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# Effect of soluble guanylyl cyclase activator and stimulator therapy on nitroglycerin-induced nitrate tolerance in rats

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ABSTRACT

Chronic nitroglycerin (GTN) anti-ischemic therapy induces side effects such as nitrate tolerance and endothelial dysfunction. Both phenomena could be based on a desensitization/oxidation of the soluble guanylyl cyclase (sGC). Therefore, the present study aims at investigating the effects of the therapy with the sGC activator BAY 60-2770 and the sGC stimulator BAY 41-8543 on side effects induced by chronic nitroglycerin treatment. Male Wistar rats were treated with nitroglycerin (100 mg/kg/d for 3.5 days, s.c. in ethanol) and BAY 60-2770 (0.5 or 2.5 mg/kg/d) or BAY 41-8543 (1 and 5 mg/kg/d) for 6 days. Therapy with BAY 60-2770 but not with BAY 41-8543 improved nitroglycerin-triggered endothelial dysfunction and nitrate tolerance, corrected the decrease in aortic nitric oxide levels, improved the cGMP dependent activation of protein kinase I in aortic tissue and reduced vascular, cardiac and whole blood oxidative stress (fluorescence and chemiluminescence assays; 3-nitrotyrosine staining). In contrast to BAY 41-8543, the vasodilator potency of BAY 60-2770 was not impaired in isolated aortic ring segments from nitrate tolerant rats. sGC activator therapy improves partially the adverse effects of nitroglycerin therapy whereas sGC stimulation has only minor beneficial effects pointing to a nitroglycerin-dependent sGC oxidation/inactivation mechanism contributing to nitrate tolerance.

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## 1. Introduction

Organic nitrates are still frequently used for the treatment of patients with angina pectoris and acute and chronic heart failure and are recommended by the guidelines for the treatment of patients with stable coronary artery disease being still symptomatic despite treatment with aspirin, beta-receptor blocker, ACE-inhibitors/AT1-receptor blockers and statins [1]. Organic nitrates dilate large coronary arteries, improve collateral blood flow and dilate capacitance vessels with a neglectable effect on peripheral arteriolar tone. Nitrates also display anti-aggregatory effects via increasing cGMP in platelets (for review see [2,3]). Despite these anti-ischemic effects when given acutely, the long-term efficacy of organic nitrates is limited by serious side effects such as the development of nitrate tolerance and endothelial dysfunction [2,3]. The mechanisms underlying these adverse effects are likely multifactorial and may involve phenomena such as pseudo-tolerance (neurohormonal counter-regulatory responses) and true vascular

*Abbreviations:* ACh, acetylcholine; ALDH-2, mitochondrial aldehyde dehydrogenase; cGK-I, cGMP-dependent protein kinase I; DHE, dihydroethidine; DHFR, dihydrofolate reductase; DMSO, dimethylsulfoxide; ECL, enhanced chemiluminescence; eNOS, endothelial nitric oxide synthase (type 3); GCH-1, GTP-cyclohydrolase-1; GTN, glyceryl trinitrate (nitroglycerin); HO-1, heme oxygenase-1; L-012, 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt; Nox, catalytic subunit of NADPH oxidase; P-VASP, vasodilator stimulated phosphoprotein (phosphorylated at Ser239); qRT-PCR, quantitative reverse transcription polymerase chain reaction; ROS, reactive oxygen species; $sGC_{\alpha1/\beta1}$, $\alpha1/\beta1$ subunit of soluble guanylyl cyclase; BAY 60-2770, sGC activator; BAY 41-8543, sGC stimulator; 3NT, 3-nitrotyrosine.

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tolerance (inhibition of organic nitrate bioactivation and downstream signaling), all of which will limit the vasodilator capacity of NO (or a related compound) released by the organic nitrate. For nitroglycerin (GTN) treatment both phenomena have been described to be secondary to the induction of oxidative stress within the vasculature [4–13], involving the formation of reactive oxygen species (ROS) and peroxynitrite within mitochondria [11,14], the cross-talk between ROS from mitochondria and NADPH oxidases [4,12], eNOS uncoupling [15,16] and oxidative impairment of the GTN bioactivating enzyme mitochondrial aldehyde dehydrogenase (ALDH-2) [17–19]. Interestingly, already in 1987 Ferid Murad's group proposed that due to the observation of tolerance and cross-tolerance to other nitrovasodilators, one explanation for this phenomenon may be a desensitization of the target enzyme sGC [20]. More recently, we have shown that chronic GTN treatment affects the expression and activity of soluble guanylyl cyclase (sGC) in vivo [21,22]. We demonstrated significant and comparable increases in the expression of the sGC subunits $sGC_{\alpha 1}$ and $sGC_{\beta 1}$ protein and mRNA in nitrate tolerant rats and rabbits [22]. While the expression of the cGMP-dependent protein kinase I (cGK-I) was not modified, we observed a strong reduction in vasodilator-stimulated phosphoprotein (VASP) serine239 phosphorylation (specific substrate of cGK-I) compatible with an inhibition of the activity of cGK-I. In vivo and in vitro treatment with vitamin C improved tolerance, reduced oxidative stress, normalized sGC expression and clearly improved cGK-I activity suggesting that increased expression of sGC in the setting of tolerance rather reflects a counter-regulatory mechanism due to chronic inhibition/desensitization of the sGC-cGK-I pathway by oxidative stress in response to GTN therapy. Peroxynitrite and superoxide, the major oxidants induced by acute and chronic GTN treatment, have been demonstrated to be potent inhibitors of the activity of the sGC [23,24]. Based on these considerations we hypothesized that two new groups of sGC targeting drugs may be able to reverse or when given concomitantly to prevent the development of tolerance but also endothelial dysfunction under chronic GTN treatment.

sGC stimulators are compounds that enhance the activation of sGC by its endogenous activator nitric oxide and sGC activators are compounds that cause sGC-dependent cGMP formation even, when the enzyme is oxidized or heme-depleted [25,26]. Several sGC activators (e.g. Cinaciguat™ (BAY 58-2667)) and stimulators (e.g. Riociguat™ (BAY 63-2521)) are so far approved for clinical use and the treatment of different forms of pulmonary hypertension and heart failure [27,28]. For detailed overview on the current clinical and pharmacological potential of sGC stimulators and activators we refer to the detailed recent review articles by Stasch and coworkers [29–31]. With the present studies we could demonstrate that the sGC activator BAY 60-2770 was able to significantly prevent tolerance or development of endothelial dysfunction mainly by reducing oxidative stress in vascular tissue, while the sGC stimulator BAY 41-8543 was virtually ineffective. These findings suggest that oxidation or removal of the heme group contributes at least in part to the tolerance/cross-tolerance phenomenon in response to chronic GTN therapy and also indicates the rational for a combination therapy with GTN and sGC activators in order to prevent tolerance and cross-tolerance.

## 2. Materials and methods

### 2.1. Materials

For isometric tension studies, GTN was used from a Nitrolingual infusion solution (1 mg/ml) from G. Pohl-Boskamp (Hohenlockstedt, Germany). For induction of nitrate tolerance, GTN was used from an ethanol solution (100 g/l) from Novasep (Leverkusen, Germany). sGC stimulator (BAY 41-8543) and sGC activator (Bay 60-2770) were a kind gift from Bayer Pharma AG (Wuppertal, Germany). L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt) was from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals including dihydroethidine (DHE) were of analytical grade and were obtained from Sigma-Aldrich, Fluka or Merck.

### 2.2. Animals and in vivo treatment

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the EU Directive 2010/63/EU and approval was granted by the Ethics Committee of the University Hospital Mainz and the Landesuntersuchungsamt Rheinland-Pfalz (Koblenz, Germany; permit number: 23 177-07/G 12-1-084). A total number of 48 male Wistar rats (6 weeks old, 300 g, Charles River Laboratories, Sulzfeld, Germany) were examined. Nitrate tolerance in male Wistar rats was induced by twice daily s.c. injection of ethanolic GTN solution (100 mg/kg/d for 3.5 days) [15]. Ethanol injections (similar volume as for ethanolic GTN solution) served as solvent control. Drugs were administrated as follows: BAY 60-2770 in DMSO (0.5 or 2.5 mg/kg/d via osmotic minipumps (Alzet model 2001) and *s.c.* infusion, 1 μl/h) and BAY 41-8543 in DMSO (1 and 5 mg/kg/d via osmotic minipumps (Alzet model 2ML1) and *s.c.* infusion, 10 μl/h) were administrated for 6 days (therapy was started 2.5 days prior to GTN treatment). Infusion of the solvent infusion (DMSO) served as control. On the day of the experiment the animals were killed under isoflurane anesthesia by transection of the diaphragm and removal of the heart and aorta.

### 2.3. Isometric tension studies

Vasodilator responses to ACh and GTN as well as to BAY 60-2770 and BAY 41-8543 were assessed with endothelium-intact isolated rat aortic rings mounted for isometric tension recordings in organ chambers, preconstricted with phenylephrine, as described previously [12,19].

### 2.4. Western blot analysis in aorta

Isolated aortic tissue was frozen and homogenized in liquid nitrogen. Proteins were separated by SDS-Page and blotted onto nitrocellulose membranes. After blocking, immunoblotting was performed with the following antibodies: monoclonal mouse α-actinin (100 kDa) as a control for loading and transfer, polyclonal rabbit sGCα1 (1:10,000, Abcam, UK), polyclonal rabbit sGCβ1 (1:500, Abcam, UK), monoclonal mouse vasodilator stimulated phosphoprotein (VASP) phosphorylated on serine239 (P-VASP, clone 16C2, 1.5 μg/ml, Calbiochem, UK), polyclonal goat cGMP-dependent protein kinase (cGK-I, 1:200, Santa Cruz Biotechnologies, USA), polyclonal rabbit ALDH-2 (1:2000, kindly provided by K.K. Ho and H. Weiner, Purdue University, West Lafayette, USA), monoclonal mouse eNOS (1:1000, BD Biosciences, USA), monoclonal mouse DHFR (1 μg/ml, Abnova Corp., Germany), monoclonal mouse GCH-1 (1 μg/ml, Abnova Corp., Germany), and monoclonal mouse HO-1 (4 μg/ml, Stressgen, San Diego, CA). Detection and quantification were performed by ECL with peroxidase conjugated anti-rabbit/mouse (1:10,000, Vector Lab., Burlingame, CA) and anti-goat (1:5000, Santa Cruz Biotechnologies, USA) secondary antibodies. Densitometric quantification of antibody-specific bands was performed with a ChemiLux Imager (CsX-1400 M, Intas, Göttingen, Germany) and Gel-Pro Analyzer software (Media Cybernetics, Bethesda, MD).

### 2.5. Nitric oxide measurement by electron paramagnetic resonance spectroscopy in aorta

Aortic nitric oxide formation was measured using EPR-based spin trapping with iron-diethyldithiocarbamate ($Fe(DETC)_2$) colloid which was freshly prepared under argon. One rat aorta was cut into ring segments of 3–4 mm length and 3 of them were placed in 1 ml Krebs-Hepes buffer on a 24-well plate on ice. The samples were stimulated with 10 μM calcium ionophore (A23187) for 2 min on ice, then 1 ml of the $Fe(DETC)_2$ colloid solution (400 μM in PBS with $Ca^{2+}/Mg^{2+}$) was

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