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4-Nitrobenzofurazans and 4-nitrobenzofuroxans: a new class of thiol-neutralising agents and potent inhibitors of nucleic acid synthesis in leucocytes

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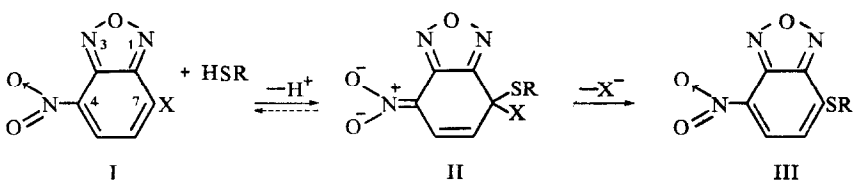
KNOCK[1] has recently drawn attention to the need for new drugs to regulate the activity of nuclear protein thiol groups intimately concerned with gene control and neoplastic proliferation. We have found that certain benz-2,1,3-oxadiazoles (benzofurazans) and their 1-*N*-oxides (benzofuroxans) would seem to offer some promise as potential anti-leukaemic drugs or immunosuppressive agents as judged by the potent effects of 4-nitrobenzofurazan (NBFZ),† 4-nitrobenzofuroxan (NBFX) and some of their derivatives upon leucocyte metabolism *in vitro*.

NBFZ and NBFX are readily prepared by nitrating benzofurazan and benzofuroxan respectively.[2] Benzofurazan (m.p. 53)[3] is obtained from benzofuroxan (m.p. 73)[4] by opening the furoxan ring with hydroxylamine in KOH and steam distilling the dipotassium salt of *o*-benzoquinone dioxime which is formed as an intermediate. The preparation and *in vitro* pharmacological activity of over 60 related compounds will be reported elsewhere.[5]



RSH = Protein—thiol or glutathione

FIG. 1. Postulated drug action of 4-nitrobenzofurazans.

Fig. 1 summarises what we believe to be the mechanism of action of these drugs (for which evidence is provided below and elsewhere[5]). Molecules activated by a 4-nitro group (I) can add nucleophiles such as a mercaptide anion at position 7 forming a Meisenheimer-type complex (II), which may exist transiently (e.g. X = Cl) or may have an appreciable lifetime (e.g. X = SCN), subsequently decomposing to yield I or III. The proportion of unchanged drug (I) and irreversibly-formed end-product

† Abbreviations used: NBFZ = 4-nitrobenzofurazan; NBFX = 4-nitrobenzofuroxan; GSH = glutathione.

(III) will depend not only on the nature of the reactant thiol but also on the nature of the 7-substituent, X. We have found that when X is a hydrogen atom or a good leaving group (chloro, azido, certain thio substituents) the drug action is largely irreversible, i.e. not readily reversed by dilution or by subsequent addition of excess thiols or by washing drug-incubated cells in fresh (drug-free) media.

Although benzofurazans and benzofuroxans possess the non-aromatic *ortho* quinonoid structure, these particular compounds can only neutralise thiols by an addition reaction; whereas *o*-quinones may both add thiols or oxidise them to the corresponding disulphides.[6] The similarities in molecular shape and size between these heterocyclic pseudoquinones and the purines may also possibly contribute to their drug activity, favouring their translocation into the cell nucleus, together with the purines.

EXPERIMENTAL

Details of the procedure used to determine drug action on polymer biosynthesis are reported elsewhere.[7] In principle, washed cells were incubated in a Krebs–Ringer phosphate medium, pH 7·4 containing added glucose (7mM) and heparin (2 I.U./ml) at a concentration of 10–15 $\times$ 10^6 cells/ml, together with drugs added as fresh solutions in *N*,*N*-dimethylformamide (10μl/ml medium) and radioactive compounds, with slow shaking in air at 37° for 30 mins. Radioactivity incorporated into the material insoluble in 8% (w/v) trichloroacetic acid (TCA) was determined after copious washing with 5% TCA and dissolution in hot 10% TCA (95° for 15 mins) or cold 0·5N NaOH.

Location of drug action

4-Nitrobenzofurazan, NBFZ (20μM) in a protein-free medium rapidly inhibited the incorporation of uridine-5-^{3}H into RNA by 85 per cent or more in the following cells: circulating lymphocytes from thoracic duct lymph (rabbit, rat) and popliteal lymph (sheep); tissue-bound lymphocytes teased from the thymus (rat, rabbit), unstimulated lymph nodes (sheep, rabbit), the bursa of Fabricius (chick) and spleen (chicken); plasma cells from swollen antigen-stimulated sheep popliteal lymph nodes; rat myeloid cells (red marrow); mixtures of macrophages and eosinophilic granulocytes (from guinea pig peritoneum); polymorphonuclear leucocytes (from guinea pig peritoneal cavity 5 hrs. after injecting peptone broth); spermatazoa (rat, human) and pig kidney epithelial cells (maintained in tissue culture). Uridine-^{3}H incorporation by the following cells was relatively insensitive (<30 per cent inhibition) to 20μM NBFZ: *Aerobacter aerogenes*, baker's yeast, *Tetrahymena pyriformis*, cultured human amnion ('U' line) cells, human epithelial (HEp-2, Hela) cells and mouse fibroblasts (L-929 line). The incorporation of amino acids-^{14}C and thymidine-6^3-H by lymphocytes was also strongly inhibited by 20μM NBFZ.

This concentration of NBFZ had no effect upon the cell count when lymphocytes (from sheep popliteal lymph or chick bursa) were incubated *in vitro* and did not inibit lactate formation[8] or the incorporation of ^{32}P (from inorganic phosphate -^{32}P) or nucleosides -^{3}H into (i) a mononucleotide fraction soluble in trichloroacetic acid but coprecipitated with adenylic acid by bariumions and ethanol at pH 8·2,[9] and (ii) the acid-soluble organic phosphate fraction remaining after solvent extraction of inorganic phosphate as phosphomolybdic acid.[10] These findings suggest that low concentrations of NBFZ do not primarily inhibit the (extranuclear?) energy-yielding reactions in lymphocytes (glycolysis, oxidative phosphorylation) although at higher concentrations (100μM) NBFZ did depress lactate formation.

ATP synthesis by phosphorylating rat liver mitochondria (oxidising succinate)[11] was 40 per cent inhibited by NBFZ at 20μM but mitochondrial respiration (and the added yeast hexokinase activity) were not affected by 400μM NBZ. The *in vitro* incorporation of inorganic sulphate-^{35}S into mucopolysaccharide sulphates by slices of bovine nasal cartilage, which is sensitive to thiol reagents (e.g. iodoacetate),[12] was 45 per cent inhibited by 20μM NBFZ. The proteolytic digestion of azoalbumen by crystalline papain (pre-activated with cysteine and then freed of activator by gel filtration) which was inhibited by preincubation of the enzyme with 1 mM iodoacetate, was insensitive to 100 μM NBFZ and only 35 per cent inhibited by 1 mM *N*-ethylmalemide. Jackbean urease (determined with Nessler's reagent)[13] was insensitive to less than 1 mM NBFZ, NBFX or *N*-ethylmaleimide but was inhibited by 0·1–1·0 mM 1,2-naphthoquinone.[14] These two latter compounds and ninhydrin inhibit thiol enzymes[6, 14–16] and, like NBFZ and NBFX, they each irreversibly inhibited uridine incorporation by lymphocytes (Table 1). This table also shows that both the 4-nitro group and the oxadiazole ring are required for NBFZ and NBFX to exhibit this type of drug action.

Preincubating the active compounds, listed in Table 1, with glutathione (GSH) for 10 mins in 0·1 M sodium phosphate, pH 7·4 before adding them to the cells, abolished their drug activity. Much greater amounts of GSH (30–50 eqts.) were required to render the 7-thio derivatives of NBFZ and NBFX inactive, by this procedure, than were required to 'neutralise' NBFZ, NBFX, ninhydrin and *N*-ethylmaleimide (needing only 5 eqts. or less of GSH). On preincubating 7-thio derivatives of

TABLE 1. INHIBITION OF URIDINE-5-^{3}H INCORPORATION INTO SHEEP LYMPHOCYTE RNA *in vitro* BY SOME BENZOFURAZANS (BFZ), BENZOFUROXANS (BFX) AND OTHER THIOL-NEUTRALISING AGENTS

Data for calculating the percentage inhibition (±5%) was obtained from triplicate incubations of each drug with cells

Compound (m.p.)	Concn (μM)	Inhib. (%)	Compound	Concn (μM)	Inhib. (%)
4-Nitrobenzofurazan, NBFZ (93–4°)	5	55	5-Nitrobenzofurazan	100	50
* 5-Methyl-NBFZ (83–4°)	50	25	5-Nitrobenzofuroxan	50	65
* 7-Thiocyanato-NBFZ (97–8°)	20	95	4-Nitrobenz-2,1,3-thiadiazole	200	30
* 7-Thiophenyl-NBFZ (156–7°)	20	90	3-Nitrobenz-1,2-quinonedioxime	100	50
* 7-Thiobenzyl-NBFZ (114–5°)	20	85	* 4-Ethoxycarbonyl-BFX (107·5–8°)	400	40
4-Nitrobenzofuroxan, NBFX (142–3°)	5	25	7-Nitro-3-methylanthranil	400	55
	20	90	Ninhydrin	100	80
5-Methyl-NBFX (98–100°)	50	45	1,2-Naphthoquinone	40	80
7-Methyl-NBFX (167–8°)	100	65	*N*-Ethylmaleimide	20	90

* New compound (with correct chemical analysis).

NBFZ with excess GSH, these compounds became noticeably more water-soluble and the 7-thio substituent was rapidly liberated into solution. [The equivalent amount of the appropriate 7-thio substituent, e.g. thiophenol, showed not more than 10 per cent of the drug activity of the corresponding 7-thio NBFZ]. Drug action was also neutralized by preincubation with other thiols e.g. 2-mercaptoethanol, cysteine and its methyl ester, D. L-penicillamine (which were however less effective mole per mole than GSH) but not by preincubation with cystine or alanine methyl ester. All these thiols rapidly formed colourations with colourless solutions of NBFZ at pH 7·4 and NBFZ slowly stained cartilage slices and cell-free lymph a greyish brown. Cartilage slices which had been preincubated for 15 mins with 0·5 mM iodoacetate or 0·2 mM *N*-ethylmaleimide in 0·1 M sodium phosphate, pH 7·4 stained very much less, or not at all, with NBFZ (0·05–0·2 mM) added subsequently, indicating that all three drugs probably react with the same functional group(s) in this tissue.

In summary, NBFZ and NBFX and some of their derivatives are novel drugs which can rapidly inactivate biologically essential thiol groups under neutral conditions at room temperature. The absolute drug activity of these potent compounds can be regulated by introducing appropriate substituents in the molecule: thus the *in vitro* activity is (a) diminished by a substituent at position 5 in the nucleus (which sterically hinders the 4-nitro group and consequently the formation of a coplanar intermediate, II) and (b) can be accentuated by certain lipophilic 7-thiosubstituents or equivalent 'leaving groups'. It remains to be established whether these drugs inhibit nucleic acid and protein biosynthesis in leucocytes by (i) direct reaction with nuclear protein thiols (participating in nuclear oxidative phosphorylation or polymer biosynthesis) or (ii) by neutralising essential non-protein thiols such as glutathione and, amongst other possibilities, thereby potentiating one of the natural inhibitors of cellular proliferation, 'Retine' (a ketoaldehyde normally destroyed by the glutathione-dependent enzyme, glyoxalase) [17]

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Uptake *in vivo* and *in vitro* of actinomycin D by mouse leukemias as factors in survival

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THE ANTIBIOTIC, actinomycin D, has been used in the treatment of animal and human neoplasms.[1] The drug was shown to bind with guanine residues in DNA, leading to inhibition of DNA-dependent RNA polymerase;[1–3] the structure of the DNA-drug complex has been partly elucidated.[4–6] In addition to inhibition of RNA synthesis, the antibiotic has been shown to interfere with other metabolic processes in cells.[7–9] The disposition of actinomycin D in animals has been studied.[10–11] The drug was mainly found in kidney, liver, and spleen; in liver, the drug was concentrated in the nuclear fraction. Resistance to actinomycin D in bacterial [12–15] and mammalian[16–17] cells was found to be related to barriers to drug uptake.

We examined uptake of actinomycin D by six mouse leukemias, which varied in response to the drug from almost complete resistance to "cures". Sources of these tumors and methods of cell isolation have been described.[18, 19]

Data on the effect of actinomycin D on survival of tumor-bearing animals were obtained as follows. Animals were treated with actinomycin D (50 μg/kg) by i.p. injection from day 1 to day 10 after