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In vitro inhibition of human erythrocyte glutathione reductase by some new organic nitrates

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ABSTRACT

Glutathione reductase (GR), is responsible for the existence of GSH molecule, a crucial antioxidant against oxidative stress reagents. The antimalarial activities of some redox active compounds are attributed to their inhibition of antioxidant flavoenzyme glutathione reductase, and inhibitors are therefore expected to be useful for the treatment of malaria. Twelve organic nitrate derivatives were synthesized and treated with human erythrocyte GR. The molecules were identified as strong GR inhibitors and novel antimalaria candidates.

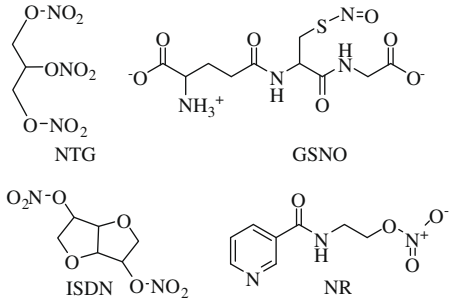
For many years, Nitroglycerin (NTG) and other organic nitrates have been the mainstay of cardiovascular therapy[1] and of particular benefit in the treatment of diangina pectoris,[2] unstable angina[3] and the early stages of acute myocardial infarction.[4] They have shown real efficacy in coronary atherosclerosis, hypercholesterolemia or other blood vessel wall disorders involving endothelial dysfunction[5,6] and reduced vasodilator capacity of the coronary arteries[7] (Fig. 1).

Glutathione reductase (GR; NADPH: oxidized glutathione oxidoreductase, EC 1.6.4.2), a flavoprotein, is an important enzyme which catalyzes the conversion of oxidized glutathione into the reduced form. GR enables several vital functions of the cell, such as the detoxification of free radicals and reactive oxygen species as well as protein and DNA biosynthesis, by maintaining a high ratio of GSH/GSSG.[8,9] It is a target enzyme for antimalarial and antitumor drugs,[10] and studies on the enzyme are therefore important for drug development.[10c]

One may expect an increase in the antiplasmodial activity of nitroaromatic and quinoidal compounds with their redox potential. However, the antimalarial activity of some redox active compounds, such as 10-arylizoalloxazines[11] and methylene blue,[12] was also attributed to their inhibition of antioxidant flavoenzyme glutathione reductase, which catalyzes the reduction of glutathione disulfide (GSSG) at the expense of NADPH. It is assumed that both human erythrocyte and *Plasmodium falciparum* GR play important roles for the intraerythrocyte growth of parasites, protecting them from oxidative stress.[10c,11,12] Since nitroaromatic and quinoidal compounds may efficiently inhibit GR from various sources,[13–15] it is necessary to assess the relative importance of redox cycling and GR inhibition in their antiplasmodial activity.[10c,14–18]

In our work, toward the discovery of novel GR inhibitors, we synthesized novel organic nitrate derivatives (Fig. 3). Compounds were evaluated for their ability to inhibit human erythrocyte GR. Inhibition is reported as IC_{50} and K_i (μM) and the results are the average of at least three independent experiments (Fig. 2).

The rationale of investigating nitro compounds as GR inhibitors is due to the fact that the simple molecules have been shown to be inhibitor of human GR.[12a] Grellier et al. showed the antiplasmodial activity of a series of homologous nitroaromatic compounds,[19]



NTG: Nitroglycerin, **GSNO:** S-Nitrosoglutathione, **ISDN:** Isosorbide dinitrate, **NR:** Nicorandil

Figure 1. Structure of some organic nitrates.

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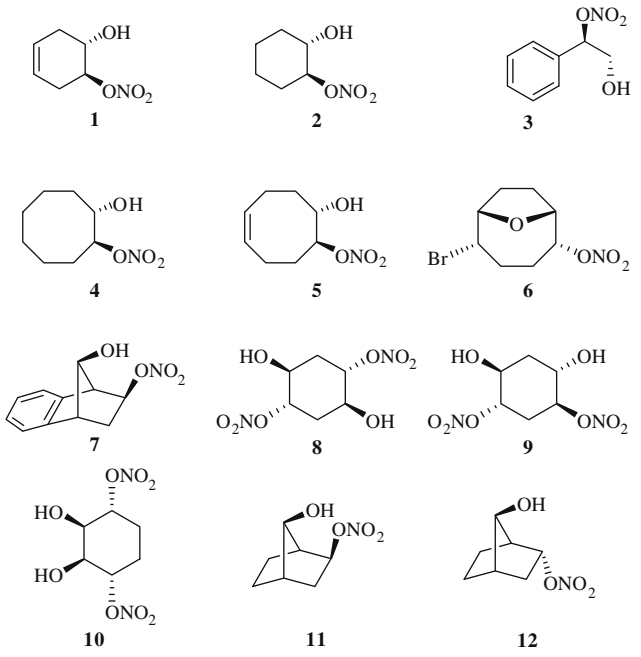

Figure 2. Structure of the investigated molecules.

which were either strong or weak inhibitors of erythrocyte GR. For this purpose, that study selected the derivatives of 2-(59-nitrofuryl-vinyl)-quinoline-4-carbonic acid, which possess a broad spectrum of bactericidal and antiparasitic activities,[20] and inhibit yeast GR[14a] and *Trypanosoma congolense* trypanothione reductase.[14c]

The purification of human erythrocyte GR was performed using simple two-step method by 2′,5′-ADP Sepharose-4B affinity gel chromatography and Sephadex G-200 gel filtration chromatography.[21,22] Human erythrocyte glutathione reductase was purified, 2633-fold with a specific activity of 26.33 EU mg^{-1} and overall yield of 32..[21–26] β-hydroxy nitrates were synthesized using ring-opening reaction of epoxides with $Bi(NO_3)_3{\cdot}5H_2O$, which was used both as catalyst and reagent.[27] Some of the nitrate derivatives have previously been synthesized by other groups as well.[28] Inhibitory effects of organic nitrates on the enzyme activity were tested under in vitro conditions; K_i values were calculated from Lineweaver–Burk graphs[29] and given in Table 1.

We report here the first study on the inhibitory effects of the organic nitrates **1–12** on human erythrocyte GR. The previous reports by Becker et al.[11c,12] investigated other nitro derivatives (including arylizoalloxazines) by using Beutler's method, monitoring GR inhibition.[30] Data of Table 1 show inhibition of hGR by compounds **1–12**.

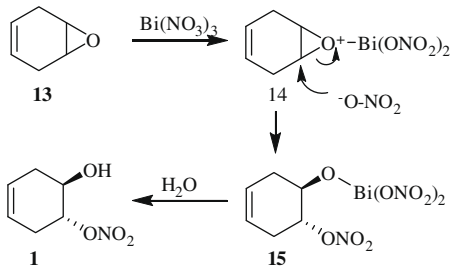

Figure 3. Preparation of organic nitrates.

Compounds **1–12** behaved as strong inhibitors for GR, with K_i values in the range of 11.7–44.3 μM. *trans*-(1*S*(*R*),8*S*(*R*),*Z*)-8-hydroxycyclooct-4-enyl nitrate **5** was relatively ineffective GR inhibitor (K_I: 44.3 μM), similarly to the structurally related compounds **1** and **2** (K_I: 43.7 μM). A second group of derivatives, including **3, 4** and **6–12**, showed stronger inhibitory activity as compared to the previously mentioned organic nitrates, with K_I values of 11.7–25.6 μM, (Table 1). Thus, the nature of the groups in *ortho*- and *para*- to the cyclic OH moiety strongly influences GR inhibitory activity of the molecules. It is also interesting to note that hydroxybicyclo [2.2.1]heptan-2-yl nitrate derivatives **11–12** were much better hGR inhibitors as compared to the corresponding *trans*-(1*S*(*R*),2*S*(*R*))-2-hydroxycyclohexyl nitrate (**2**) and *trans*-(1*S*(*R*),8*S*(*R*),*Z*)-8-hydroxycyclooct-4-enyl nitrate (**5**) from which they were prepared. Kinetic investigations (Lineweaver–Burke plots, data not shown) indicate that similarly to some antibiotics and analgesic drugs,[22–24] all the investigated organic nitrates act as noncompetitive inhibitors. These results suggest that protein sulphydryl groups are the target for inhibition by organic nitrates. The differences in the kinetics of inactivation of trypanothione reductase and glutathione reductase could reflect differences in the crystal structures of the disulphide-binding sites of the enzymes. The active site of trypanothione reductase is wider and possesses a hydrophobic and negatively charged region[31] that accommodates the spermidine moiety of its substrate,[32] whereas that of glutathione reductase is much narrower and contains a positively charged and hydrophilic region[33] that interacts with the glycine carboxylates of glutathione disulphide.[34] Molecular modelling studies show that in glutathione reductase, the disulphide-binding site is narrower, mainly due to Arg-A330, Arg-A20 and Asn-A100, whereas in trypanothione reductase, residues that are found at equivalent positions are hydrophobic and generally smaller. In both pockets, there is a His near to the phenyl ring of the drug which could serve to stabilise this aromatic moiety, while a Tyr close to the melaminyl ring may form electrostatic interactions with the considerable number of nitrogens. These additional noncovalent

Table 1
K_i and IC_{50} values obtained from regression analysis graphs for GR in the presence of different inhibitors concentrations

Inhibitor	IC_{50} value (μM)	K_i value (μM)	Inhibition type
trans-(1*S*(*R*),6*S*(*R*))-6-Hydroxycyclohex-3-enyl nitrate (**1**)	23	35.1	Noncompetitive
trans-(1*S*(*R*),2*S*(*R*))-2-Hydroxycyclohexyl nitrate (**2**):	21	43.7	Noncompetitive
trans-(*R*(*S*))-2-Hydroxy-1-phenylethyl nitrate (**3**):	16	25.6	Noncompetitive
trans-(1*S*(*R*),2*S*(*R*))-2-Hydroxycyclooctyl nitrate **(4):**	14.2	21.9	Noncompetitive
trans-(1*S*(*R*),8*S*(*R*),*Z*)-8-Hydroxycyclooct-4-enyl nitrate (**5**)	29	44.3	Noncompetitive
(1*S*(*R*),2*S*(*R*),5*R*(*S*),6*R*(*S*))-5-Bromo-9-oxabicyclo[4.2.1] nonan-2-yl nitrate (**6**)	11	21.5	Noncompetitive
9(*R*(*S*))-Hydroxy-1,2,3,4-tetrahydro-1,4-methano-naphthalen-2(*R*(*S*)-yl nitrate (**7**)	10.3	18.8	Noncompetitive
(1*R*(*S*),2*R*(*S*),4*R*(*S*),5*R*(*S*))-2,5-Dihydroxycyclo-hexane-1,4-diyl dinitrate (**8**)	8.71	18.4	Noncompetitive
(1*S*(*R*),3*S*(*R*),4*S*(*R*),6*S*(*R*))-4,6-Dihydroxycyclo-hexane-1,3-diyl dinitrate (**9**)	8.1	17.9	Noncompetitive
(1*R*(*S*),2*R*(*S*),3*S*(*R*),4*S*(*R*))-2,3-Dihydroxycyclo-hexane-1,4-diyl dinitrate (**10**)	7.17	17.4	Noncompetitive
(2*S*(*R*),7*R*(*S*))-7-Hydroxybicyclo[2.2.1] heptan-2-yl nitrate (**11**)	7.13	13.1	Noncompetitive
(2*R*(*S*),7*R*(*S*))-7-Hydroxybicyclo[2.2.1]heptan-2-yl nitrate (**12**)	6.81	11.7	Noncompetitive

interactions could thus serve to stabilise the initial monothioarsane enzyme inhibitor complex.[33–35]

Cunningham et al. demonstrated relatively weaker inhibitory activity of phenylarsenoxide and arsenite against both enzymes in their study.[35] However, non-covalent interactions per se are insufficient to allow inhibition by analogues such as sodium melarsen or *p*-[(4,6-diamino-*s*-triazin-2-yl)amino] benzoic acid ethyl ester that lack the trivalent arsenic atom. The most striking difference between glutathione reductase and trypanothione reductase is the 13-fold lower K_i involving the time-dependent rearrangement to form the dithioarsane adduct.[35] Comparison of the models of trypanothione reductase and glutathione reductase with the arsenical bound to the disulphide exchange thiol would suggest that the charge-transfer thiol is indeed less accessible in glutathione reductase due to the narrower active-site cleft.[35]

As mentioned above, the active site of GR is narrow and positively charged, and it might therefore be inhibited because of the interaction with the hydroxyl groups on organic nitrates. Contrarily, the active site of typanothion reductase is wider and negatively charged. This may allow organic nitrates to bind this enzyme with positively charged nitrogen atoms in a wider region, and to inhibit typanothion reductase at lower concentrations. Therefore, we propose that the organic nitrates used in this study, which are also cyclitol derivatives, may be potential antimalaria drugs.

Organic nitrates **1–12** used in this study affect the activity of human erythrocyte GR due to the presence of the different functional groups (–OH, $–ONO_2$ and –Br) present in their mono and bicyclic scaffold. Our findings here indicate thus another class of possible GR inhibitors of interest, in addition to the well-known chloroquine and aminoquinoline derivatives bearing bulky in their molecules. Indeed, some new organic nitrates investigated here showed effective hGR inhibitory activity, in the low micromolar range, by the Beutler's method[30] which usually gives K_I-s an order of magnitude higher. These findings point out that substituted nitro compounds may be used as leads for generating potent GR inhibitors. This approach may also be useful in the design and exploitation of trypanocidal quinones and nitroaromatics.

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trans-(1S(R),2S(R))-2-Hydroxycyclohexyl nitrate (**2**): The product **2** was obtained from cyclohexenepoxide (1.00 g, 10.21 mmol) and $Bi(NO_3)_3{\cdot}5H_2O$ (4.95 g, 10.21 mmol) as described above by typical procedure in 5 min. The residue was submitted to column chromatography (silica gel, 30 g) eluting with ethyl acetate/hexane (10:90). While the first elution gave the unidentified product(s) (500 mg), the last eluate provided the product **2** as colourless oil (1.04 g, 63%). ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.77 (ddd, J = 13.6, 9.1, 4.6 Hz, $CHONO_2$, 1H), 3.61 (ddd, J = 13.6, 9.1, 4.6 Hz, CHOH, 1H), 2.89–2.84 (m, OH, 1H), 2.20–2.10 (m, CH_2, 2H), 1.80–1.50 (m, CH_2, 2H), 1.49–1.20 (m, CH_2, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 89.21, 72.41, 35.06, 30.70, 25.73, 25.43; IR (CH_2Cl_2, cm^{-1}) 3573, 3400, 3039, 2908, 2854, 1631, 1557, 1440, 1348, 1314, 1277, 1213, 1101, 1071, 1040, 996, 973, 867, 755. Anal. Calcd for $C_6H_{11}NO_4$: C, 44.72; H, 6.88; N, 8.69. Found: C, 44.91; H, 6.58; N, 8.60.
trans-(R(S))-2-Hydroxy-1-phenylethyl nitrate (**3**): The product **3** (colourless oil, 520 mg, 98%) was prepared as described above by typical procedure for 20 min by starting Styrene epoxide (350 mg, 2.89 mmol) and $Bi(NO_3)_3{\cdot}5H_2O$ (1.40 g, 2.89 mmol). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40–7.36, (m, aryl, 5H), 5.93 (dd, J = 8.4, 3.9 Hz, $CHONO_2$, 1H), 3.97 (dd, J = 12.6, 8.4 Hz, A part of AB system, CH_2OH, 1H), 3.86 (dd, J = 12.6, 3.9 Hz, A part of AB system, CH_2OH, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 134.73, 129.68, 129.23, 126.88, 86.16, 64.18; IR (CH_2Cl_2, cm^{-1}) 3451, 3064, 3032, 2926, 1727, 1706, 1632, 1603, 1575, 1496, 1454, 1413, 1374, 1277, 1255, 1135, 1072, 1027, 753. Anal. Calcd for $C_8H_9NO_4$: C, 52.46; H, 4.95; N, 7.65. Found: C, 52.80; H, 4.99; N, 7.77.
trans-(1S(R),2S(R))-2-Hydroxycyclooctyl nitrate (**4**): The the mixture of cyclooctene epoxide (300 mg, 2.38 mmol) and $Bi(NO_3)_3{\cdot}5H_2O$ (1.16 g, 2.38 mmol) in 3 mL of CH_2Cl_2 was refluxed for 16 h. After the filtration, the residue was submitted to column chromatography (silica gel, 30 g) eluting with ethyl acetate/hexane (10:90). The elution gave the product **4** as colourless oil (380 mg, 84%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.08 (ddd, J = 16.1, 8.5, 2.6 Hz, $CHONO_2$, 1H), 3.86 (ddd, J = 16.1, 8.5, 2.6 Hz, CHOH, 1H), 2.31 (m, OH, 1H), 1.96–1.41 (m, CH_2, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 89.94, 71.49, 30.33, 29.22, 26.16, 25.56, 25.22, 22.73; IR (CH_2Cl_2, cm^{-1}) 3575, 3400, 2930, 2861, 1623, 1467, 1449, 1362, 1280, 1213, 1149, 1115, 1089, 1053, 1012, 994, 866, 757. Anal. Calcd for $C_8H_{15}NO_4$: C, 50.78; H, 7.99; N, 7.40. Found: C, 50.47; H, 8.10, 7.45.
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