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INHIBITION OF T CELL MITOGENESIS BY NITROFURANS

CARMEN MERCADO, FÉLIX MOLINA, JUAN NAVAS, CARMEN QUIÑONES and E. H. EYLAR*

Department of Biochemistry, Ponce School of Medicine, Ponce, Puerto Rico 00732

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Abstract—A group of nitrofurans (5-nitro-2-furaldehyde, nifuroxime, nitrofurazone, nitrofurantoin, 5-nitro-2-furoic acid and 2-nitrofuran) were evaluated for inhibition of mitogenesis (DNA synthesis) in human peripheral blood T cells. T cells, either triggered by phorbol myristate acetate (PMA) or in the presence of accessory cells, were activated with a specified mitogen [phytohemagglutin (PHA), concanavalin A (ConA), or anti-CD3] and the amount of tritiated thymidine incorporated into DNA was determined. The results obtained indicate that nitrofurans inhibit mitogenesis irrespective of activator. 5-Nitro-2-furaldehyde was much more inhibitory than the other compounds, while 2-nitrofuran was less inhibitory. When the aldehyde group (5-nitro-2-furaldehyde) was replaced by a carboxyl group (5-nitro-2-furoic acid), the inhibitory activity was also reduced greatly. These results show that while the nitro group alone confers inhibitory activity to the furan ring, the group at the 2 position is crucial. In general, the mitogenic response of purified T cells (lacking accessory cells) triggered by PMA (phorbol ester) was inhibited less than that of the T cell–accessory cell system. With the latter, 50% inhibition of T cell mitogenesis was achieved by nifuroxime, nitrofurazone, and nitrofurantoin at 45–51 and 34–39 μM with PHA and ConA respectively. When purified T cells were used, the values were 71–85 and 55–60 μM respectively. For a given drug concentration, mitogenesis was more inhibited when induced by ConA or anti-CD3 than by PHA. The importance of using a single cell system (purified T cells) was emphasized by the interesting finding that only this system showed enhancement of mitogenesis, up to 35–40% at low drug levels. With the exception of the nitrofuraldehyde, the nitrofurans at strongly inhibitory levels were only moderately cytotoxic, exhibiting 62–85% cell survival after exposure to drug for 68 hr. Our results suggest that nitrofurans inhibit T cell mitogenesis by a relatively non-toxic mechanism; these results are comparable to those obtained for mammalian cells under aerobic conditions.

Nitrofurans exhibit antibiotic activity against a range of organisms as diverse as bacteria, yeasts, and algae [1–4]. These agents have been widely used in clinical and veterinary practice, and for agricultural and industrial purposes. Nitrofurans also possess mutagenic and often carcinogenic properties [5–7]. Studies show that they react with DNA to form complexes, induce interstrand cross-links, and produce alkali-labile lesions [8–11]. Nitrofurans also interfere with nucleic acid synthesis [12, 13]. Strains of *Escherichia coli* impaired in DNA repair functions are more sensitive to nitrofurantoin [14]. In mammalian cells nitrofurantoin reduces and supresses the activity of natural killer cells [15], and inhibits the incorporation of thymidine into DNA of human T cells stimulated with phytohemagglutin (PHA†) [16]. Its use also has been associated with a severe lung disease characterized by an increase in T lymphocytes of the T-helper subset CD-4 [17]. Removal of the nitro group eliminates mutagenic activity as well as effects on cellular immunity [18].

The activation of T cells, which ultimately leads to mitogenesis, involves a complex cascade of reactions affecting the gene expression of more than 70 proteins including interleukin-2 and its receptor [19, 20]. In view of the reports on the sensitivity of nucleic acids to nitrofurans, we thought T cell mitogenesis might provide a good model to study the effects of nitrofurans on gene expression and DNA synthesis. In the present study, we investigated the effects of various 5-nitrofurans on the mitogenic response and viability of purified human peripheral blood T cells, activated by lectins PHA and concanavalin A (ConA) and monoclonal anti-CD3, in the presence of either accessory cells or phorbol ester (PMA). Inhibition of mitogenesis was observed for most nitrofurans studied, irrespective of activator used. This inhibition was not due to cell death because, with the exception of 5-nitro-2-furaldehyde, the nitrofurans showed only modest cytotoxicity at levels that were 90–100% inhibitory for mitogenesis.

MATERIALS AND METHODS

Reagents. Phorbol myristate acetate (PMA), ConA and the myeloperoxidase kit were obtained from the Sigma Chemical Co.; PHA was obtained from Wellcome Laboratories. Mouse monoclonal anti-human CD3 (OKT3) was obtained from the Ortho Corp. Fetal calf serum (FCS) and RPMI medium were obtained from the Hyclone Corp. Lymphokwik reagent was obtained from the One Lambda Corp. and tritiated thymidine from the ICN Corp. Nitrofurans were obtained from the Aldrich and the Sigma Chemical Co., and were used directly or purified by recrystallization.

* Correspondence: E. H. Eylar, Department of Biochemistry, Ponce School of Medicine, PO Box 7004, Ponce, PR 00732.

† Abbreviations: PHA, phytohemagglutin; ConA, concanavalin A; PMA, phorbol myristate acetate; PBS, phosphate-buffered saline; IL-1, interleukin-1; HF cells, mononuclear cell fraction obtained by Ficoll–Hypaque centrifugation containing both T cells and accessory cells; and DMSO, dimethyl sulfoxide.

Preparation of nitrofurans. Solutions of each nitrofuran were prepared in either 5% DMSO or ethanol plus PBS. A stock solution was prepared by dissolving the drug in 0.5 mL of DMSO and 9.5 mL of PBS. These were stored at 4° in the dark. The nitrofuraldehyde was prepared fresh for each experiment. Stock solutions of the other chemicals were prepared monthly.

Preparation of purified T cells. T cells were prepared from heparinized human blood obtained from healthy donors, 20–35 years of age [21]. Twenty milliliters of blood was mixed with 10 mL of PBS; the mononuclear cells were isolated by centrifugation two times over Ficoll–Hypaque and subsequently referred to as HF cells. Approximately $2–3 \times 10^7$ mononuclear cells were obtained at this stage, and were used to prepare purified T cells free from macrophages and B cells as follows. The HF cells (approximately 0.9×10^7) were washed twice in microfuge tubes with PBS and then treated with 0.8 mL Lymphokwik for 55 min at 37° according to the manufacturer's instructions [22]. The solution was then overlayed with 0.2 mL PBS, and centrifuged at 3800 rpm for 2 min in an IEC Centra-4B centrifuge. The dead cells were removed, and the cell pellet was suspended in 5 mL PBS. The cells were washed twice in PBS and suspended in supplemented RPMI medium (with 9% FCS). Typically, T cells were recovered in 45–65% yield. The B cell contamination was evaluated using fluorescein-labeled goat anti-human IgG and fluorescent microscopy as previously described [23]. Macrophage contamination was evaluated by the myeloperoxidase reaction and account for less than 0.1% of the cells in the purified T cell populations and 5–10% in the HF cells (T cell–accessory cell system). Additional evidence for the removal of accessory cells was shown by the mitogenic response using PHA; purified T cells (minus PMA) showed only 0–5% of the response of HF cells. Generally, no B cells were observed in the purified T cell preparations. From 3–6% B cells were observed in the mononuclear (HF) cell preparations.

Mitogenic assay. An aliquot of 50 μL of cells (5×10^4) was placed in RPMI medium containing heat-inactivated fetal calf serum (9% FCS) for a total volume of 0.1 mL and incubated for 62 or 86 hr at 37° over 6% CO_2, using Corning or KF microtiter plates (flat bottom, 96 wells). Cell stimulation was performed with optimal concentrations of PHA (0.25 μg/mL) and ConA (2.5 μg/mL) for activation of human T cells as previously established [24]. In purified T cells, lacking the accessory cells required for activation, PMA (1.25 ng/mL) was added to promote activation. Nitrofurans were added approximately 5 min prior to the mitogen. At 16 hr before harvesting, 0.5 μCi of tritiated thymidine in 25 μL was added and incubated for 86 hr (sometimes 62 hr). The cells were harvested and washed on glasspaper, and the amount of radioactivity was counted in an LKB Betaplate or a Beckman liquid scintillation counter. Each measurement was performed in triplicate and the mean ± SEM determined.

RESULTS

The mitogenic response of HF cells (T cells and accessory cells) to PHA and ConA at various nitrofuran concentrations (concentration–response curve) is shown in Fig. 1 for a typical experiment. The mitogenic response of T cells induced by ConA and PHA was inhibited by the nitrofurans as shown by the decreased incorporation of tritiated thymidine with increasing drug concentration. Whether activated by PHA or ConA, the drug concentration range of inhibition of mitogenesis was quite narrow. All drugs showed 90–100% inhibition at 25 μg/mL, with the exception of 5-nitro-2-furoic acid. No inhibition was observed at concentrations below 2–3 μg/mL. 5-Nitro-2-furoic acid was less inhibitory than the other compounds and at 25 μg/mL caused less than 20% inhibition. The nitrofuraldehyde was clearly most inhibitory. Nitrofurantoin, nitrofurazone and 2-nitrofuran showed similar behavior on activation with either ConA or PHA. The results

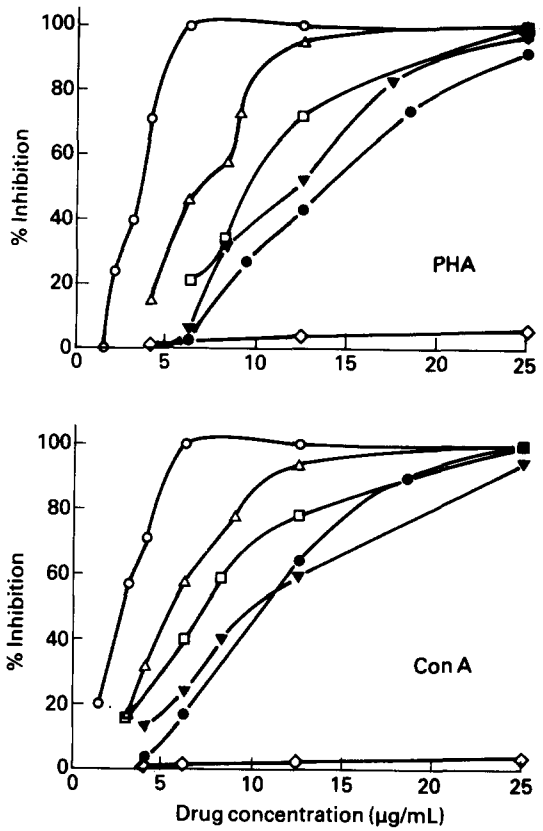

Fig. 1. Inhibition of PHA- and ConA-induced mitogenesis by nitrofurans. The inhibition of T cell mitogenesis with the HF cell preparation is shown for PHA (upper panel) and ConA (lower panel). The mitogenic response is shown in the presence of nitrofuraldehyde (○), nifuroxime (△), nitrofurazone (□), nitrofurantoin (▼), 2-nitrofuran (●), and 5-nitro-2-furoic acid (◇), respectively. PHA (0.25 μg/mL) and ConA (2.5 μg/mL) were used at optimal concentrations under conditions described in Materials and Methods. The percent inhibition refers to the reduction in mitogenic response (incorporation of tritiated thymidine) compared to controls in the absence of drugs. In general, the cpm achieved with PHA and ConA per 5×10^4 cells/well was 80,000–140,000 for PHA and 70,000–90,000 for ConA.

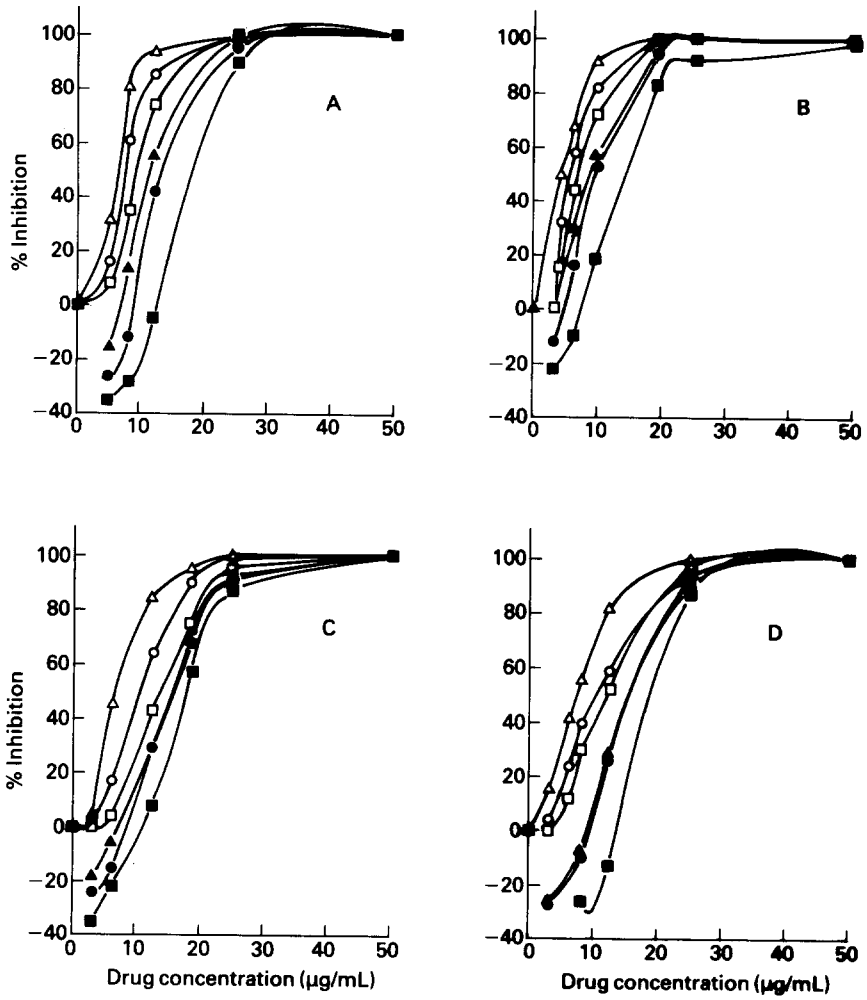

Fig. 2. T cell mitogenesis inhibited by nitrofurans. The inhibition of T cell mitogenesis with the HF cell preparation (open symbols) and the purified T cells (closed symbols) are compared for four drugs: nitrofurazone (A), nifuroxime (B), 2-nitrofuran (C), and nitrofurantoin (D). Mitogenesis was induced with PHA (0.25 μg/mL, squares), ConA (2.5 μg/mL, circles) or OKT3 (25–50 ng/mL, triangles). With purified T cells (5×10^4) having 1.25 ng/mL of PMA, the tritiated thymidine incorporated was generally 50,000–90,000 cpm with each of the activators. With the HF cells (5×10^4), the cpm varied from 60,000 to 120,000 for all three activators. The values below zero percent inhibition refer to percent stimulation over control values. The mitogenic assay was performed as described in Materials and Methods.

were reproducible and were independent of the solvent used to dissolve the drugs (ethanol or DMSO) and the time of incubation (62 or 86 hr).

The mitogenic responses of HF cells and purified T cells to the three activators (PHA, ConA, and OKT3) at various drug concentrations are shown for each drug in Fig. 2. In all cases, the mitogenic response at a given drug concentration was inhibited more for the HF cells than that of the purified T cells with PMA. In other words, the T cells were less inhibited when triggered by PMA than by accessory cells. An outstanding feature of the studies of T cells triggered with PMA was the stimulation observed at low concentrations, sometimes as much as a 35–40% increase, and not found with HF cells (Fig. 2). This finding was consistently observed in the six separate experiments performed with every drug. In all experiments, each drug concentration was evaluated in triplicate; the resultant mean showed a ±SEM of 6–18%. Similarly, stimulation of DNA synthesis was observed at low drug concentrations when mitogenesis was evaluated after 62 hr as well as 86 hr (data not shown).

The degree of mitogenic inhibition at a given drug concentration was greater for ConA and OKT3 activation than for PHA. The response to OKT3 was generally inhibited somewhat more than that for ConA as shown in Fig. 2 and Table 1, where the drug concentrations producing 50% inhibition are tabulated. On a molar basis, the nitrofuraldehyde was most inhibitory and the 2-nitrofuran was 2.5 to 3 times less inhibitory. Nifuroxime, nitrofurazone, and nitrofurantoin showed approximately the same degree of inhibition.

The cytotoxicity of the nitrofurans towards T lymphocytes, as judged by cell killing and uptake of

Table 1. Nitrofuran concentration producing 50% inhibition of mitogenesis

	IC_{50} (μM)					
	HF			Purified T		
Drug	PHA	ConA	OKT3	PHA	ConA	OKT3
5-Nitro-2-furaldehyde	24.8	19.2	14.2	26.2	19.2	14.9
Nitrofurazone	51.0	33.8	27.3	85.8	67.6	60.6
Nitrofurantoin	48.3	39.0	29.4	71.4	63.0	60.0
Nifuroxime	44.8	35.9	24.4	82.0	68.9	54.5
2-Nitrofuran	141	99.1	59.3	157	141	140

The nitrofuran concentration producing 50% inhibition of mitogenesis using HF cells or purified T cells was determined from the data shown in Fig. 2. Induction of mitogenesis was achieved with PHA, ConA or OKT3 as described in Materials and Methods.

Table 2. Cytotoxicity of nitrofurans

Drug	Concn (μg/mL)	Cells surviving after 20 hr	44 hr	68 hr
5-Nitro-2-furaldehyde	6.25	58	40	29
	3.12	75	64	48
Nitrofurazone	25	91	87	78
	12.5	92	87	80
Nifuroxime	25	87	77	62
	12.5	91	83	75
Nitrofurantoin	25	92	84	77
	12.5	95	87	81
2-Nitrofuran	25	92	85	79
5-Nitrofuroic acid	25	93	90	85
Control (no drug)		94	89	86

Nonproliferating HF cells (T cells plus accessory cells) were incubated in 24-well microtiter plates as described in Materials and Methods for various time periods with nitrofurans, but without activators (PHA, ConA), and then counted microscopically in the presence of 0.5% trypan blue. Approximately 100–200 cells per sample were counted. The percent cells surviving refers to the number of live cells (unstained) over the total cells counted times 100. The table shows the average results from four separate experiments. For 5-nitro-2-furaldehyde and 2-nitrofuran, the average result from two separate experiments is shown.

trypan blue, was evaluated and the results are shown in Table 2. At 25 μg/mL, where the inhibition of HF cells was 95–100% and for T cells was 87–100%, the nitrofurans showed only moderate cytotoxicity. Some cytotoxicity was observed with nitrofurazone and nifuroxime at 25 μg/mL after 68 hr, but at 12.5 μg/mL the cytotoxicity was near that of the control. Nitrofurantoin likewise was only mildly cytotoxic, and 5-nitro-2-furoic acid showed no toxicity. The 5-nitro-2-furaldehyde was highly toxic even at 6.25 μg/mL; however, at 3.12 μg/mL in which 57% of mitogenic activity was inhibited, 64% of cells were surviving after 2 days. These results show that the cytotoxicity of the nitrofurans is generally much less than the degree of inhibition of mitogenicity for a given drug concentration.

DISCUSSION

Our results show that the nitrofurans effectively inhibited T cell mitogenesis induced by PHA, Con A or anti-CD3 either in the presence of accessory cells, which are required for activation, or in the absence of accessory cells where activation is promoted by PMA. 5-Nitro-2-furaldehyde was the most inhibitory, while 5-nitro-2-furoic acid was the least. The other four nitrofurans showed similar concentration curves that reached zero inhibition at 1–4 μg/mL and on a molar basis showed 50% inhibition at approximately the same concentration with the exception of 2-nitrofuran which was 2.5 to 3 times less active.

The nitrofurans are of interest because of their antimicrobial, their mutagenic, and in some cases their carcinogenic activity. In comparison with other studies, a concentration–response inhibition of mitogenesis by nitrofurantoin was reported for HF cells that is similar to what we report in this paper for human T cells [16]. It was further shown that nitrofurantoin (at 25 μg/mL) had little effect on protein synthesis or chemotaxis in neutrophils which suggests that DNA may be the major target for nitrofuran action. When compared to nitrofuran studies performed with microorganisms, our results show that the inhibition of mitogenesis in human T cells occurred at drug levels similar to those that

inhibit DNA synthesis and growth in bacteria under aerobic conditions.*

It was consistently found with all nitrofurans studied that the T cells, lacking accessory cells, were more resistant to inhibition than the T cell–accessory cell combination. These results suggest that the participation of macrophage, which is required for T cell activation [25, 26], may also be affected by these compounds. Alternately, the data may reflect a more resistant condition of protein kinase C in the presence of PMA which is absent in the T cell–accessory cell system. Enhancement of the metabolic activation of the drug in the presence of the macrophage is also a possibility. Thus, we can conclude that the T cell can show a different response to nitrofurans in the presence of PMA (purified T cells lacking accessory cells) or accessory cells (the T cell–accessory cell system).

The purified T cell system, at relatively low drug concentrations, showed enhanced mitogenesis rather than inhibition, a phenomenon not observed with the T cell–accessory cell system. We might speculate that at low concentrations the hydrophobic nature of the drugs affects the crucial enzyme protein kinase C in terms of its nonpolar interactions with membrane lipids or PMA [27, 28]. This enzyme binds to PMA and the plasma membrane as an early critical step in the sequence of transducing events [29], and plays a role in receptor protein phosphorylation [30]. Enhanced binding of PMA may enhance activation events with the purified T cells, but not with the HF cells where events are mediated by macrophages.

In general, except for the nitrofuraldehyde, the nitrofurans were only mildly toxic even after prolonged exposure (2–3 days). Both nitrofurazone and nitrofurantoin were only slightly toxic whereas nifuroxime, which showed basically the same degree of inhibition, was definitely more toxic. These results suggest that the inhibitory effect on mitogenesis is not primarily a consequence of cytotoxicity. Our results are in agreement with a study [31] of mouse L929 cells exposed to nitrofuran under aerobic conditions which showed that inhibition of DNA synthesis was not generally associated with cytotoxic effects. Anaerobic conditions enhance cytotoxicity presumably through alkali-labile lesions and DNA strand breaks [32].

In a study of cytotoxicity with Chinese hamster cells (aerobic), a correlation was found between the reduction potential of various nitro compounds and 50% cell survival [33]. In our study, a correlation was also observed between inhibition of the mitogenic response and the half-wave reduction potential of nitrofurans. The aldehyde having the least negative reduction potential was much more inhibitory than the 5-nitro-2-furoic acid with the highest negative reduction potential. Nitrofurantoin, nifuroxime, and nitrofurazone, with very similar reduction potentials [34], were intermediate in inhibitory activity (Table 1).

Consideration of structure–inhibition relationships has shown conclusively that the nitro group imparts to the nitrofurans the major mutagenic, carcinogenic and immunosuppressive activity [18]. The nitro group is also required for biological activity of nitrofurans *in vivo* where nitroreduction and superoxide formation take place in an aerobic environment [35]. However, the 2-nitrofuran itself is 2.5 to 3 times less inhibitory than the nitrofurans having the azomethine linkage at the 2 position (nitrofurantoin, nifuroxime, nitrofurazone) and 6 to 7 times less inhibitory than 5-nitro-2-furaldehyde. Although these compounds are apparently converted into the aldehyde [36], they are much less toxic or inhibitory than the aldehyde itself. Interestingly, a carboxyl group (5-nitro-2-furoic acid) greatly mitigates the activity of the nitro group. This is not due to poor permeability because other studies show that this compound can enhance mitogenesis markedly under appropriate conditions.† Therefore, the inhibition of mitogenic activity by nitrofurans is not the result of cell killing.

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