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Nonacidic Inhibitors of Human Microsomal Prostaglandin Synthase 1 (mPGES 1) Identified by a Multistep Virtual Screening Protocol

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Microsomal prostaglandin E_2-synthase (mPGES-1) is a target for future anti-inflammatory drugs. Inhibitors of mPGES-1 mimicking prostaglandin E_2 often interact with cyclooxygenases (COXs) 1 and 2, leading to unwanted side effects. Selective inhibitors of mPGES-1 can be obtained by deliberate abdication of the acidic groups, which are an important feature of COX inhibition. Here, we present a successful virtual screening study that results in a potent nonacidic mPGES-1 inhibitor lacking COX inhibition.

Introduction

Today's most frequently used nonsteroidal anti-inflammatory drugs (NSAIDs[a]) are associated with gastrointestinal and cardiovascular side effects.[1−3] Especially patients suffering from chronic inflammatory diseases (e.g., rheumatoid arthritis), which are under permanent treatment with NSAIDs, are exposed to a risk of gastrointestinal ulcerations and cardiovascular complications.[4,5] Thus, there is a need for safer anti-inflammatory drugs.

As most NSAIDs act as COX-inhibitors, one possible approach to circumvent NSAID-related side effects is to block enzymes more selectively downstream of COX in the arachidonic acid cascade. The idea is to selectively inhibit the release of proinflammatory prostaglandins while maintaining the biosynthesis of prostaglandins with housekeeping functions. Prostaglandin E_2 **1** (PGE_2, Chart 1), which is one of the most prominent proinflammatory mediators released from the arachidonic acid cascade, is produced by three distinct PGE synthases: cytosolic prostaglandin E_2 synthase (cPGES) and two microsomal prostaglandin E_2 synthases (mPGES-1 and mPGES-2). The structure of mPGES-1 was determined by electron crystallography by Jegerschöld and co-workers.[6] In contrast to mPGES-2 and cPGES, the expression of mPGES-1 is inducible and therefore responsible for the release of PGE_2 as a reaction to inflammatory stimuli. Selective inhibition of mPGES-1 might therefore be a promising approach for the design of effective anti-inflammatory drugs lacking NSAID-related side effects.[7,8]

The development of mPGES-1-inhibitors is still in its infancy. Notably, the use of established murine pain models to evaluate promising compounds in vivo is complicated because of low similarity of the human and murine enzymes.[9] Although several compounds have been identified in vitro, none of them have reached clinical development so far. One of the first mPGES-1-inhibitors was the sulfonamide derivative **2** (NS-398).[10] Originally characterized as a COX-2-inhibitor, **2** exemplifies one of the major problems of the design of mPGES-1 inhibitors, namely, lack of selectivity against structurally related enzymes. Subsequently reported indole derivatives such as **3** (MK-886) showed the same problem, as **3** is an inhibitor of the 5-lipoxygenase (5-LO) activating protein (FLAP), which is (like mPGES-1) a member of the MAPEG family.[10] Recently, a class of pirinixic acid derivatives has been reported as dual mPGES-1/5-LO inhibitors.[11] As these unselective compounds are carboxylic acids resembling the structure of the endogenous substrate prostaglandin H_2, one approach to overcome the selectivity problem might be the search for nonacidic mPGES-1 inhibitors. The first representative of this class is the phenanthreneimidazole **4** (MF63). **4** has no structural relation to the previously reported carboxylic acids and displays high activity (IC_{50} = 1.3 nM) and selectivity[12] (> 1000-fold over other prostanoid synthases). Consequently, nonacidic scaffolds for mPGES-1 inhibitors might bear a potential for drug discovery.

In this study, we present a computational approach for finding novel mPGES-1 inhibitors. In our screening, we identified **18** as an active mPGES-1 inhibitor in the submicromolar range. Compound **18** displays a high degree of structural novelty and may provide an innovative scaffold for the design of selective and potent mPGES-1-inhibitors.

Computational Methods

The libraries for virtual screening contained 360 169 compounds and were obtained from Asinex (AsinexGold, version Nov2008, 233 554 compounds, AsinexPlatinum, version

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[a]Abbreviations: mPGES-1, microsomal prostaglandin E2 synthase; COX, cyclooxygenase; NSAIDs, nonsteroidal anti-inflammatory drugs; PG, prostaglandin; cPGES, cytosolic prostaglandin E2 synthase; FLAP, 5-lipoxygenase activating protein; 5-LO, 5-lipoxygenase; FCS, fetal calf serum; IL-1β, interleukine-1 β; TNFα, tumor necrosis factor α; PBS, phosphate buffered saline; Kpi, potassium phosphate; GSH, glutathione.

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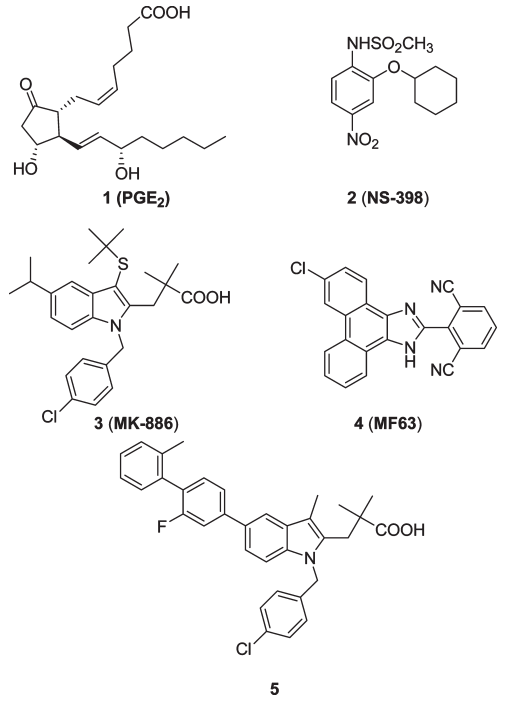
Chart 1. Published mPGES-1 Ligands



Nov2008, 126 615 compounds, Asinex, Moscow, Russia, www.asinex.com). Compound preparation included protonation of basic structures, deprotonation of acidic structures, and addition of explicit hydrogen atoms using the MOE software suite (MOE Molecular Operating Environment version 2008.10, Chemical Computing Group, Montreal, Canada, www.chemcomp.com). CATS descriptors[13] were calculated for each compound in the database. CATS is a 2D pharmacophore descriptor that considers topological distances between pairs of potential pharmacophoric points in a molecule. A toroidal self-organizing map SOM implemented in the MOLMAP software[14,15] containing 10 × 10 neurons was trained using the CATS descriptors of each Asinex library and published structures with $IC_{50} < 100$ nM.[16,17] The Asinex compounds localized in the same clusters ("neurons") as the active structures, also termed "activity islands",[15] were selected for further evaluation with a 3D screening method. Up to 250 conformers per molecule (rmsd cutoff, 0.8 Å; energy cutoff, 6 kcal/mol) were generated by MOE. The SQUIRREL method[18] was used to align the conformers to a low-energy conformer of the most potent indole **5** and phenanthrene **4** derivatives. SQUIRREL combines shape-based superposition technique with a pharmacophore scoring function. First, an approximation of solvent-accessible surface is calculated and decomposed into surface patches with nearly equal local curvature, called "shapelets".[19] Then the "shapelets" of the query and target molecule are superimposed to identify areas of similar shape. The resulting shape-based alignment is assigned a score which is based on a fuzzy pharmacophore function.[20] The 4 × 200 Asinex compounds with the highest SQUIRREL scores resulting from the screening of AsinexGold and AsinexPlatinum database with **4** and **5**, respectively, were chosen for scaffold analysis and visual examination. The SAReport module of MOE software was used to cluster the molecules according to the underlying chemical scaffold.

Cell Biological Methods

Cells and Reagents. HeLa (human cervix carcinoma) cells were purchased from Deutsche Sammlung für Mikroorganismen and Zellkulturen (DSMZ, Braunschweig, Germany) and incubated in RPMI medium 1640, containing high glucose, GlutaMAX, 10% fetal calf serum (FCS), 100 units/mL penicillin G, and 100 μg/mL streptomycin, which were purchased from Invitrogen (Germany). Cells were cultured at 37 °C in an atmosphere containing 5% CO_2. Recombinant human interleukin-1 β (IL-1β) and recombinant human tumor necrosis factor α (TNFα) were purchased from PeproTech (London, U.K.). All chemical compounds provided by Asinex (Asinex, Moscow, Russia) exhibit ≥95% purity determined by manufacturer using LC−MS.

mPGES-1 Activity Assay. To investigate the inhibitory activity of the different compounds found by computational methods on the mPGES-1 enzyme in vitro, the microsomal fraction of HeLa cells was prepared. Approximately 4×10^6 cells were incubated for 24 h at 37 °C in medium containing 10% FCS. The medium was removed, and cells were stimulated with IL-1β (1 ng/mL) + TNFα (5 ng/mL) for 16 h. After being washed with 10 mL of phosphate buffered saline (PBS), cells were scraped in 2 mL of PBS and centrifuged at 2500*g* for 2 min at 4 °C. Cell pellets were resuspended in 600 μL of potassium phosphate buffer (Kpi buffer, 0.1 M, pH 7.4), containing 1× Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), sucrose (0.25 M), and reduced glutathione (GSH, 1 mM). Samples were sonificated and centrifuged at 170000*g* for 1 h at 4 °C. The microsomal fraction (pellet) was resuspended in 50 μL Kpi buffer (0.1 M, pH 7.4) containing 1× Complete and reduced GSH (2.5 mM), and total protein content was measured using the Bradford method.[21]

The mPGES-1 activity assay was performed as described by Thoren et al.[22] Briefly, 0.15 mg/mL protein was incubated with various concentrations of **6**−**22** (0.001−30 μM) for 30 min on ice. The reaction was initiated with 20 μM PGH_2 (Larodan, Malmö, Sweden) and terminated after 1 min by adding a stop solution containing 40 mM iron chloride ($FeCl_2$) and 80 mM citric acid. After solid phase extraction the amount of produced PGE_2 was measured by LC−MS/MS analysis as described previously.[23] The IC_{50} was calculated using SigmaPlot 11 software (Systat Software Inc., San Jose, CA) by fitting the four-parameter logistic curve.

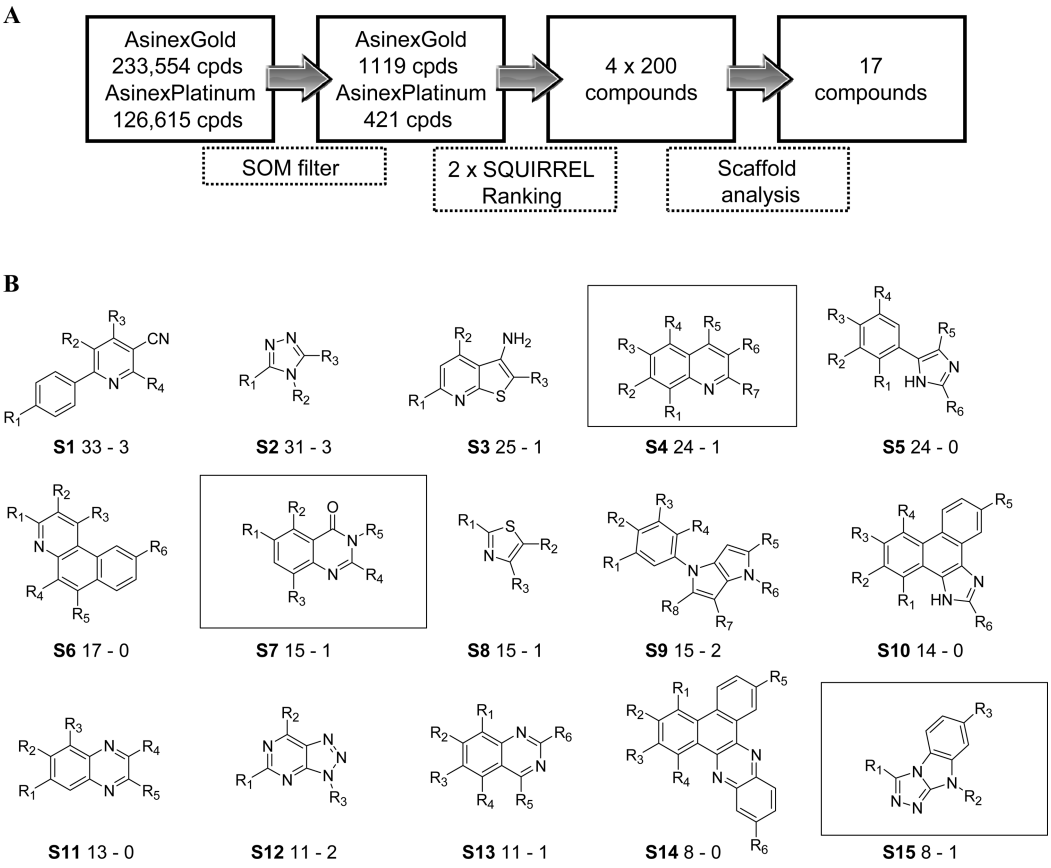
COX-Inhibitor Screening Assay. Inhibition of COX-1 (ovine) and COX-2 (human recombinant) activity by **18** was measured using a COX inhibitor screening assay kit (Cayman Chemicals, Ann Arbor, MI, according to the manufacturer's protocol. SC-560 (5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl-1-pyrazole), a selective COX-1 inhibitor, and celecoxib, a selective COX-2 inhibitor, were used as positive controls. The COX assay is based on the measurement of the amount of PGE_2, PGD_2, and $PGF_{2\alpha}$ produced by $SnCl_2$ reduction of COX-derived PGH_2. The amounts of these prostaglandins were quantified by LC−MS/MS analysis as described previously.[24]

Results and Discussion

Two distinct highly potent classes of mPGES-1 inhibitors are available at present: phenathrene[16] and indole[17] derivatives. These two compound classes provide a good starting point for a ligand-based virtual screening study. Although the electron crystallographic structure released by Jegerschöld and co-workers[6] provides intriguing insights into the catalytic mechanism of mPGES-1, the binding site adopts the so-called closed conformation, which makes the straightforward application of established receptor-based virtual screening techniques rather difficult. Therefore, we decided to follow the multistep ligand-based strategy.

The training of the self-organizing map revealed that the indole-based compounds and the phenanthrene derivatives occupy different activity islands (see Supporting Information). The reason for using the SOM filter was the dramatic

Chart 2. (A) Schematic Overview of the Virtual Screening Procedure and (B) Enriched Scaffolds Found by Virtual Screening[a]



[a] For part B, the first number indicates number of representatives in the 4 top 200 lists. The second number is the amount of ordered compounds. Scaffolds with active representatives are framed.

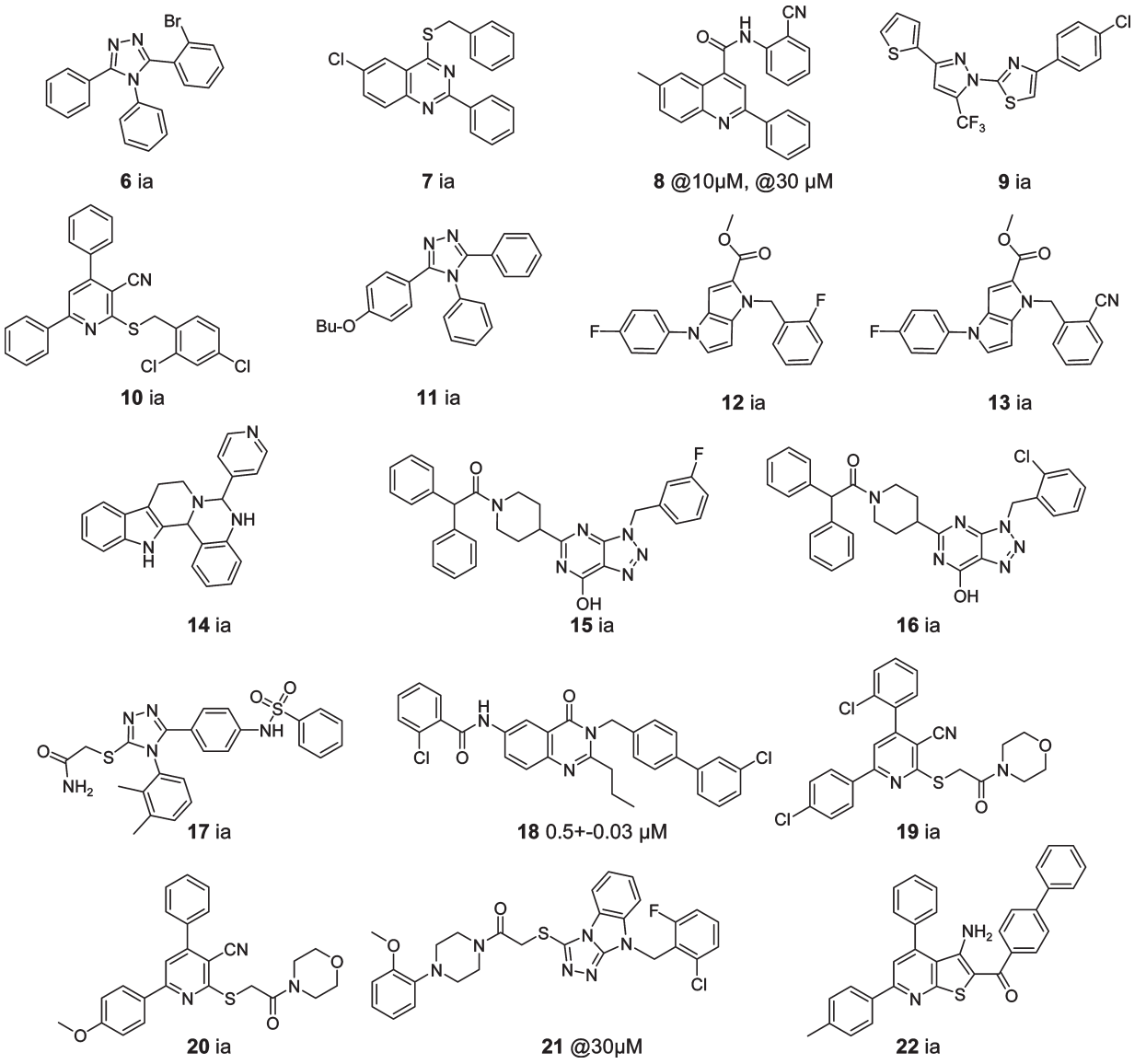
reduction of the computational time for 3D conformer generation because only 1540 out of 360 169 compounds have passed this screening step. The screening compounds projected to these activity islands were ranked by their similarity to the most potent substances from both chemical classes, namely, **4** and **5**, using the shape and pharmacophore based screening software SQUIRREL. Manual examination of the screening results clustered by chemical scaffold revealed that structures with the same scaffold were enriched using both chemically different reference structures. Chart 2A gives a schematic overview of the single steps of the virtual screening protocol.

We regarded every scaffold that had more than five representatives among the 200 compounds with the highest similarity to the reference structures as "enriched". Chart 2B is an overview of all recognized scaffolds. From each scaffold, one or several top-ranked structures were selected for further in vitro evaluation. Scaffolds **S5**, **S6**, **S10**, **S11**, and **S14** were excluded from the compound selection because of the structural similarity to phenanthrene-derived structures previously described by Côte and co-workers.[10] The examination of the enriched scaffolds revealed several nitrogen-based heterocyclic compounds. Compounds that are not covered by one of the scaffolds shown in Chart 2B were regarded as a singletons. Multiple compounds from scaffolds **S1**, **S2**, **S9**, and **S12** we ordered from Asinex to minimize the chance of the occurrence of false positives/negatives in the screening. Indole derivative **14** was highly similar to the phenanthrene-derived reference structure **4**, therefore, it was selected for screening although it was a singelton.

The compounds shown in Chart 3 were evaluated in vitro for their ability to inhibit mPGES-1 activity in concentrations of 1.5, 15, and 30 μM. HeLa cells express cPGES and mPGES-2 constitutively, whereas mPGES-1 is inducible after stimulation with TNFα and IL-1β.[19] As it is known that mPGES-1 is coupled to COX-2 and mainly responsible for PGE_2 production after stimulation of cells with IL-1β and TNFα,[25] we tested the inhibitory effect of **6**−**22** (Chart 3) on mPGES-1 activity using microsomal fractions of HeLa cells after stimulation of cells with IL-1β and TNFα. To verify if mPGES-1 was induced after stimulation of HeLa cells, we performed a Western blot from the microsomal fraction and looked for mPGES-1 protein expression. mPGES-1 was highly inducible when HeLa cells were treated with IL-1β and TNFα (see Supporting Information). The mPGES-1 assay was performed with **6**−**22**. Only compounds that exhibited a concentration dependent inhibition of PGE_2 production and at least 40% inhibition at the highest concentration were regarded as "active". Compounds **8**, **18**, and **21** showed $>40\%$ inhibition at 30 μM in a concentration-dependent manner (Figure 1A). IC_{50} calculation was performed for active **18**, yielding an IC_{50} of 0.5 ± 0.03 μM (Figure 1B).

The undesired cross-reactivity against COX-1 and COX-2 was investigated in vitro for **18**. Compound **18** did not exhibit inhibitory activity on both cyclooxygenases at 1, 10, and 30 μM. The lack of acidic groups that could be widely found in previous reported mPGES-1-inhibitors might thereby be a valid strategy to develop mPGES-1 inhibitors without COX cross-activity.

Chart 3. Ordered and Tested Compounds[a]



[a] The concentration at which an inhibition of PGE-2 production >40% was observed or the $IC_{50} \pm$ SE is given (ia, inactive; inhibition of PGE-2 production at 30 μM below 40%).

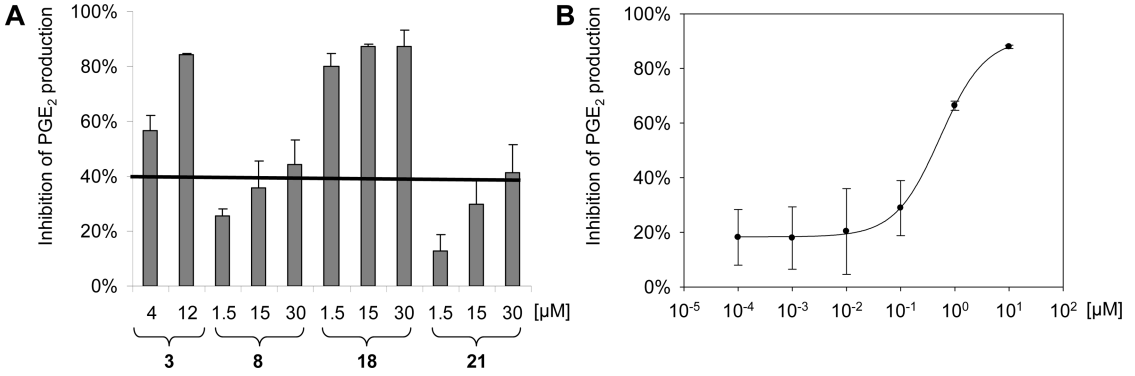


Figure 1. (A) Inhibition data for **8**, **18**, and **21**. **3** was used as positive control for mPGES-1 inhibition. All measurements were repeated at least 3 times. (B) Concentration-dependent inhibition data for **18**, used for IC_{50} determination.

Conclusions

A virtual screening protocol for identification of novel nonacidic inhibitors of mPGES-1 has been presented. The two-step strategy consists of compound clustering by a self-organizing map followed by shape and pharmacophore based screening.[26−28] It was successfully applied to finding novel scaffolds with promising inhibitory activity in a large screening compound pool. By utilizing this method, we were

able to identify three active compounds in a subset of 17 tested. These compounds represent novel scaffolds for mPGES-1 inhibitors. Compound **18** exhibits submicromolar activity on mPGES-1 and lacks activity on both COX isoforms. The discovery of **18** is a proof-of-concept for the directed exclusion of carboxylic acid-containing scaffolds to circumvent COX activity in the search of potential selective mPGES-1-inhibitors.

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Supporting Information Available: Collection of active compounds used for SOM screening, parameters for SOM training, Western blot, and ^{1}H NMR data of **8**, **18**, **21**. This material is available free of charge via the Internet at http://pubs.acs.org.

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