Similarly, replacing the chlorine at the C-5 position with a basic side chain did not have a consistent effect on the cytotoxic activity of the analogues. AP-10 was more potent than AP-3; however, AP-11 was more potent than AP-1 in CHO and K562 cells but was less potent in FaDu cells. This latter finding differs from previous observations by Showalter *et al* (3), but is similar to results obtained by Hartley *et al* (2).

The anthrapyrazole analogues had a broad range of activity in inhibiting the decatenation activity of topoisomerase II with IC_{50} values ranging from 0.2±0.1 to 48.4±7.8 μM. Losoxantrone and piroxantrone were the most potent inhibitors of topoisomerase II activity. A tertiary amine in the basic side chain at N-2 appeared to increase the inhibition of topoisomerase II activity, but a chlorine at the R_5 position did not have a consistent effect. Moving the chlorine from C-5 to C-7 appeared to increase the inhibition of decatenation. However, there was no significant correlation between the cytotoxic activity of the anthrapyrazoles analogues and their ability to inhibit decatenation by topoisomerase II.

This study demonstrated that structural changes on the basic side chain at N-2 and at C-5, C-7 can significantly affect the cytotoxic activity of anthrapyrazoles as well as their ability to inhibit topoisomerase II activity. However, we were unable to identify consistent effects for specific changes. Studies with additional anthrapyrazole analogues may provide a clearer understanding of how various structural factors influence the activity of these agents. Unlike the clinically tested anthrapyrazoles, losoxantrone and piroxantrone, which appear to produce their anticancer effects by inhibiting topoisomerase II activity (4), the cytotoxic activity of the anthrapyrazole analogues investigated in this study did not correlate with their ability to inhibit topoisomerase II decatenation activity. This suggests that these analogues may produce cytotoxic effects by inhibiting other forms of topoisomerase II activity or by other mechanisms.

Acknowledgements

Supported by grants from CancerCare Manitoba Foundation (AB), the National Cancer Institute of Canada with funds from the Canadian Cancer Society (AB), the Canadian Institutes of Health Research (BBH), the Canada Research Chairs Program (BBH), a Canada Research Chair in Drug Development (BBH), the Robert A. Welch Foundation (Grant AF-0005) (FSG) and the Herbert and Kate Dishman Endowment at Southwestern University (FSG).

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