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GLUTATHIONE POTENTIATES cGMP SYNTHESIS INDUCED BY THE TWO PHENYLFUROXANCARBONITRILE ISOMERS IN RFL-6 CELLS.

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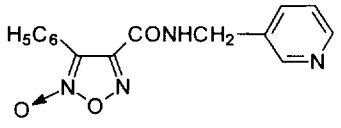
Abstract: The cellular mechanism of bioactivation underlying guanylate cyclase activation by the pair of phenylfuroxancarbonitrile isomers **2a, b** was investigated. In cultured rat lung fibroblast (RFL-6 cells) it was principally dependent on intracellular thiols.

Furoxans (1,2,5-oxadiazole 2-oxide derivatives) are compounds which possess NO-like pharmacological activities.[1] In cell-free systems they are able to activate soluble guanylate cyclase (sGC), in the presence of thiol cofactors.[2,3] This behaviour can be explained by the finding that they can release nitric oxide by reaction with -SH groups. The NO release is not thiol specific. In more complex systems, like cells and tissues, the biotransformation of furoxan to NO has been only scantily studied. With a selected series of furoxancarboxamides, NO release in segments of rabbit femoral artery was found to be not thiol-dependent, and enzymatic NO generation may be involved. Indeed the vasodilator effect of N-(3-pyridylmethyl)-3-phenyl-4-furoxancarboxamide **1** was reduced by pretreatment of the segments with SKF525a (30 μM), a well known cytochrome P-450 inhibitor.[4]

Recently we studied a large number of 3,4-disubstituted furoxans for their NO releasing properties as well as for their vasodilating and antiplatelet activity.[3,5] The pair of phenylfuroxancarbonitrile isomers **2a** and **2b** were among the most active compounds we found in this screening.



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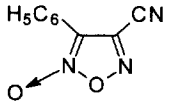
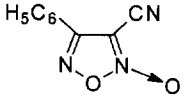
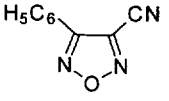


2a



2b



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In particular **2b,** which recently has become a commercial product, attracted our attention for its efficiency as activator of the rat lung sGC in the presence of cysteine, its potency as vasodilator on strips of rat thoracic aorta precontracted with noradrenaline, and as inhibitor of platelet aggregation.[6] For this derivative we suggested possible mechanisms of NO release under the action of thiophenol.

The present study primarily focuses on ability of derivatives **2a** and **2b** to increase the cytosolic levels of cGMP in rat lung fibroblast RFL-6 cells[7] under different experimental conditions: 1) under basal conditions; 2) after raising and lowering the intracellular reduced glutathione (GSH) content by incubating the cells with exogenous GSH and 1-chloro-2,4-dinitrobenzene (CDNB) respectively; 3) after incubating the cells with SKF525a. The levels of cGMP in the cells were also determined in the presence of 4-phenyl-3-furazancarbonitrile **2**, which was synthesised for control purpose, since it is unable to release NO. The major aim of the work was to check the importance of intracellular thiols and enzymatic transformations in RFL-6 cells as far as NO production by selected furoxans is concerned.

Derivative **2b** was synthesised starting from the corresponding oxime by the action of thionyl chloride, according to the procedure we previously described[6]; **2a** was prepared in similar manner (mp 81°C, petroleum ether, lit.[8], 81°C). Derivative **2** was obtained from **2a** or **2b** (0.94 g, 5 mmol) dissolved in trimethylphosphite (5 ml). The reaction solution was refluxed for 3 hours and then poured into cold 2 N hydrochloric acid (50 ml). The white precipitated formed was collected by filtration, washed with water and dried (yield 90%). Mp 42-43°C, methanol/water. Microanalyses, C,N,H, within 0.4% of theoretical values. ^{1}H-, ^{13}C-NMR, IR, mass spectrum in keeping with the structure.

Basal level of cGMP was significantly enhanced in a concentration-dependent manner by incubating RFL-6 cells with derivatives **2a** and **2b** (Fig. 1).[9] Derivative **2b** was more effective than derivative **2a** in stimulating cGMP production. By contrast no significant effect on the basal level of cGMP was found by incubating cells with the furazan analogue **2** (100 μM), which is unable to release NO.

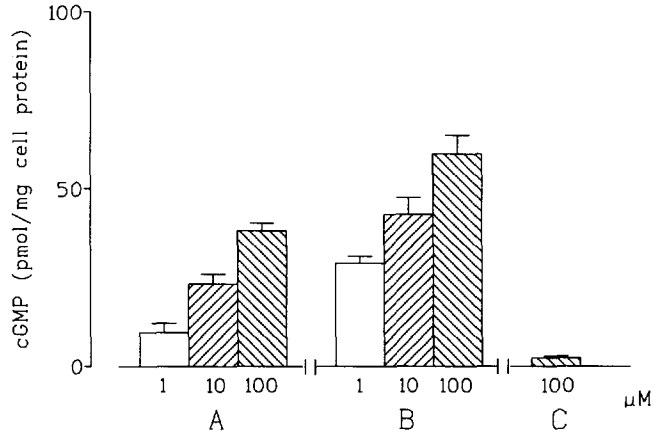

Fig 1 Concentration-dependent increase of intracellular cGMP in RFL-6 cells produced by: A) compound 2a; B) compound 2b; C) compound 2. Basal cGMP level was 1.9 ± 0.7 pmol/mg cell protein. Values are means ± S.E. (n=4).

When RFL-6 cells were incubated for 2 hours with Ham's F12 medium containing 2 mM GSH, the amount of soluble thiols increased by about 50% (Fig. 2 panel a). A similar result has been obtained in bovine[10] and human endothelial cells.[11] On the contrary incubation of cells with CDNB, which forms covalent adduct with GSH lowered the amount of soluble thiols in a concentration-dependent way (Fig. 2 panel b).[12]

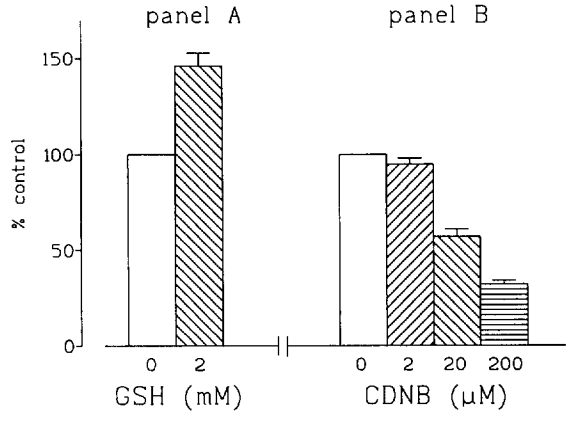

Fig.2 panel a) RFL-6 replenishment with reduced glutathione (GSH). After 2 h incubation in Ham's F12 alone (control assumed as 100%) or Ham's F12 containing 2mM GSH, cells were washed twice and tested for soluble thiol content. Values are means ± S.E. (n=5); panel b) effect of 1-chloro-2,4-dinitrobenzene (CDNB) on intracellular thiol level in RFL-6 cells. After 30 min incubation in Ham's F12 containing CDNB at the indicated concentrations, cells were washed twice and tested for reduced soluble thiol content. Values are means ± S.E. (n=5).

When RFL-6 cells pretreated with CDNB at concentration 2 μM, 20 μM, 200 μM were incubated with **2b** (100 μM), a concentration-dependent decrease in cGMP level was observed (Fig. 3).

A similar situation occurred with **2a** (Fig. 4). cGMP synthesis induced by **2b** and **2a** (100 μM) was increased when the experiments were performed with RFL-6 cells thiol-loaded by a pretreatment with 2mM GSH (Fig. 3, 4).

The biotransformation of the pair of phenylfuroxancarbonitrile isomers responsible for the increase of cGMP is not cytochrome P-450-dependent. In fact treatment of the cells with SKF525a and then with **2b** and **2a** (100 μM) did not cause any significant variation in the increase of intracellular levels of cGMP induced by these compounds when used alone (Fig. 3,4).[13]

In conclusion this work demonstrates that bioactivation of the two phenylfuroxancarbonitrile isomers in RFL-6 cells is principally due to their interaction with intracellular sulfhydryl groups and, differently from other NO donors like glyceryl trinitrate [14], is independent from cytochrome P-450 activity. So far, the intracellular GSH concentration regulates the amount of NO released.

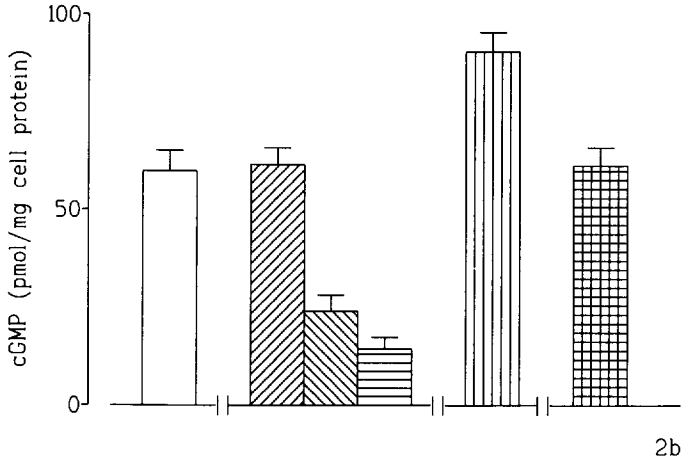

Fig.3. Effect on guanylate cyclase activation induced by compound 2b 100μM alone (□) and after treatment with: CDNB 2μM (▨), CDNB 20 μM (▧), CDNB 200 μM (▤), GSH 2mM (▥), SKF 525a 100μM (▦). Values are means ± S.E. (n=4).

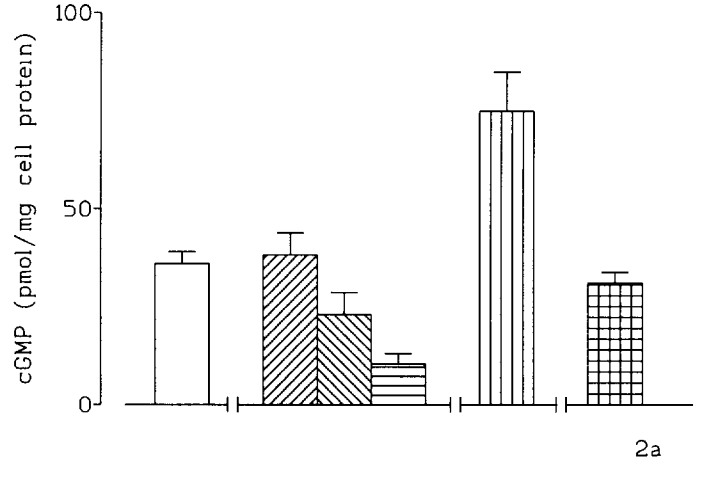

Fig.4. Effect on guanylate cyclase activation induced by compound 2a 100μM alone (□) and after treatment with: CDNB 2μM (▨), CDNB 20 μM (▧), CDNB 200 μM (▤), GSH 2mM (▥), SKF 525a 100μM (▦). Values are means ± S.E. (n=4).

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7. *Cell culture*. RFL-6 cell (a rat lung fibroblasts cell line) were maintained in Ham's F12 medium, supplemented with 20% fetal calf serum. The cells were grown in a humidified incubator at 37°C and 5% CO_2.

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9. *cGMP determination*. Cells grown to confluence in 35-mm diameter Petri dishes were washed twice with a balanced salt solution (PBS: NaCl 150 mM + Na_2HPO_4 129 mM, buffered to pH 7.4) and then incubated for 30 min at 37°C in 1 ml of Ham's F-12 medium containing 0.5 mM isobuthylmethylxantine (IBMX). After this period the supernatant was aspirated and dishes were washed twice with PBS. Derivatives under study dissolved in DMSO were diluted in Hepes-buffer (Hepes buffer: NaCl 145 mM, KCl 5 mM, $MgSO_4$ 1 mM, $CaCl_2$ 1 mM, Hepes-Na 10 mM, glucose 10 mM pH 7.4) at different concentrations and immediately added to the dishes. Incubation was stopped after 15 minutes by aspirating the supernatant and adding 300 µl of ethanol. When ethanol was evaporated, 0.4 ml of Tris-EDTA buffer (Tris-HCl 50 mM + EDTA 4 mM, pH 7.5) were added. After 10 minutes 100 µl supernantant were tested for the level of cGMP by immunoassay with a cGMP [^{3}H]assay system (Amersham, Bucks, UK).

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12. *CDNB-mediated thiol depletion. GSH loading and intracellular thiol measurement.* Cell monolayers in 35-mm diameter Petri dishes were incubated at 37°C for 30 minutes with Ham's F 12 medium containing IBMX (0.5 mM) and either 1-chloro-2,4-dinitrobenzene (CDNB) or ethanol as control. After this period cells were washed twice with PBS buffer and tested for cGMP or thiol content. GSH loading was performed by incubating cells in 35-mm diameter Petri dishes for 90 minutes with Ham's F 12 medium containing 2 mM GSH . After this time period IBMX 0.5 was added to the same solution and incubation was prosecuted for 30 minutes more. To extract glutathione, ethanol (300 µl) was added to washed monolayers and, after solvent evaporation, 1 ml of PBS-EDTA-ARG buffer (NaCl 150 mM, Na_2HPO_4 129 mM, EDTA 4 mM, L-Arg 5 mM, pH 7.4) was added. The intracellular level of soluble thiols was measured in the extracts by the 5,5'-dithiobis(dinitrobenzoic acid) (DTNB) colorimetric method.[15]

13. *cGMP determination in the presence of SKF525a.* Part of the cells pretreated with IBMX and washed with PBS were exposed for 10 minutes to 100 µM SKF525a diluted in PBS. After this time period, furoxan derivatives were added to the same balanced salt solution and the experiment was performed as previously described for cGMP determination.

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