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# Rational design of soluble guanylate cyclase (sGC) activators and modified synthesis of HMR-1766 (Ataciguat)

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*S u m m a r y. The heme enzyme soluble Guanylate Cyclase (sGC) is a pivotal amplification target in the NO-sGC-cGMP pathway, converting guanosine-5´-triphoshate (GTP) to cyclic guanosine-3´,5´-monophosphate (cGMP) in response to its major physiological stimulant, nitric oxide (NO). However, several pathologies are etiologically linked with loss of the heme moiety by sGC, resulting in unresponsiveness to NO and ensuing dysfunction of the whole axis. We report the synthesis of HMR-1766 (Ataciguat), a heme-independent sGC "activator" which binds in the heme pocket of sGC, following a modified approach described in the original patent and the ability of our product to increase cGMP levels in the cancer cell line LNCaP, following oxidation of the sGC heme. Furthermore, we describe a docking virtual screening-based strategy, which enables the identification of novel, similar-acting compounds as putative therapeutic molecules.*

## INTRODUCTION

The endogenous signaling molecule nitric oxide (NO) plays a diverse and critical role in the homeostatic control and in disease pathophysiology in a number of systems, including (but not limited to) the cardiovascular and central nervous system and the gastrointestinal tract [1-5].

Its best-characterized and most broadly accepted mode of action is the activation of the heme-linked enzyme sGC, production of cyclic guanosine monophosphate (cGMP) and triggering of subsequent biochemical cascades *via* cGMP-dependent and -independent mechanisms [2, 3].

Based on this mode of action, the functional modification of molecular targets that are involved in cGMP level determination has been a *bona fide* translational approach for decades.

In fact, glyceryl trinitrate, in use for over a century and a half now, as well as sodium nitroprusside and other NO donors, induce their marked anti-anginal vasodilatatory effects by stimulation of sGC [2, 3, 5].

As a complementary therapeutic tack, prevention of cGMP degradation by inhibition of phosphodiesterase 5 (PDE5) activity has resulted in approved drugs such as sildenafil and tadalafil, which are being used in erectile dysfunction and in pulmonary hypertension but are also undergoing expansion of their initial therapeutic applications [2, 6, 7].

The success of these therapeutic interventions relies on undisturbed functionality of the endogenous NO-sGC-cGMP axis, i.e. on levels of NO able to provide the basis for the additive effect of NO donors or on cGMP levels amenable to further magnification by PDE inhibitors. However, in many diseases, exemplified by chronic heart failure or certain forms of pulmonary hypertension, distinct components of the NO-sGC-cGMP axis are dysfunctional or impaired, contributing to the pathology and invalidating these therapeutic approaches. In such situations, there is, for example, either a) reduced production or bioavailability of NO and/or b)increased oxidation and dissociation of the critical $Fe^{+2}$ of the heme moiety of the sGC enzyme, unresponsiveness of sGC to NO and NO donors and increased degradation of the sGC apoenzyme, all these resulting in reduced cGMP levels and subsequent signaling [2, 8]. To overcome this pathological dysfunction of the NO-sGC-cGMP axis, additional therapeutic strategies have recently focused on small molecules able to modulate sGC activity in either of two distinct modes: they can either synergize with endogenously generated NO and stabilize its interaction with the functional enzyme to boost the reduced activity of the holoenzyme (these are called “stimulators”) or are able to interact with sGC in an NO- and heme-independent fashion, thus both stabilizing the heme-free or heme-oxidized sGC as well as increasing its enzymatic activity (these are called “activators”) [2, 8-10].

Despite the best efforts from both academic and pharmaceutical industry research groups and the intense evaluation of several such sGC modulators in clinical trials, only one stimulator molecule, Riociguat, has been approved for the clinic up to date, indicating that there is still significant unmet therapeutic need for similar drugs targeting the NO-sGC-cGMP axis [2, 9, 11-14]. For this reason, our long-term goal is to rationally design, synthesize and assay new molecular entities able to act as sGC activators. We report herein on a) a slightly modified, improved chemical synthesis of HMR-1766 (5-Chloro-2-((5-chlorothiophene)-2-sulfonamido)-*N*-(4-(morpholinosulfonyl)phenyl)benzamide [27] a well-described sGC activator molecule [8, 15-17], b) the basic characterization the bioactivity of thus synthesized HMR-1766, in order to use it as a “positive Control” in subsequent studies and finally c) our initial *in silico* screening efforts to identify small molecules mimicking the spatial arrangement of the porphyrin group and of the activator BAY 60-2770 [18] bound in the sGCβ subunit’s heme pocket, to utilize them in the design of novel sGC activators.

## METHODS

### Synthesis of HMR-1766

All reagents were obtained commercially from Alfa Aesar, Sigma–Aldrich or Merck and used without further purification. Reactions involving moisture-sensitive reactants were conducted in flame-dried glassware under an atmosphere of argon. Reagents and anhydrous solvents were transferred via syringe. Analytical TLC was performed on Merck Silica gel 60 $F_{254}$ on precoated silica gel plates. Visualization was accomplished under UV light (254 and 365 nm), by exposure to iodine vapors and by use of Seebach staining solution. Flash column chromatography was performed on silica gel (SDS 60A, 40–63 μm). NMR spectra ($^{1}H$ and $^{13}C$) were recorded on a Bruker DPX 400 MHz (400 MHz for $^{1}H$), Avance III HD Ascend TM spectrometer or a Bruker Avance III High-Definition four-channel 700 MHz (700 MHz for $^{1}H$, 176 MHz for $^{13}C$) spectrometer. The chemical shifts ($\delta$) are reported in parts per million (ppm). The residual solvent signal is used as internal standard. The following abbreviations are used for the proton spectra multiplicities: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), broad signal (br) and combinations thereof. Coupling constants (*J*) are given in Hertz [Hz].

**5-Chloro-2-nitrobenzoyl chloride (2):** 5-chloro-2-nitrobenzoic acid (1 g, 0.049mmol, 1.0 equiv) was treated with freshly distilled thionyl chloride (4.4 ml, 0.060 mmol, 1.2 equiv.) at 90 ˚C for 16 h. Excess of $SOCl_2$ was removed under vacuum. The crude compound **2** was used to the next step without further purification.

**5-Chloro-*N*-(4-(morpholinosulfonyl)phenyl)-2-nitrobenzamide (4):** A mixture of **2** (900 mg, 4.47 mmol, 1.2 equiv.) and **3** (897 mg, 3.7 mmol, 1 equiv.) in toluene (2.9 mL) was stirred at 80 °C for 16 h. After completion of the reaction, the mixture was cooled to room temperature, diluted with EtOAc (10 mL) and quenched with aq. saturated $NaHCO_3$ (15 mL) and EtOAc (10 mL). The two layers were separated and the aqueous phase was washed with EtOAc (3 × 15 mL). The combined organic layers were dried with anhydrous $Na_2SO_4$ and concentrated under reduced pressure. The crude product was purified by silica gel

flash column chromatography (n-hexane/EtOAc, 10:0 to 8:2) to give **4** (1.3 g, 3.05 mmol, 83 %) as a white powder.

$^1$H NMR ($CD_3OD$, 400 MHz) *δ* 7.71 (dd, *J* = 8.2, 0.8 Hz, 2H), 7.44 (d, *J* = 8.8 Hz, 2H), 7.33 – 7.28 (m, 2H), 7.23 – 7.17 (m, 1H), 3.39 – 3.31 (m, 4H), 2.63 – 2.55 (m, 4H) ppm.

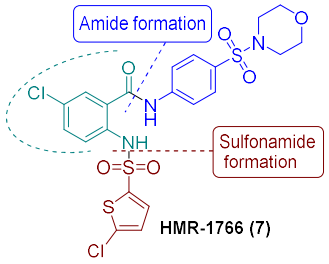
**2-Amino-5-chloro-*N*-(4-(morpholinosulfonyl)phenyl)benzamide (5)**: To a degassed solution of **4** (500 mg, 1.17 mmol, 1.0 equiv.) in dry THF (17 mL) 10 % palladium on activated carbon (125 mg, 0.117 mmol, 0.1 equiv.) was added. The reaction mixture was stirred for 3 h at room temperature under $H_2$-atmosphere (balloon). After completion of the reaction, the mixture was filtered through a Celite pad and the filtered cake was washed thoroughly with THF (3 × 10 mL) and EtOAc (3 × 10 mL). The solvent was removed under reduced pressure to give a residue which was recrystallized from EtOAc. Compound **5** (335 mg, 0.84 mmol, 72%) was obtained as a white powder.

$^1$H NMR ($CDCl_3$, 400 MHz) *δ* 7.76 (d, *J* = 8.7 Hz, 2H), 7.57 (d, *J* = 8.8 Hz, 2H), 7.49 (d, *J* = 2.3 Hz, 1H), 7.08 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.61 (d, *J* = 8.8 Hz, 1H), 3.90 (s, 2H), 3.65 – 3.56 (m, 4H), 2.90 – 2.83 (m, 4H) ppm.

**5-Chloro-2-((5-chlorothiophene)-2-sulfonamido)-*N*-(4-(morpholinosulfonyl)phenyl)benzamide (HMR-1766, 7) (Picture 1)**: A mixture of **5** (30 mg, 0.076 mmol, 2 equiv.) and **6** (8 mg, 0.038 mmol, 1 equiv.) in acetonitrile (0.25 mL) was stirred at 90 °C for 3 days in a sealed tube. After completion of the reaction, the reaction mixture was cooled to room temperature, filtered and the filtered solid was washed with $CH_3CN$ (5 x 3 mL). The crude residue was purified by silica gel flash column chromatography (*n*-hexane/EtOAc, 9:1 to 5:5) to provide **7** (15 mg, 0.026 mmol, 70 %) as a white solid.

$^1$H NMR ($CD_3COCD_3$, 700 MHz) *δ* 10.19 (bs, 1H), 8.02 (dd, J = 8.7, 2.2 Hz, 2H), 7.94 (t, J = 2.3 Hz, 1H), 7.81 (d, J = 8.5 Hz, 2H), 7.71 (d, J = 8.7 Hz, 1H), 7.66 (d, J = 11.5 Hz, 1H), 7.36 (d, J = 4.1 Hz, 1H), 6.98 (d, J = 4.0 Hz, 1H), 3.70 (t, J = 4.7 Hz, 4H), 2.97 (t, J = 4.7 Hz, 4H).

$^{13}$C NMR ($CD_3COCD_3$, 176 MHz, Acetone-d6) *δ* 166.74, 166.68, 143.37, 143.26, 137.79, 133.77, 133.53, 131.63, 129.90 (2xC), 129.44, 128.63, 127.17, 125.97, 121.31, 121.23(2xC), 66.63 (2xC), 47.13 (2xC) ppm.




*Picture 1: Key steps towards the synthesis of HMR-1766 (**7**).*

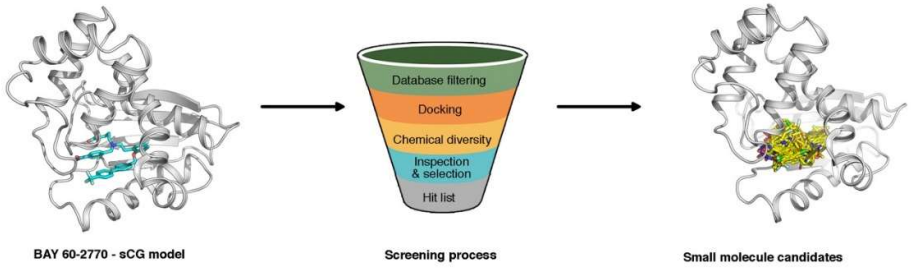
Docking virtual screening

Cell-based cGMP assay

The ability of compounds to increase cGMP levels was assayed in the cancer cell line LNCaP, which is able to respond to sGC modulators [19]. The cells were plated in 48-well plates in DMEM growth media (Gibco-Thermo Fisher Scientific) supplemented with penicillin/streptomycin and Fetal Bovine Serum. 48h later, upon reaching confluence, the cell monolayers were exposed to serum-free medium containing 0.1% BSA for 2 hours (all concentrations given are final, in the well). Subsequently, cells were treated for 20 min with the heme-oxidizing compound [1, 2, 5] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) [19], 10μM or with vehicle dimethylsulfoxide (DMSO). At the end of this period, the cells were washed once and the medium was replaced with DMEM without any additions, save the phosphodiesterase inhibitor isobutyl-methylxanthine (IBMX) at 1mM. 5 min later, vehicle Control (CTL) or the test compounds sodium nitroprusside (SNP, positive control) or HMR-1766 (synthesized in-house as described above) were added at 10μM. After 15 min, media were discarded and 0.1M HCl was added in the wells, to stop the reaction and to extract the cellular cGMP. The retrieved extracts were used to determine cGMP by a competition ELISA kit (Cayman Chemicals, Ann Arbor, MI, USA). NaOH 0.1M was added to the wells to solubilize the protein, determined by a Micro BCA Protein Assay kit (Thermo Scientific) in order to normalize the cGMP levels for cell load. LNCaP cells were originally obtained from the ATCC, USA. Unless otherwise specified, reagents and chemicals were ordered from Sigma-Aldrich (Antisel, Greece).

In order to identify new compounds that act as activators of human sGC, we constructed a homology model based on the crystal structure of the activator BAY 60-2770 bound to the Nostoc H-NOX domain [20]. Based on this model, a docking virtual screening process was implemented using the ZINC database, a compendium of commercially available chemical structures in a computer-searchable format [21] enabling fast and efficient identification of small molecule compounds. Following a filtering of the initial database, a shorter list of negatively charged molecules was obtained, similar to previously identified activators [22]. Using a robust process of docking, defining a grid box with dimensions of 25x25x25 Å, a search space of 1 binding mode and a search parameter of 8 [23]. A list of 5000 putative molecular candidates was sorted on the basis of the complementarity to the heme-binding site, docking score and structural features (Picture 2).

Clustering of the best-scoring molecules was made using physico-chemical and structural properties and represented in 3D space using CheS-Map [24].




*Picture 2. Schematic representation of the computational process of constructing a homology model of BAY 60-2770 bound to sGC, virtual screening and final set of small molecule compounds*

## RESULTS AND DISCUSSION

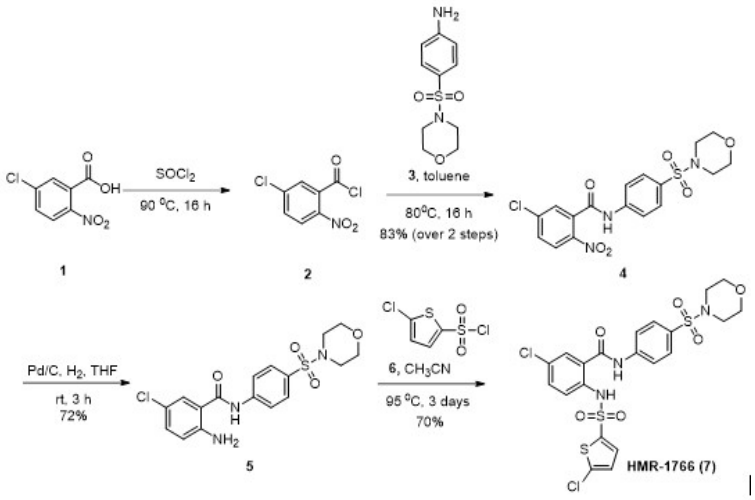
### Modified synthesis of HMR-1766

The synthesis of HMR-1766 (Ataciguat) (**7**) was initially described in a patent by Hoechst Marion Roussel Gmbh but provided limited experimental data [27]. In 2013, Sahoo et al. introduced a Ru(II)-catalyzed methylphenyl sulfoximine-aided intermolecular *o*-C–H amidation of arenes that was successfully employed for the synthesis of **7**, with an overall yield that did not exceeded 22% regarding the main steps of the synthetic scheme [25].

Herein, we present a modified, simple approach for the synthesis of **7** that is based on the initially described in the patent and which utilizes low cost, commercially available reagents and easily accessible intermediates. As it is depicted in Scheme 1, the synthetic route towards **7** involves as key steps the amide coupling between an appropriately substituted benzoic acid derivative with a 4-(morpholinosulfonyl)aniline followed by sulfonamidation with an appropriately substituted thiophene precursor.

Commercially available 5-chloro-2-nitrobenzoic acid **1** was selected as the starting material for our synthetic endeavors (Picture 3). Treatment of **1** with thionyl chloride ($SOCl_2$) afforded the benzoyl choride over $H_2$ atmosphere furnished the corresponding aniline derivative **5** with a 72% yield. Finally, sulfonamide coupling between aniline **5** and 5-chlorothiophene-2-sulfonyl chloride **6** [26] provided the HMR-1766 (**7**) product **7** with a 70% yield.

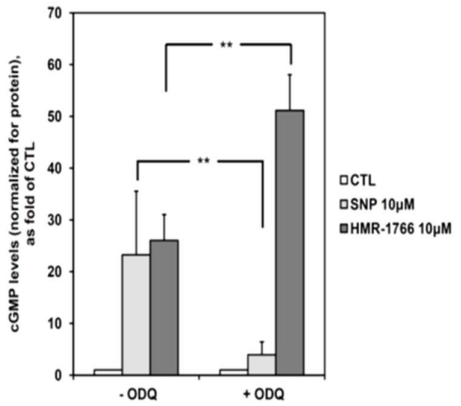
Overall, we achieved to synthesize **7** in four steps and 42% overall yield. Notably, our approach was successfully applied by avoiding the utilization of complicated intermediates, expensive catalysts and cumbersome purification methods, thus enabling the production of **7** in adequate quantities and its exploitation as a “positive Control sGC activator” in our bio-assays, as demonstrated below.




*Picture 3: Synthesis of HMR-1766.*

Comparison of the cGMP-raising effects of SNP and HRM-1766

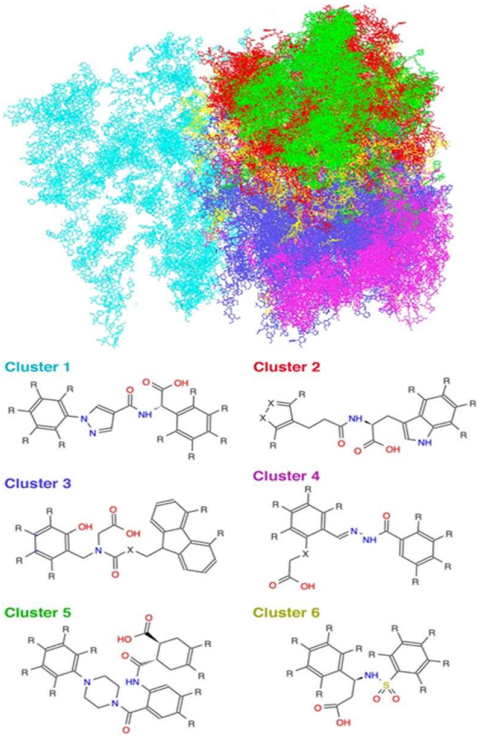
The basal (CTL) cGMP levels in LNCaP cells were not significantly affected by treatment with the oxidant ODQ (1.96±1.05 and 1.49±0.48 pmol/µg protein in cells untreated and treated with ODQ, respectively, P=0.21, N=10 wells). SNP 10µM, an NO donor whose activity requires the presence of non-oxidized heme associated with sGC, raised cGMP levels in naïve (ODQ-untreated) LNCaP cells by 23.2-fold compared to Control. This stimulation was, as expected for NO and NO donors, largely depressed in cells pre-treated with the heme-oxidizing compound ODQ, and was reduced to 4-fold of CTL (Picture 4, P=0.009, effect with/out ODQ). In-house synthesized HMR-1766 increased cGMP levels similarly to SNP (by 26-fold) in LNCaP cells in the absence of ODQ pretreatment. However, in contrast to SNP, the effect of HMR-1766 was almost doubled in cells pretreated with ODQ, reaching 51-fold (Picture 4, P=0.001, effect with/out ODQ), demonstrating that the in-house synthesized HMR-1766 compound can indeed act as an NO- and heme-independent sGC activator.




*Picture 4: Confluent LNCaP cells were untreated (-ODQ) or pretreated (+ODQ) for 20 min with 10 µM of the heme oxidant ODQ and were subsequently exposed to 1mM IBMX for 5 min. They were then treated for 15 min with vehicle (CTL) or 10 µM of either SNP or HMR-1766. Cellular cGMP was determined using a commercial kit and was normalized for protein content. Data are expressed as means ± standard deviations of fold- increases compared to vehicle (CTL) of 6-10 independent determinations (wells) per group. P values were determined by 2-tailed Student's t-test. **: P<0.01.*

Docking virtual screening

The methodology for identifying novel active compounds required a filtering of the ZINC database, resulting in a shorter list of negatively charged molecules. Using a robust docking process on a subset of putative molecular candidates, these were sorted on the basis of their complementarity to the heme-binding site, docking score and structural features. In order to validate the results of this screening process, the best-scoring molecules were clustered using physico-chemical and structural properties and were represented in 3D space (Picture 5).




*Picture 5. Clustering of the best 5000 compounds in 3D space based on their chemical similarity. Representative compound scaffolds of each cluster are depicted in the bottom of the figure, where -R depict H or mainly hydrophobic substituents, while -X stands for C, N, S or O (cluster representatives legend color corresponds to molecule coloring*

Each of the six clusters was analyzed for identifying a representative scaffold, showing that the diverse compounds from a virtual library contain similarities to the known activator BAY 60-2770 (Picture 5). All scaffolds bear a carboxyl group, but also contain a monocyclic or polycyclic aromatic ring known to be important for sGC activator-type molecules binding in the heme-binding cavity [22]. The identification of different groups highlights the diversity in the potential design of new activators using such libraries, docking and chemical similarity tools.

Our overall aim was to set a basis for the identification of novel sGC activator molecules. Herein, we describe a modified and simplified synthesis of an “archetypal” such molecule, HMR-1799 (Ataciguat), as well as the basic functional characterization of the in-house generated compound, which can serve as a standard “Control” in future bioassays. In addition, we describe an *in silico* approach, based on the crystal structure of the Nostoc H-NOX domain with the sGC activator BAY 60-2770, allowing the identification of novel molecules predicted to operate in the same fashion as BAY 60-2770 and which thus warrantee further evaluation as novel sGC activators.

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