NAFAZATROM (Bay g 6575) INHIBITION OF TUMOR CELL LIPOXYGENASE ACTIVITY AND CELLULAR PROLIFERATION

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1. Introduction

Nafazatrom has been reported to possess significant antithrombotic and thrombolytic activity in model systems of experimental thrombosis [1]. The mechanism of action for these antithrombotic effects appears related to the ability of the drug to stimulate prostacyclin (PGI_2) production by the vascular wall [2]. Nafazatrom has been reported to possess significant antimetastatic activity [3] presumably due to its ability to enhance PGI_2 production, as PGI_2 itself is a potent antimetastatic agent [4]. Considering the effect of nafazatrom on arachidonic acid metabolism [5] and the suspected importance of these metabolites in tumor growth [6] we examined the effects of nafazatrom on tumor cell proliferation in vitro and in vivo and on tumor cell arachidonic acid metabolism. We report here that nafazatrom inhibits tumor cell lipoxygenase activity but not prostaglandin endoperoxide synthetase (cyclooxygenase + hydroperoxidase). Further, this compound was found to be a potent cytostatic agent against the B16 amelanotic melanoma (B16a).

2. Materials and methods

The B16a was propagated by subcutaneous implantation in male syngeneic C57BL/6J mice (Jackson Lab., Bar Harbor ME). Tumors (0.5–1.5 g) were removed and disaggregated to yield monodispersed cells [7]. Freshly dispersed B16a tumor cells were adapted for growth in tissue culture medium [8] allowed to grow to confluency in primary culture then subcultured one time for use in these experiments. The cells were counted in a hemocytometer. Viabilities were determined by vital dye exclusion and lactate dehydrogenase (LDH) release as determined fluorometrically [9].

For DNA, RNA or protein synthesis experiments the tumor cells were further purified by centrifugal elutriation as in [7]. [^{3}H]Thymidine (New England Nuclear, 80 Ci/mmol) incorporation into acid-insoluble DNA, [^{3}H]uridine (New England Nuclear, 56 Ci/mmol) incorporation into acid-insoluble RNA, and [^{3}H]leucine (New England Nuclear, 39 Ci/mmol) incorporation into protein were determined as in [10].

Arachidonic acid metabolism was measured in the microsomal pellet (indomethacin-sensitive cyclooxygenase activity) and microsomal supernatant (lipoxygenase activity) from B16a tumors. Tumors were homogenized in 250 mM d-mannitol, 1 mM EDTA and 0.4% fatty acid free bovine serum albumin in 100 mM phosphate buffer (pH 7.8) (PBS). The supernatant from 2 centrifugations (8500 × *g*, 10 min) was recentrifuged at 100 000 × *g* for 45 min. The pellets were resuspended in PBS and 3 mg palmitic acid was added to each 5 ml supernatant. Pellet or supernatant (1 ml) was preincubated with the drugs for 5 min at 37°C in a shaking water bath. Then 5 nmol hematin, 5 nmol epinephrine and 1.9 nmol [^{14}C]arachidonic acid were incubated for 15 min at 37°C. The assays were terminated by lowering the pH to 3.5 with 1 N HCl. Samples were extracted in ethyl acetate and dried under N_2 and then chromatographed by TLC in hexane:diethyl ether:acetic acid (50:50:1). TLC plates were scanned with a Berthold radiochromatogram scanner.

Nafazatrom (Bay g 6757; 1-[(2-β-napthyloxy)ethyl]-3-methyl-2-pyrazolin-5-one; fig.1) was dissolved in 0.1 N NaOH and diluted in culture medium or 0.9% NaCl to final concentrations.

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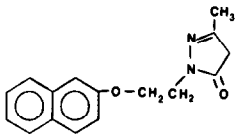

1-[2-(β-napthyloxy)ethyl]-3-methyl-2-pyrazolin-5-one (Bay g 6575, Bayer Pharmaceutical)

Fig.1. Chemical structure of nafazatrom.

3. Results

The effects of nafazatrom on proliferation of B16a cells in culture were examined over a period of 14 days. Media was changed and drug added every other day. A dose-dependent decrease in cell proliferation was observed with a minimal effective dose of 0.1 μg/ml (fig.2). This effect of nafazatrom was not due to cytotoxicity as determined by vital dye exclusion and LDH release (fig.2). Nafazatrom also decreased [^{3}H]thymidine incorporation into DNA. Exposure of B16a tumor cells to nafazatrom for 4 h produced a dose-dependent decrease in DNA synthesis with an ED_{50} of 0.6 μg/ml (fig.3). After a 4 h exposure to nafazatrom, RNA synthesis was inhibited in parallel with inhibition of DNA synthesis. Protein synthesis was unaffected at this time interval (fig.4).

In a preliminary in vivo experiment animals were injected subcutaneously with 10^5 B16a tumor cells and treated with Nafazatrom (sc) at 0.8 mg/kg for 21 days. Final tumor weight in treated animals (0.49 ± 0.27 g; n = 12) was significantly decreased from untreated controls (3.98 ± 0.39 g; n = 12).

Nafazatrom inhibited the formation of monohydroxy fatty acids synthesized from the lipoxygenase pathway by B16a cells (fig.5, ED_{50} = 3 μM). This

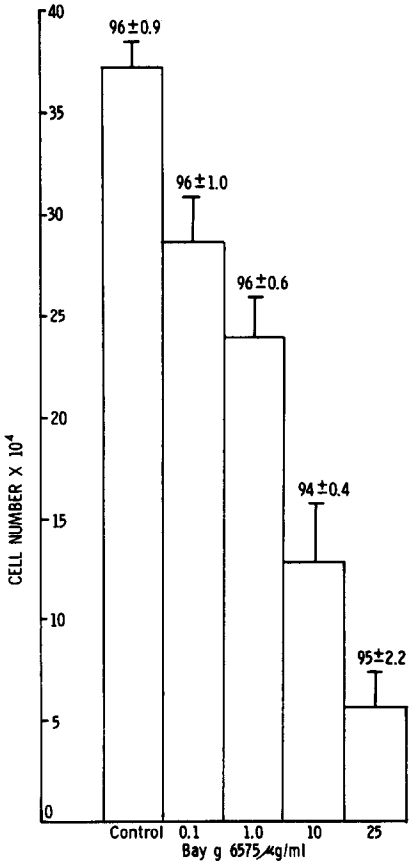

Fig.2. Effects of nafazatrom on proliferation of B16a cells in culture. The numbers above each bar represent final cell viabilities determined by vital dye exclusion. Similarly, there was no difference in LDH release between controls and nafazatrom-treated cells (not shown). Values are expressed as x ± SEM; n = 5.

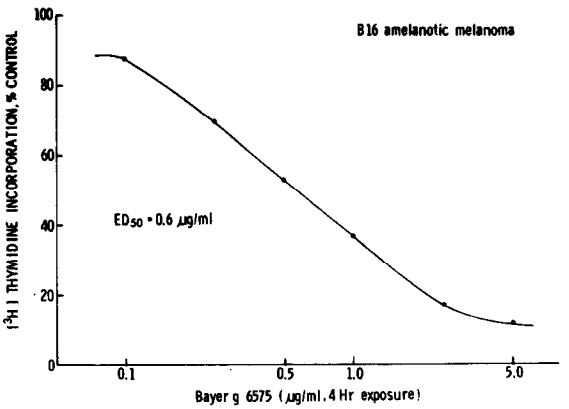

Fig.3. Dose-dependent inhibition of DNA synthesis ([^{3}H]-thymidine incorporation) by B16a cells following exposure to nafazatrom. n = 4.

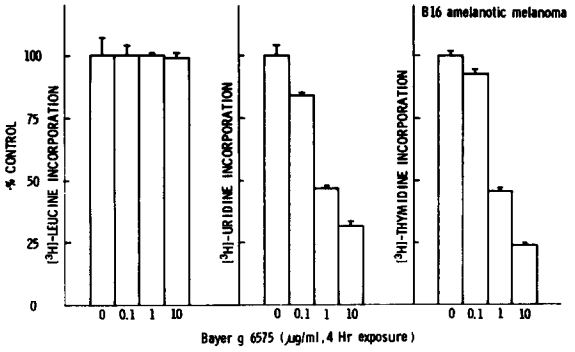

Fig.4. Effects of nafazatrom on protein ([^{3}H]leucine), RNA ([^{3}H]uridine) and DNA ([^{3}H]thymidine) synthesis by B16a cells: x ± SEM; n = 4.

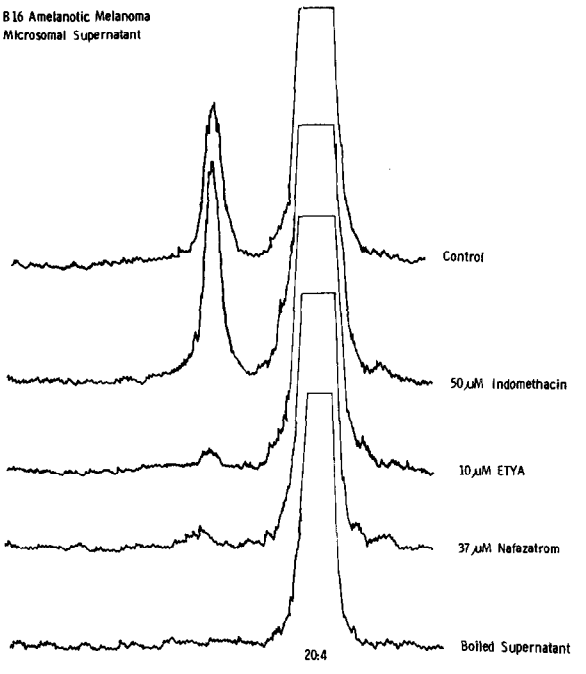

Fig.5. Radiochromatogram scans of [^{14}C]arachidonic acid (20:4) conversion into monohydroxy fatty acids by the microsomal supernatant from B16a tumor cells. Boiled supernatant scan indicates that this conversion was enzymatic. ETYA and nafazatrom inhibited conversion of 20:4 into monohydroxy fatty acids but indomethacin did not.

product was not inhibited by indomethacin (50 μM), a cyclooxygenase inhibitor, but was inhibited by 5,8,11,14-eicosatetraynoic acid (ETYA; $ED_{50} = 0.9$ μM), an inhibitor of both the lipoxygenase and cyclooxygenase pathways. Subsequent analysis resolved the monohydroxy fatty acid peak shown in fig.5 into 2 components one of which comigrated with authentic 5-hydroxyeicosatetraenoic acid (5-HETE), a metabolite of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and the second comigrated with authentic 12-hydroxyeicosatetraenoic acid (12-HETE), a metabolite of 12-hydroperoxyeicosatetraenoic acid (12-HPETE) (not shown). Nafazatrom at the doses studied did not inhibit formation of cyclooxygenase products.

4. Discussion

Nafazatrom demonstrated inhibition of tumor cell proliferation in vitro and tumor growth in vivo. The effectiveness of this compound both in vivo and in vitro at low doses suggests its potential as a cancer chemotherapeutic agent. Nafazatrom unlike current chemotherapeutic agents [11] does not appear to be a cytotoxic agent at its effective dose(s). In animal models nafazatrom is not toxic at 10 g/kg acute exposure [1] and no toxicity has been reported following administration to human volunteers [2].

The mechanism by which nafazatrom inhibits tumor cell proliferation is unclear. However, we have observed that nafazatrom behaves as a lipoxygenase inhibitor and therefore would inhibit the conversion of arachidonic acid to hydroperoxyeicosatetraenoic acid isomers (5-,8-,9-,11-,12- and 15-HPETEs). It has been observed that the B16a melanoma (this study) and the Lewis lung carcinoma (unpublished) produce at least 2 lipoxygenase products (5-HPETE and 12-HPETE). These hydroperoxy fatty acids have been demonstrated to increase cellular cGMP [12–14], a compound which has been described as a positive signal for cellular proliferation [15,16]. We speculate that the ability of nafazatrom to inhibit tumor cell proliferation and tumor growth may be related to its activity as a lipoxygenase inhibitor.

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