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Cytostatic activity of pharmacological concentrations of indomethacin in cell cultures and inactivity of closely related compounds

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Indomethacin and other nonsteroidal anti-inflammatory drugs arrest proliferation of tumor cells *in vivo* [1] and of transformed and nontransformed cells in culture [2–4] in the G_1 phase of the cell cycle [4]. The cytostatic activities of these drugs parallel their anti-inflammatory activities [2, 4]. The action is not cytotoxic; cultures can be inhibited from 4 [3, 4] to 9 days (unpublished data) without loss of cell viability or the ability to resume growth. Once the drug is removed, growth proceeds synchronously through the remainder of the cycle [4] and is accompanied by well-defined changes in amino acid transport [5] and DNA synthesis [4].

In general, drug levels higher than those observed therapeutically were required for these actions, although a major proportion (80 per cent) of the drug was bound to serum protein in the culture medium [4]. In this paper, we report that in BRL-T rat hepatoma cultures, which can be grown in the presence of low serum concentrations, indomethacin was cytostatic at levels near those observed in plasma after pharmacological doses of drug. We also report that cytostatic activity was not retained by compounds resembling structural fragments of indomethacin (I), such as 5-methoxy-methylindoleacetic acid (II), chlorobenzoic acid (III) or indoleacetic acid (IV) (Fig. 1), nor was it observed with the anti-inflammatory steroid drug, dexamethasone.

Rat hepatoma (HTC) chemically transformed cultures and BRL-T hepatocytes (cloned from the liver of a 5-week-old Buffalo rat) [6] were grown as described elsewhere [4]. Suspensions of cells (50×10^3 cells/ml) in Eagle's medium with 10% fetal calf serum were dispensed (1 ml) into individual wells of Costar tissue culture cluster plates (16 mm diameter wells, Cat. No. 3524, Cambridge, MA). Individual plates contained 1 row (6 wells) of cultures as controls (no drug), and the remaining rows were treated with drugs as indicated. Dexamethasone acetate was dissolved in dimethylsulfoxide and added in volumes of 10 μl to each culture. Other drugs were dissolved in 200 μl dimethylsulfoxide, neutralized where necessary with an equivalent amount of 1 N NaOH, and then diluted with 10 ml of the Eagle's medium. Aliquots (100 μl) of the drug solution were added to the cultures on day 2 of culture growth, a stage when the cultures were entering into exponential growth ($100–150 \times 10^3$ cells/well). The cultures were incubated for an additional 2 days, and cell count was determined by trypsinization and counting in a Neubauer counting chamber [4]. All incubations were carried out at 37° in an atmosphere of 95% O_2 and 5% CO_2. Media were prepared by the Media Unit, Division of Research Services, NIH, and solutions were sterilized by filtration. Values are expressed as means ± S.E. of six cultures.

Chemicals were obtained from the following sources: indomethacin (I) and dexamethasone acetate from the Sigma Chemical Co. (St. Louis, MO) and indoleacetic acid (IV), 5-methoxy-methylindoleacetic acid (II), and *p*-chlorobenzoic acid (III) from the Aldrich Chemical Co. (Milwaukee, WI).

The data for HTC cultures are summarized in Fig. 2 (A and B). Indomethacin inhibited culture growth in a dose-dependent fashion over the concentration range of 0.1 to 0.4 mM with an ED_{50} of 0.29 mM. *p*-Chlorobenzoic acid (III) and 5-methoxy-methylindoleacetic acid (II), as well as indoleacetic acid (IV) (Fig. 1), were inactive or weakly inhibitory at concentrations of 2 mM (Fig. 2A). Compounds I and II were inactive individually or in combination. Dexamethasone had no cytostatic activity and did not potentiate the effects of indomethacin (Fig. 2B).

Unlike the HTC cultures, the rat hepatoma BRL-T cell line grew in media containing 3% fetal calf serum at a growth rate similar to that observed in 10% fetal calf serum (Fig. 3A). Growth of the rat hepatoma BRL-T cell was inhibited by indomethacin in a dose-dependent manner (Fig. 3B) with an ED_{50} of 0.15 mM in the presence of 10% fetal calf serum. The values for ED_{50} decreased in linear fashion with decreasing concentrations of fetal calf serum (insert, Fig. 3). Extrapolation of the data indicated an ED_{50} of 0.05 mM in the absence of serum.

All the nonsteroidal anti-inflammatory drugs possess a lipophilic group, generally a substituted phenyl or heteroaryl group, and an acetic acid side chain or acidic moiety with a pK_a value of about 4.5. A hypothetical receptor structure based on structure–activity relationships of several families of anti-inflammatory drugs [7, 8] and of the indomethacin series [9] has been postulated to contain three sites: a cationic site to accommodate the acidic group, adjacent flat areas to accommodate the aromatic ring(s), and below these a trough to which the benzoyl group of indomethacin or comparable groups of other anti-inflammatory drugs [8] fit. With the finding that many of the anti-inflammatory drugs are potent inhibitors of prostaglandin synthesis [10], the suggestion has been made that the receptor can also accommodate prostaglandin E_2 [8].

The "fragments" of indomethacin tested here neither

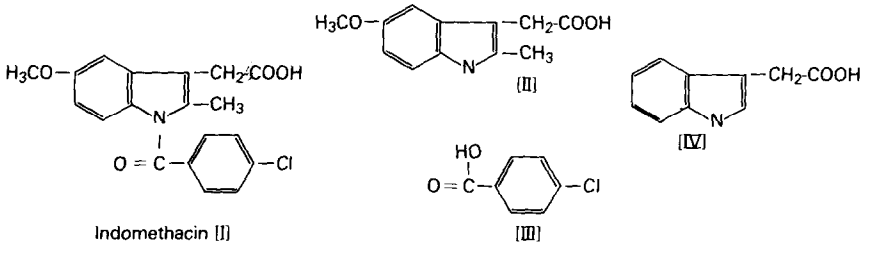

Fig. 1. Structures of indomethacin and related compounds tested for their cytostatic activity.

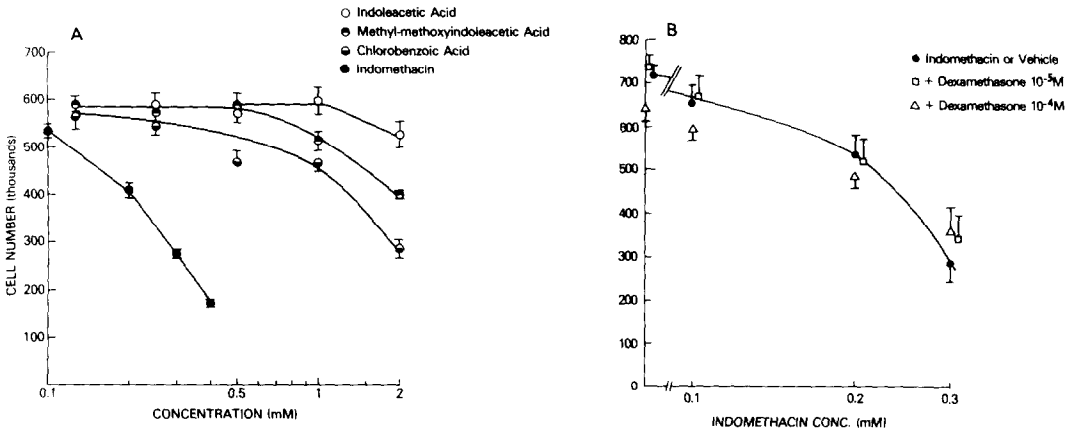

Fig. 2. Effects of indomethacin and related compounds (A) and dexamethasone and indomethacin (B) on the growth of HTC cultures. The compounds were added on day 2 of culture growth. Cell counts on day 2 were 61,000 ± 3500 for the experiment shown in panel A and 123,000 ± 3400 for that shown in panel B. Cell numbers were determined 2 days later. Each value is the mean count ± S.E.M. of six cultures.

fulfill all three requirements for the receptor fit nor retain cytostatic or anti-inflammatory activity. Of the more than 700 indole compounds screened, only indomethacin was reported to possess anti-inflammatory activity [11]. As reported elsewhere, all of the nonsteroidal anti-inflammatory drugs tested inhibit culture growth in a dose-dependent fashion [2, 4]. Introduction of a hydrophilic group into the aromatic ring, as in gentisic acid, reduces the anti-inflammatory and cytostatic activity of the compound, whereas halogenated compounds, such as meclofenamic acid and indomethacin, which are potent anti-inflammatory agents *in vivo* [12] are also the most potent cytostatic agents so far tested [1]. The cyostatic activity is restricted to the acidic nonsteroidal anti-inflammatory drugs. Neither dexamethasone (present data) nor the basic prostaglandin synthesis inhibitors are cytostatic [13]. In addition, the action of indomethacin is not antagonized by exogenous prostaglandins or their precursor arachidonic acid [13].

The anti-inflammatory drugs are largely ionized at plasma pH, but sufficient non-ionized drug remains to allow rapid equilibration of drug across lipid cell membranes [14, 15]. The drug distributes between the intra- and extracellular spaces according to the difference in pH, and under physiological conditions this leads to greater concentrations of drug within cells. As predicted from theoretical considerations [14], a small decrease in the pH will lead to much greater concentrations of drug within the cell, and this has been observed experimentally by us in cell cultures [16] and by Brune *et al.* [14, 15] in inflammatory exudates.

The inhibition of growth by indomethacin and other anti-inflammatory drugs has now been demonstrated with a variety of cell types. These include the rat hepatoma (HTC and BRL-T) cell lines (Ref. [3] and this paper), the nontransformed human fibroblasts [2, 4], mouse L-fibroblasts and mitogen stimulated rat lymphocytes (unpublished data). Inhibition was observed with indomethacin concentrations of 0.1 to 0.4 mM, but binding of drug to serum albumin [17] diminished the level of free drug in the

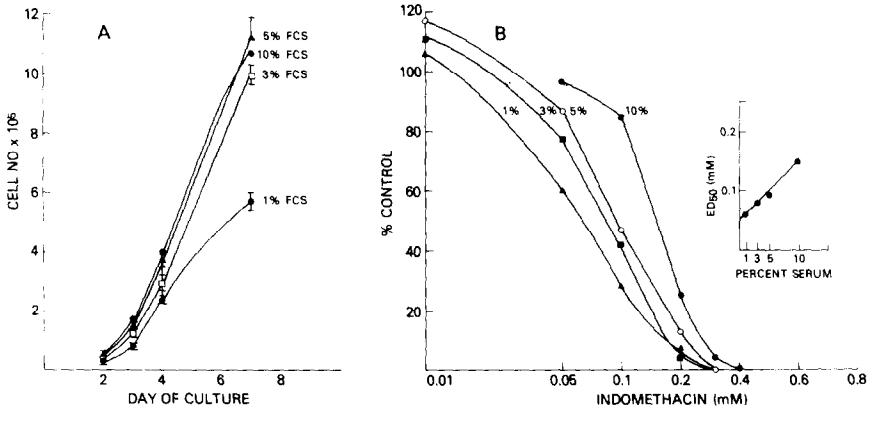

Fig. 3. Cytostatic activity of indomethacin in cultures of BRL-T cells grown in media with various concentrations of fetal calf serum. The protocol of this study was identical to that in Fig. 2. The growth curves of control cultures grown in media with different serum concentrations are shown in panel A and inhibition produced by indomethacin at different serum concentrations is shown in panel B. The insert shows the relationship between ED_{50} for indomethacin and serum concentration. In panel B, cell count on day 2, when indomethacin was added to the cultures, ranged from 39,000 ± 5500 to 52,000 ± 3000 cells/well for cultures grown in 1 and 10% fetal calf serum respectively. On day 4, when the experiment was completed, cell counts ranged from 250,000 ± 13,000 to 400,000 ± 26,000 cells/well for control cultures (no indomethacin added) grown in 1 and 10 per cent fetal calf serum respectively.

culture medium [4]. When BRL-T cell cultures were grown in medium with low serum content, marked inhibition of growth occurred with 50 μM indomethacin (Fig. 3) or with concentrations which approach those reported for total drug levels in plasma of animals receiving pharmacological doses of the drug or in humans receiving high (200 mg) doses of indomethacin [2, 18].

Laboratory of Cellular Metabolism
National Heart, Lung, and Blood Institute
National Institutes of Health
Bethesda, MD 20205, *U.S.A.*

BARBARA M. BAYER*
MICHAEL A. BEAVEN

* Address all correspondence to: Dr. Barbara M. Bayer, National Heart, Lung and Blood Institute, National Institutes of Health, Building 10, Room 5N323, Bethesda, MD 20205, U.S.A.

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