

The effect of inhibitors of arachidonic acid metabolism on proliferation and death of tumor cells

Yu.N. Korystov*, V.V. Shaposhnikova, M.Kh. Levitman, A.A. Kudryavtsev, L.N. Kublik, A.A. Narimanov, O.E. Orlova

Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region, 142292, Russia

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Abstract The effect of inhibitors of arachidonic acid metabolism on proliferation and death of tumor P-388 cells in a broad concentration range was studied. Cell proliferation was estimated by the metaphase frequency and the proportion of cells in S phase; cell death was determined from lysis, staining of cells with trypan blue, nuclear damage, percentage of cells with subdiploid DNA and the type of DNA fragmentation. It was shown that low concentrations of phospholipase A₂ and lipoxygenase inhibitors stimulated the proliferation of P-388 cells. At higher concentrations, the inhibitors suppressed cell proliferation by blocking the G₁-S transition and induced cell death of the apoptosis type. Indomethacin, an inhibitor of cyclooxygenase, did not initiate cell death, nor did it affect the proliferation of P-388 cells at concentrations of up to 10 µM.

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Key words: Arachidonic acid metabolism; Apoptosis; Proliferation; Tumor cell

1. Introduction

Membrane phospholipids are the source of various intra- and intercellular messengers that control and mediate proliferation, death, and other cellular responses [1,2]. Phospholipase A₂ (PLA₂), a catalyzer of the hydrolysis of choline and ethanolamine glycerophospholipids, is the key enzyme responsible for the release of arachidonic acid (AA) in different signal reactions in mammalian cells [3–5]. The released AA is subsequently oxidized either by cyclooxygenase to form cyclic products such as prostaglandins, or by lipoxygenase giving rise to linear chain products such as lipoxins and leukotrienes [5,6]. Previously we have shown that 4-bromophenacyl bromide (BPB), a PLA₂ inhibitor [3], and nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor [7], prevent apoptosis in irradiated thymocytes [8–10]. At the same time, in some tumor cells, the blockage of the lipoxygenase pathway of AA metabolism induces apoptosis [11]. In addition, lipoxygenase inhibitors suppressed the proliferation of tumor cells [12–14]. The differences in the effects of drugs on normal and tumor cells are essential for effective antitumor therapy. To our knowledge, no study has been conducted to monitor the effects of PLA₂ inhibitors on tumor cells in this context. Moreover, it is unclear whether the induction of apoptosis by lipoxygenase inhibitors is a phenomenon common to tumor cells. This prompted us to study the effects of BPB,

NDGA, and indomethacin on death, nuclear condensation and DNA degradation of lympholeukemic P-388 line cells.

2. Materials and methods

Lympholeukemic P-388 cells were cultivated in the abdominal cavity of male DBA₂ mice (20 g). The number of cells inoculated into one mouse was 2×10^6 . After seven days, cells ($3\text{--}6 \times 10^8$) were withdrawn from the abdominal cavity, precipitated by centrifugation ($400 \times g$, 10 min), and washed with Hanks' solution, pH 7.2.

Cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 10 mM HEPES (pH 7.2) and gentamicin (40 µg/ml). Cells were incubated at 37°C in a humidified incubator with 5% CO₂ in plastic dishes (diameter 3 cm) at a concentration of 10^6 cells/ml. Cells did not adhere to plastic.

Cell death was estimated by a decrease in cell number (cell lysis) and by staining of cells in a 0.04% trypan blue solution (Serva) after 22 h incubation. The effect of the drug was determined by the criterion of cell survival:

$$S = \frac{N_1}{N_0} 100\%$$

where N_1 is the final concentration of unstained cells, which can be higher or lower than N_0 , depending on the extent of proliferation and death of cells in the population.

Nuclear damage was evaluated by chromatin condensation (nuclear pycnosis) and fragmentation of condensed chromatin (nuclear fragmentation). The damaged nuclei were identified by fixation with acetic acid-ethanol (1:3) and Giemsa staining after 8 and 22 h incubation. The frequency of nuclear damage was calculated under the microscope at $600\times$ magnification by examining 500–1000 cells in each preparation.

The metaphase frequency was calculated under the microscope at $600\times$ magnification by examining 1000 cells in each preparation that

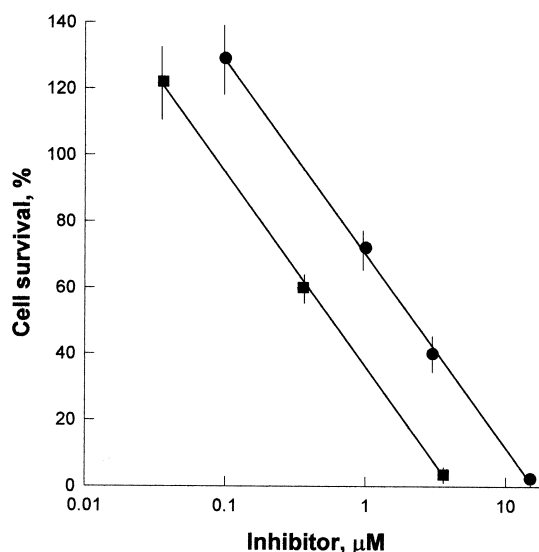


Fig. 1. Effect of BPB (■) and NDGA (●) on cell survival. $n = 6$.

*Corresponding author. Fax: (7) (095) 924-04-93.

Abbreviations: AA, arachidonic acid; BPB, 4-bromophenacyl bromide; NDGA, nordihydroguaiaretic acid; PLA₂, phospholipase A₂

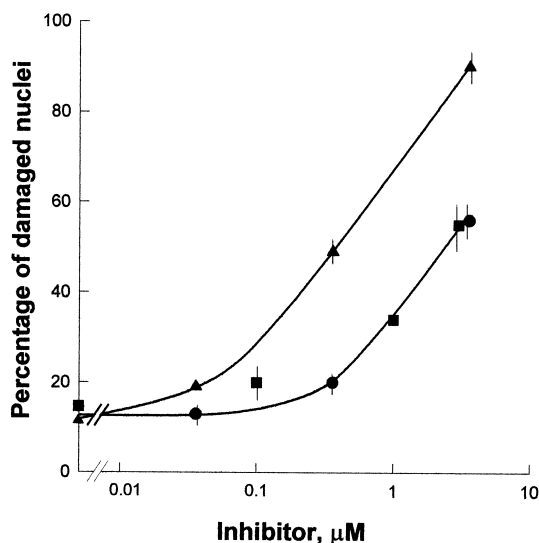


Fig. 2. Effect of BPB (●,▲) and NDGA (■) on nuclear damage at 8 h (▲) and 22 h (●,■) incubation. $n=5$.

were fixed in a mixture of methanol and acetic acid (3:1) and stained by the method of Giemsa. The percentage of cells with a subdiploid set of DNA (sub- G_1) was determined on a flow cytometer (laboratory setup). Cells fixed with cold 70% ethanol were stained, transferred into phosphate buffer (pH 7.2), and stained with Hoechst 33258 (Serva) for 5 min (2 $\mu\text{g}/\text{ml}$). In each series, 20 000–50 000 cells were assayed. The distribution of cells with respect to the DNA content in each stage of the cycle was determined from experimental histograms.

DNA was isolated by the method of Maniatis [15] with minor modifications. Washed cells (10^6) were lysed for 15 min at room temperature in a solution containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and 1% SDS. Then NaCl was added to a final concentration of 1 M, and the solution was kept for 30 min at 4°C. To purify DNA from proteins, an equal volume of phenol (pH 8.0) was added to the lysate. The mixture was centrifuged ($1000\times g$, 5 min), the aqueous phase was collected, and an equal volume of chloroform was added. After centrifugation ($1000\times g$, 5 min), DNA was freed from salts by reprecipitation with isopropanol (with a volume equal to the volume of the sample) followed by 80% ethanol. The precipitate was dissolved in 10 μl of H_2O and 2 μl of RNA solution (1 mg/ml) (Sigma), and the solution was incubated for 1 h at 50°C. Then the samples were stained with bromophenol blue (Serva). DNA was separated in 1.8% agarose for 2.5 h at $U=5$ V/cm. After staining with ethidium bromide gels were photographed in UV light.

BPB (Sigma), indomethacin (Serva) and NDGA (Serva) were added to the cell suspension prior to incubation. Stock solutions of these drugs in DMSO were used. The points in the graphs are the means $\pm$ standard deviation. The number of assays (n) for each experiment per series is given in the figure legends.

3. Results and discussion

We studied the effects of various inhibitors of AA metabolism on cell survival in a wide range of concentrations (Fig. 1). The results showed that BPB and NDGA significantly

Table 1
Effect of BPB (3.6 μM) and NDGA (3 μM) on the distribution of DNA content in P-388 cells

Conditions	DNA content			
	Sub- G_1	G_1	S	$G_2\text{M}$
Control	12 $\pm$ 2	38 $\pm$ 4	37 $\pm$ 2	13 $\pm$ 2
BPB (3.6 μM)	38 $\pm$ 3	38 $\pm$ 4	20 $\pm$ 3	4 $\pm$ 1
NDGA (3 μM)	32 $\pm$ 3	38 $\pm$ 4	23 $\pm$ 1	7 $\pm$ 1

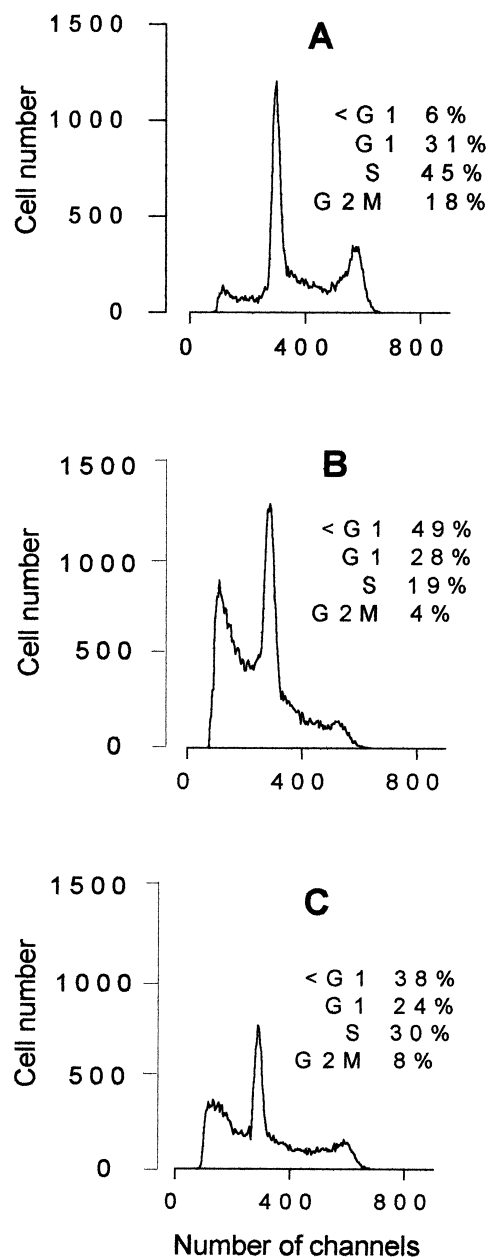


Fig. 3. Distribution of P-388 cells with respect to DNA content in control (A), after 22 h incubation with BPB (3.6 μM) (B) and NDGA (3 μM) (C).

changed the cell survival in a concentration-dependent manner. At low concentrations of the inhibitors (BPB < 0.08 μM , NDGA < 0.3 μM), the cell survival increased compared with untreated control; at higher concentrations, the cell survival decreased proportionally to the log of concentration. BPB was more effective than NDGA: the IC_{50} was 0.6 μM for BPB and 2.2 μM for NDGA. At the same time, indomethacin, which is known to cause a specific blockage of cyclooxygenase at a concentration of 1 μM [16], had no effect on cell survival at concentrations of up to 10 μM : the cell survival was $100 \pm 6\%$ at 1 μM and $98 \pm 3\%$ at 10 μM . Taken together, these data suggest that some optimal level of arachidonic acid and lipooxygenase metabolites is necessary for the growth and viability of tumor cells. It is possible that the level of these metabolites under control conditions was higher than the optimal

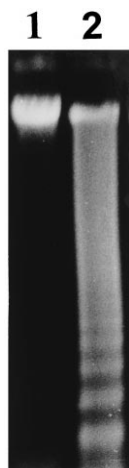


Fig. 4. DNA electrophoretograms of control cells (1) and cells treated for 6-h with NDGA (10 μ M) (2).

level and low doses of inhibitors increased the cell survival. As the inhibitor concentration increased, the level of metabolites dropped below the optimum and cell survival decreased. The decrease in cell survival might be due to both the suppression of cell division and the induction of cell death.

The metaphase index, a criterion of cell proliferation, increased from $1.5 \pm 0.3\%$ in control to $2.6 \pm 0.6\%$ in cells treated with $0.036 \mu\text{M}$ BPB and decreased to $0.6 \pm 0.2\%$ in cells treated with $3.6 \mu\text{M}$ BPB. High inhibitor concentrations also induced cell damage. Fig. 2 shows the effect of BPB and NDGA on the extent of nuclear damage. The percentage of damaged nuclei increased in a concentration-dependent manner. The time dependence was reverse: the nuclear damage after 8 h incubation was higher than after 22 h incubation, which could be explained by lysis of damaged cells.

Fig. 3 shows the distribution of cells with respect to DNA content, as determined by flow cytometry. After treatment with inhibitor, the proportion of cells with subdiploid DNA increased and the proportion of S phase cells decreased. These

changes are presented in Table 1. The data presented in Table 1 imply that the inhibitors blocked the transition from G_1 to S and induced DNA degradation in G_1 . These processes were equal since the portion of G_1 cells was not changed. Fig. 4 shows that DNA fragmentation was internucleosomal, indicating that the lipoxygenase inhibitor initiated apoptosis in P-388 cells. Thus, low concentrations of the inhibitors of PLA₂ (BPB) and lipoxygenase (NDGA) ($0.03\text{--}0.04 \mu\text{M}$ for BPB and $0.1 \mu\text{M}$ for NDGA) stimulate proliferation, and higher concentrations induce apoptosis of tumor cells and block the G_1 -S transition.

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