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PRELIMINARY COMMUNICATION

MITOXANTRONE AND BISANTRENE INHIBITION OF PLATELET AGGREGATION AND PROSTAGLANDIN E_2 PRODUCTION IN VITRO

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Mitoxantrone (MXN), a novel anthracenedione antineoplastic agent, displays antitumor efficacy equal or superior to that of adriamycin against several cultured tumor cell lines and against murine tumors *in vivo* (1,2). MXN (Novantrone), identified as a therapeutically promising antineoplastic agent, is presently employed in England, Europe and Canada, and is in phase II and III clinical trials in the United States. MXN has produced a good response in patients with refractory metastatic breast cancer and is effective in the treatment of leukemias (3-6). Bisantrene (BA) has exhibited antitumor activity in a variety of animal tumor models and has been employed in phase I and II clinical trials (7).

A complex array of factors influence the metastatic spread of a tumor. One aspect of tumor metastasis which has received considerable attention is the association of circulating tumor cells with host platelets (8) to form platelet-tumor cell aggregates which adhere to vascular intima. It has been reported that tumor cells are capable of initiating platelet aggregation *in vitro* (9), and ultrastructural studies have demonstrated that tumor emboli are surrounded by platelets *in vivo* (10-12). Prostacyclin (PGI_2) and inhibitors of thromboxane synthesis which decrease platelet aggregation have been shown to diminish tumor metastasis in experimental animal models (13-17). More recent studies employing calcium channel blockers as inhibitors of platelet aggregation have shown that these compounds have inhibited platelet-enhanced tumor cell adhesion to cultured endothelial cells and have produced a substantial reduction in spontaneous metastasis in B16a tumor-treated mice (18).

In view of the proposed role for platelet aggregation in tumor metastasis and since MXN and BA are administered intravenously thereby exposing formed blood elements to elevated concentrations of drug, the effects of MXN and BA on platelet aggregation and PG biosynthesis have been examined. Results presented in this communication provide evidence that the antineoplastic agents MXN and BA inhibited collagen-stimulated platelet aggregation and PGE_2 production in platelets *in vitro*, a finding which may be of significance in the clinical use of these drugs.

METHODS

Samples of human blood were obtained from healthy adult volunteers with no history of blood dyscrasias and who had not used non-steroidal anti-inflammatory agents for the previous 7 days. Blood was withdrawn into 20 ml Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) containing 80 mg sodium citrate in 1 ml water. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 150-200×g for 15 min at room temperature with removal of the PRP layer (19). Platelet-poor plasma (PPP) was prepared by centrifugation of an aliquot of blood at approximately 1000×g for 5 min with removal of the PPP layer. Human collagen was a gift of Dr. Ennio Rossi, Department of Medicine, Northwestern University School of Medicine. BA and MXN were supplied by the Natural Products Branch and Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Silver Springs, MD. Stock solutions of MXN or BA were made with either distilled water or isotonic (0.9%) saline.

Platelet aggregation studies were performed using a platelet aggregometer (Chrono-Log Corporation, Havertown, PA). A 1.5 ml cuvette, containing 0.30 ml PRP, 10 μl of drug and a stirring bar, was placed in the aggregometer at 37°. Various concentrations of collagen (0.024 ml) were added to stimulate aggregation. The baseline reading was performed using PPP containing 10 μl of drug at the same concentration as that used for the aggregation. Control aggregation rates were determined using 10 μl isotonic saline in place of drug.

For quantitation of PGE_2 production, 1 ml of PRP with 33 μl of MXN or BA was incubated in a polypropylene tube at 37° for 5 min in an oscillating water bath to allow for equilibration. Then 81 μl of collagen was added followed by an additional 5 min incubation. The reaction was terminated by freezing the incubation tube in a dry ice/acetone bath. Samples were analyzed for PGE_2 content using [^{125}I]PGE_2 radioimmunoassay (New England Nuclear, Boston, MA) after Bond-elut extraction (Analytichem International, Harbor Beach, CA). Control incubations were performed under identical conditions using 33 μl of isotonic saline in place of the drug. Basal values for PGE_2 production were obtained using platelets that were not challenged with collagen.

RESULTS

The effects of MXN and BA on collagen-induced platelet aggregation were examined. Both MXN and BA caused a concentration-dependent decrease in platelet aggregation. Figure 1, panel I, presents the results of a series of light transmission experiments on collagen-induced platelet aggregation. Tracing A is the control sample (absence of drug) and shows the extent of platelet aggregation produced by 0.72 μg/ml of collagen. Tracings B and C show the effects of increasing concentrations of MXN on collagen-induced platelet aggregation performed under identical conditions as control. Virtually complete inhibition

of aggregation was observed at 160 μM MXN (curve C), while a small change relative to control was observed at 32 μM (curve B). Figure 1, panel II, shows the results of comparable experiments on platelets using BA at 16 μM (curve B) and 32 μM (curve C). Both concentrations of BA caused a significant decrease in the extent of collagen-induced platelet aggregation, with 32 μM BA producing complete inhibition of aggregation.

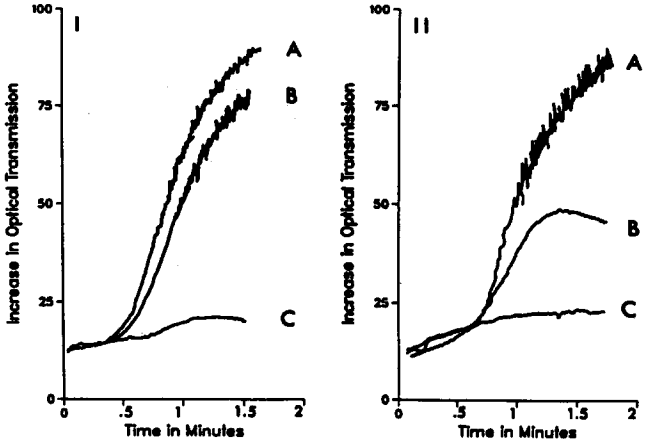

Fig. 1. Inhibition of collagen-induced aggregation of human platelets by mitoxantrone and bisantrene. Platelet aggregation is expressed as arbitrary units of optical transmission. Concentrations of mitoxantrone (panel I) are A=none, B=32 μM, and C=160 μM. Concentrations of bisantrene (panel II) are A=none, B=16 μM, and C=32 μM.

Drug effects on collagen-induced platelet aggregation rates were quantified. Table 1 contains the results of experiments using platelets obtained from three different human subjects. The platelets were challenged with two different collagen levels (0.54 and 1.1 μg/ml), and the degree of inhibition of platelet aggregation by MXN and BA varied with the level of collagen challenge to platelets. When platelets were stimulated with 0.54 μg/ml collagen, inhibition of platelet aggregation was 42 and 96% of control at 32 and 160 μM MXN, respectively. In contrast, an elevated level of collagen (1.1 μg/ml) resulted in a decreased inhibition of platelet aggregation, with 11 and 62% inhibition occurring at 32 and 160 μM MXN respectively. Comparable changes were observed with BA. BA at 16 and 32 μM produced 57 and 92% inhibition at 0.54 μg/ml collagen, respectively, and 31 and 80% inhibition at 1.1 μg/ml collagen. For comparison, in this system which employed collagen (1.1 μg/ml)-stimulated platelets, indomethacin produced 75% inhibition of the platelet aggregation rate at a concentration of 1.6 μM. In addition to collagen, platelet aggregation may be stimulated by a variety of other agents including ADP and epinephrine. Epinephrine-induced platelet aggregation is biphasic, and PG and thromboxane synthesis occurs primarily during the second wave of aggregation. MXN and BA also inhibited the second wave of platelet aggregation after stimulation with either epinephrine or ADP (P. Frank and R. F. Novak, unpublished).

Table 1. Effects of mitoxantrone and bisantrene on platelet aggregation

Treatment	Concentration (μM)	Inhibition of platelet aggregation (% control)	
		Collagen concentration 1.1 μg/ml	0.54 μg/ml
Mitoxantrone	32	11 ± 15*	42 ± 6
	160	62 ± 12	96 ± 7
Bisantrene	16	31 ± 12	57 ± 19
	32	80 ± 9	92 ± 8

* Average ± standard deviation of samples obtained from three different donors.

Additional studies were conducted to monitor the effects of comparable concentrations of MXN and BA on the production of PGE_2 by collagen-stimulated (1.4 μg/ml) platelets. Table 2 contains the results of experiments which included the same platelet preparations as were used for the platelet aggregation studies. Both MXN and BA inhibited the production of PGE_2 in a concentration-dependent manner; BA appeared to be more potent than MXN. The production of PGE_2 by collagen-stimulated (1.4 μg/ml) platelets was 37 and 5% of control when incubated at 32 and 160 μM MXN, respectively, whereas the production of PGE_2 was diminished to 18% of control when incubated with BA at 32 μM.

Table 2. Effects of mitoxantrone and bisantrene on PGE_2 production in collagen-stimulated platelets*

Treatment	Concentration (μM)	% Control	% Inhibition
None		100	0
Mitoxantrone	32	37 ± 24+	63 ± 24
	160	5 ± 9	95 ± 9
Bisantrene	16	48 ± 39	52 ± 39
	32	18 ± 23	82 ± 23

* Collagen stimulation at 1.4 μg/ml.
+ Average ± standard deviation of samples obtained from five different donors.

DISCUSSION

Previous work in our laboratory revealed that MXN inhibited both basal and drug-stimulated lipid peroxidation in a variety of subcellular fractions including cardiac sarcosomes and mitochondria (20) and that inhibition occurred via termination of lipid hydroperoxide-dependent initiation and propagation reactions (21). Since various antioxidants have been shown to inhibit PG biosynthesis in sheep vesicular gland preparations (22), the effects of MXN and BA on platelet aggregation and PG production were characterized.

Both MXN and BA were effective inhibitors of collagen-stimulated platelet aggregation, and the degree of inhibition varied inversely with the concentration of collagen employed. MXN exhibited a dose-response relationship for inhibition of aggregation and PGE_2 formation which spanned a 5-fold concentration range from 32 to 160 μM. In contrast, BA exhibited a steep dose-response relationship with inhibition occurring over the relatively narrow concentration range of 16 to 32 μM. The antiaggregatory effects of these drugs were greater than their abilities to inhibit PGE_2 synthesis. Further work is in progress to characterize the effects of MXN and BA on thromboxane and PG production in platelets and other preparations.

Intravenous administration of MXN to patients as a 15 min bolus (1-3 mg/m^2) or 30 min infusion (12 mg/m^2) resulted in peak plasma levels of ~30-40 ng/ml (~0.01 μM)(23,24). Plasma levels of 100-200 ng/ml have been reported within 15 min of MXN infusion (25). Intraperitoneal administration of MXN at 12-14 mg/m^2 resulted in an average peak plasma level of 36 ng/ml, with individual levels ranging from 1.4-138 ng/ml (26). MXN exhibits a long terminal elimination half-life from plasma (>40 hrs) with plasma levels of 5-10 ng/ml present up to 20 hours (23,26). MXN distributes into deep tissue compartments from which it is slowly released (24,26). BA (260-280 mg/m^2 i.v. over 1 hr) gave plasma concentrations of 0.5-1.0 μg/ml (~1-2 μM) with an elimination half-life comparable to that of MXN (27).

The concentration of[^{14}C]MXN in formed blood elements (e.g. red blood cells) was consistently greater (2-to 10-fold) than that present in plasma (24,26). Given the elimination kinetics and elevated levels of MXN in red blood cells, it follows that the concentration of MXN achieved in platelets may be significantly greater than that in plasma. Moreover, the use of intermittent multiple dosing regimens and/or disease-induced increases in elimination half-life may expose platelets to elevated levels of MXN for longer periods of time.

It has been demonstrated recently that calcium channel blockers of the dihydropyridine class inhibit tumor cell-induced platelet aggregation as well as platelet-enhanced tumor cell adhesion to plastic or endothelial cells *in vitro* (18). Further, it has been suggested that compounds which inhibit interactions among tumor cells, platelets and endothelial cells *in vitro* may possess antimetastatic properties when administered *in vivo*. Indeed, experiments conducted on the dihydropyridine calcium channel blockers showed that these agents inhibit metastases significantly in animal models (18). The results of our experiments support the conclusion that MXN and BA are effective inhibitors of platelet aggregation and PGE_2 production *in vitro*; inhibition of platelet aggregation which is dependent on other stimuli and/or components involved in the aggregation process is being evaluated. Although other drugs possess the ability to inhibit platelet aggregation, MXN is unique in that it is an effective antineoplastic agent which also inhibits platelet aggregation; other congeners of

MXN with diminished toxicity may display similar inhibitory properties. These data may be of significance since MXN (Novantrone) is presently in clinical trials and may be of interest given the suggested role for platelets in the metastatic spread of cancer.

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