# Discovery of novel non-acidic microsomal prostaglandin E2 synthase-1 (mPGES-1) inhibitors through a multi-step virtual screening protocol

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**Abstract**

Microsomal prostaglandin E2 synthase-1 (mPGES-1) is considered as potential therapeutic target for the treatment of inflammatory diseases and certain types of cancer. In order to explore novel areas in the chemical space and to capture novel scaffolds for mPGES-1 inhibition, we applied a virtual screening protocol, which comprises molecular docking, fingerprints-based clustering with diversity-based selection, protein-ligand interactions fingerprints, and molecular dynamics (MD) simulations with Molecular Mechanic–Poisson Boltzmann Surface Area (MM-PBSA) calculation. The hits identified by use of our protocol were carefully analyzed to ensure the selection of novel scaffolds, which make stable interactions with key residues in the mPGES-1 binding pocket and inhibit the catalytic activity of the enzyme. As a result, we hereby successfully discovered two promising chemotypes **6** and **8** as non-acidic mPGES-1 inhibitors with IC50 of 1.15 and 1.25 μM, respectively. A minimal structural optimization of **8** resulted in compounds **15**, **19** and **20** with promising improvement in the inhibitory activity (IC50 = 0.3-0.6 μM). The unprecedented chemical structures of **6** and **8**, which are prone to further derivatization reveal a new and attractive field for the development of mPGES-1 inhibitors with potential anti-inflammatory and anticancer properties.

*Keywords*: Microsomal prostaglandin E2 synthase-1; prostaglandin; virtual screening; docking; molecular dynamics; inflammation.

# 1. Introduction

The microsomal prostaglandin E2 synthase-1 (mPGES-1) has been the object of extensive research for next generation of anti-inflammatory drugs during the last two decades [1, 2]. mPGES-1 is the terminal enzyme in the biosynthesis of PGE2, which is the main mediator of acute and chronic inflammation, fever and pain (Figure 1) [1]. After its first discovery by Samuelsson and co-workers [3], mPGES-1 has rapidly become an attractive target for pharmacological intervention of inflammatory diseases, such as arthritis, atherosclerosis, neuro-degenerative diseases and cancer [2, 4-6]. The biosynthesis of PGE2 is initiated by the liberation of arachidonic acid (AA) from membrane phospholipids by cytosolic phospholipase A2. Cyclooxygenases (COX-1/2) then convert free AA to PGH2, which is the junction point for the biosynthesis of several structurally related PGs that are formed from PGH2 by the action of their respective PG synthases. The PGs biosynthesized by these pathways include PGE2, PGD2, PGF2α, PGI2 (prostacyclin) and thromboxane A2 (TXA2) [1]. The isomerization of PGH2 to PGE2 is carried out by PGE2 synthases (PGES), which include three members such as cytosolic PGE2 synthase (cPGES) and two microsomal PGE2 synthases (mPGES-1 and mPGES-2) (Figure 1). While both cPGES and mPGES-2 are constitutively expressed in a variety of tissues, mPGES-1, which is functionally coupled with cyclooxygenase-2 (COX-2), is strongly up-regulated under inflammatory conditions [1]. Therefore, it is believed that selective inhibition of mPGES-1 would selectively interfere with the biosynthesis of pro-inflammatory PGE2, while intervention with COX-1/2 by nonsteroidal anti-inflammatory drugs (NSAIDs, such as indomethacin or ibuprofen) prevents the production of all PGs. Hence, inhibition of mPGES-1 has been a developing pharmacological strategy for next generation NSAIDs that allow selective interference with pro-inflammatory PGE2, and is anticipated to overcome the common side effects (i.e. gastrointestinal and cardiovascular) observed with current NSAIDs due to suppression of homeostatic prostanoids.

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**Figure 1.** The Arachidonic acid pathway

Figure 2 demonstrates several promising scaffolds that were developed as mPGES-1 inhibitors during the last decade [7]. For example, indole-carboxylic acid derivatives, which evolved from the structure of the 5-lipoxygenase-activating protein (FLAP) inhibitor MK-886, effectively inhibit mPGES-1 activity *in vitro* [8]. Other potent mPGES-1 inhibitors comprise various chemical scaffolds such as phenanthrene-imidazoles [9, 10], 4-biarylimidazoles [11], tri-substituted urea derivatives [12], imidazoquinolines [13, 14], dihydropyrimidines [15], and benzoxazole-piperidines [16, 17]. Although, these mPGES-1 inhibitors with distinct chemical classes have been identified, none has been proven clinically useful so far due to various problems (e.g. strong plasma protein binding and high lipophilicity) as reviewed elsewhere [7, 18]. Up to now, only a single clinical trial with an mPGES-1 inhibitor (i.e. LY3023703 by Eli Lilly) has been reported [19], while second compound from Glenmark Pharmaceuticals, namely GRC-27864, has recently entered phase I clinical development for the potential treatment of chronic inflammatory disorders such as osteoarthritis and rheumatoid arthritis (Clinical Trials Identifier: NCT02179645). To our knowledge, no structural information regarding both compounds has yet been disclosed. Therefore, developing new inhibitors of mPGES-1 with distinct scaffolds is a major challenge in anti-inflammatory drug development. Based on these considerations, we hereby report the identification of new and potent inhibitors of human mPGES-1 with various scaffolds through a multi-step virtual screening study. Newly identified chemotypes are prone to further optimizations for the development of potent mPGES-1 inhibitors as anti-inflammatory and anticancer agents.

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**Figure 2.** The main scaffolds of potent mPGES-1 inhibitors developed during the last decade.

**2. Results and discussion**

Virtual screening (VS) and molecular docking are well-established computational methods that are appropriate to predict the binding interaction of small molecules with experimental protein structures, and to retrieve putative small-molecule inhibitors from large chemical databases [20]. However, some factors remain as challenging obstacles for obtaining a true prediction of the ligand-protein binding-affinity such as protein plasticity, water-mediated binding interactions, presence of structural water molecules in the active site and solvent entropy. Considering of all these factors, we herein describe a multi-step VS protocol (Figure 3), which combines molecular docking, the protein-ligand interactions profile, molecular dynamics (MD) simulations, and Molecular Mechanic–Poisson Boltzmann Surface Area (MM-PBSA) energy calculation in order to increase the virtual screening accuracy. Our final objective is to identify novel small-molecule mPGES-1 inhibitors with no cross-activity against COXs. The selection step in our protocol depended on preserving some key protein-ligand interactions as noticed in the four mPGES-1-inhibitor crystal structures (4YL0, 4YL1, 4YL3, 4YK5) by investigating their stability using MD simulations followed by MM-PBSA calculations.

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**Figure 3.** The workflow of the applied VS protocol

*2.1. Analysis of protein-ligand interactions in reported crystal structures of mPGES-1*

The recent elucidation of the first crystal structure of mPGES-1 in active form (PDB ID: 4AL1) [21] was rapidly followed by elucidation of further high-resolution structures of human mPGES-1-inhibitor complexes (PDB ID: 4BMP, 4YK5, 4YL1, 4YL3 and 4YL0) [22] to support the rational design of novel mPGES-1 inhibitors [23-25]. With this aim, we retrospectively analyzed the reliability of a variety of available mPGES-1 crystal structures for use in our virtual screening study (Table 1).

**Table 1**. Important protein-ligand interactions for co-crystallized inhibitors in the published mPGES-1 crystal structures **4YL0**, **4YL1**, **4YL3**, and **4YK5**.



Careful analysis of the binding interactions of the co-crystallized inhibitors with mPGES-1 led to the recognition of a binding groove and important residues participating in the protein-ligand interactions. For example, x-ray crystal structures with PDB ID: 4YL0 and 4YL3 represent the mPGES-1 structures bound to a phenanthrene-imidazole derivative (MF-63) and a brominated biaryl-imidazole derivative, respectively (see Figure S1A-B for chemical structures). Both inhibitors bind between two mPGES-1 monomers, *i.e.*, monomer A and monomer B, and more precisely, between two helical turns of α-4 from monomer A and α-1 from monomer B [21, 26]. By examining the binding interactions, we mostly noticed strong hydrogen bonds in the more polar region close to residues S127 (from monomer A), H53 and R52 (from monomer B), while aromatic rings extended inside both the deep hydrophobic groove and binding groove above the glutathione (GSH). Therefore, strong hydrophobic interactions are made with different residues such as A123 and S127 side chains (monomer A) and R38, L39, F44, D49, H53, and R53 side chains (monomer B) (Table 1).

In the case of MF63 (Figure S1A) in the crystal structure 4YL0, one of the imidazole's nitrogens contributes also to a hydrogen bond with H53, while the other may be involved either with hydrogen bond to GSH or with a bridge of water molecules connected with one of the ligand's cyanide groups (Figure 4A). The planar aromatic tetracycle is involved with multiple hydrophobic interactions with residues P124, S127 and V128 from monomer A. In case of the brominated biaryl-imidazole derivative (Figure S1B) in 4YL3, the bi-aryl hydrophobic tail interacts with P124, S127, V128, T131, L132 and L135 from monomer A (Figure 4B). In both structures, water molecules mediate protein-ligand interactions in order to fill distances and satisfy hydrogen bond's donors/acceptors in both the ligand and the receptor (Figure 4, Table 1) [21, 22]. Other X-ray crystal structures (PDB ID: 4YK5 and 4YL1) have captured the binding of two indole-carboxylic acid derivatives (Figure S1C-D) to mPGES-1, showing double salt bridges between the inhibitor's carboxylate groups and the side chain of R52 from monomer B (Table 1, Figure S2). In both structures, bridging water helps in mediating another interaction between the carboxylate and the neighboring residue H53. The aromatic ring, attached to the indole's nitrogen, extends in the binding groove above GSH, making hydrophobic interactions with the side chains of R38, L39, and F44 (from monomer B) and also with GSH. The fluorinated biaryl extension goes down the hydrophobic binding pocket making strong hydrophobic interaction with V128, Y130, and T131 (from monomer A) and on the other side with Y28 and I32 (from monomer B) (Figure S2, Table 1).

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**Figure 4.** X-ray binding mode analysis of A) the phenanthrene-imidazole derivative (MF-63**,** Figure S1A) inside the binding pocket of mPGES-1 (PDB ID: 4YL0); the residue R52 from 4YL0 (in orange) is superimposed on the same residue from 4YL3 (the pink side chain) in order to show the difference of the side-chain conformation. B) The biaryl-imidazol derivative (Figure S1B) inside the binding pocket of mPGES-1 (PDB ID: 4YL3).

## *2.2. Molecular docking and enrichment studies*

We tested the reliability of the Glide docking program (Schrödinger suite 2016) if the observed binding modes of inhibitor ligands in mPGES-1 crystal complexes (PDB ID: 4YK5, 4YL0, 4YL1, and 4YL3) were reproducible by docking experiments using the respective protein structures [22]. All crystal ligands were successfully docked inside their respective crystal structures with low root-mean-square deviation (RMSD; 0 to 0.5 Å) to confirm the applicability of molecular docking experiments (Table S1 in Supplementary Information). However, the cross-docking of these four inhibitors in other crystal structures did not always produce the correct binding modes due to the distinct conformations of some flexible residues in different crystal structures. For example, the Arg52 (R52) adopts different orientations in order to make the optimal hydrogen bonds network between the specific enzyme structure and its respective ligand. The phenanthrene-imidazole ligand (MF63; Figure S1A) in 4YL0 with its voluminous aromatic tetracycle skeleton forces R52 to shift 1.15 Å outwards as compared to the same residue in structures 4YL3 and 4YL1 (Figure 4A). Consequently, docking programs may fail to dock phenanthrene-imidazole derivatives correctly to other crystal structures (4YK5, 4YL1, and 4YL3) due to a steric clash between the phenanthrene-imidazole ring and the R52, which would prevent the observed binding mode in the crystal structure 4YL0. Similarly, the indole-carboxylic acid derivative in structure 4YL1 shows a similar sensitivity for the right orientation of R52 in order to establish the salt bridge interactions. As a consequence, the correct binding mode of co-crystallized inhibitor in 4YL1 (Compd. **3** in Figure S1D) in different crystal structures was not always the top-scored solution but it appeared as the second or third-ranked docking solution (Table S1 in the Supplementary Information).

Enrichment can be generally assessed by the number of active compounds detected at a given percent of the decoy set (presumed non-binding molecules) by score-ranked poses. Enrichment was calculated at 5%, 10%, and 20% of the ranked docked decoys by applying Glide SP score for ranking (Table 2). Another enrichment metrics were also estimated for the enrichment studies such as the area under the accumulation curve (AUAC), and the area under the receiver operating characteristic (ROC) curve. The area under ROC curve generally represents the inherent capability of the docking protocol and the used crystal structure to discriminate between the actives on one hand, and the inactives and the set of decoy molecules on the other hand. The results for 4YL0 structure indicate that 47.6% of the actives are retrieved when 20% of the decoys are captured (Figure S3). That yields the highest enrichment metrics for 4YL0 structure having area under ROC curve as 0.75, while the area under ROC curve for structures 4YL3, 4YL1, and 4YK5 is 0.67, 0.64 and 0.63, respectively. That difference in the performance can be simply explained by the incorrect docking of the highly active phenanthrene-imidazole derivatives in both structures 4YL3 and 4YK5 as pointed out earlier.

**Table 2.** Enrichment metrics for glide docking of mPGES-1 inhibitors together with 1000 decoy compounds from SCHRODINGER’s decoys set.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Enrichment**  **[1000 Decoys + Inactives (IC50 ≥ 5 μM)]** | **5%**  **Decoys** | **10%**  **Decoys** | **20%**  **Decoys** | **AUAC** | **ROC** |
| **4YL0** | 16.1 | 31.2 | 47.6 | 0.72 | 0.75 |
| **4YL3** | 14.35 | 23.7 | 33.7 | 0.63 | 0.67 |
| **4YK5** | 10.45 | 18.34 | 28.3 | 0.60 | 0.63 |
| **4YL1** | 11.63 | 19.84 | 29.58 | 0.61 | 0.64 |

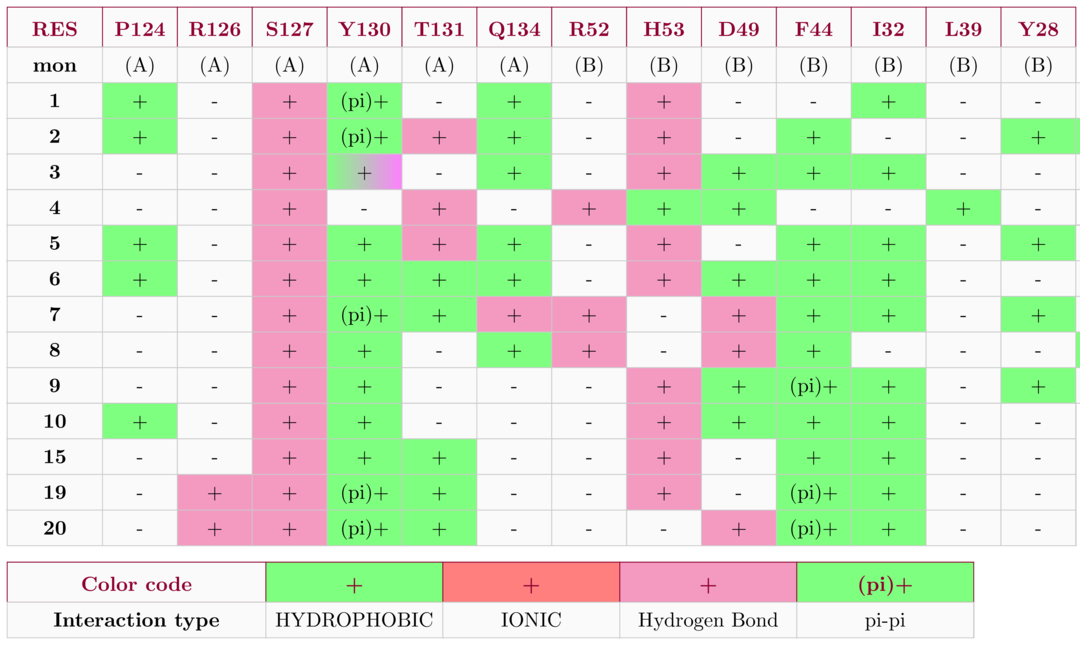
## *2.3. The virtual screening work-flow*

The general applied VS protocol includes several steps that are summarized in Figure 3. In the first step, a proper chemical library was generated starting from the MolPort screening library with adequate geometries, ionization states, conformations, and tautomers using the LigPrep module in SCHRODINGER 2016 suite. In the early step, the Lipinski’s rules of five (RO5) were applied as filter in order to establish a library with compounds displaying high drug-likeness [27]. Both crystal structures (4YL0 and 4YL3) were used in the VS study in order to take two different conformations of the R52 side chain into account. In the first structure-based VS tour, all drug-like compounds retrieved from MolPort database (about 5 mio compounds) were subjected to molecular docking to the mPGES-1 binding site using the crystal structure 4YL0. Later, the top scored compounds (40,000 compounds) were docked again inside another crystal structure, namely 4YL3, in order to take into account the two different conformations of the R52. Then, a first diversity-based selection protocol was applied to the docking poses that survived using both structural MACCS- and pharmacophore-based fingerprints application in SCHRODINGER’s canvas to obtain 1000 docking complexes with chemically diverse scaffolds. In the next step, the open-source software PLIP (protein-ligand interaction profiler) was used to process the final 1000 docking complexes for characterization of the most diverse protein-ligand interaction pattern [28]. As a result, a subset of 49 hits was selected based on diversity observed in protein-ligand interaction profiles as well as in chemical structures. Finally, ten candidates (Figure 5) were ‘cherry-picked’ from this subset based on chemical intuition and literature knowledge considering the geometrical and pharmacophoric features (i.e., non-acidic features). All selected candidates were chosen to have good similarity (Tanimoto index > 0.75) to the protein-interaction profile of the mPGES-1-co-crystallized inhibitors, which provide one hydrogen bond at least to residues of H53, R52 and S127, and at least two hydrophobic interactions with the set of residues comprising F44, P124, Y130, L39 or Y28 as presented in Table 3.Accordingly, to further investigate the stability of the binding modes of selected compounds to mPGES-1 taking into account potential ligand-induced conformational changes, we performed MD simulations (10 ns) using the 3D structure of mPGES-1 (PDB ID: 4YL3 in complex with these docked ligands), followed by MM-PBSA energy decomposition per residue as will be discussed later.

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**Figure 5.** Chemical structures of the selected VS hits. Among them, the two structures; **6** and **8**, were identified as promising mPGES-1 inhibitors in cell-free activity assays with IC50 = 1.15 and 1.25 μM respectively.

**Table 3.** The protein-ligand interactions for the VS-hits and Compound **8** derivatives docked inside mPGES-1 crystal structure 4YL3.



*2.4. MD simulations: Analysis, MM-PBSA calculation, and energy decomposition*

MD simulations were performed on the selected VS hits in order to prove the stability of the suggested binding mode and to examine the dynamic variations of the ligand-protein interactions. The time series of the RMSD of the docking complexes were under 2.6 Å over 10 ns of MD simulation, and under 0.5 Å for the simulated ligands in the putative binding pocket. To further investigate the protein-ligand interactions over the MD trajectory, the MM-PBSA method was used to estimate the binding energy and to perform energy decomposition by residue (using g\_mmpbsa tool) [29, 30]. However, an efficient algorithm for calculating the entropy term is still lacking [31-33]. Additionally, the presence of the bi-layer membrane in the case of membrane proteins would make the exact description of desolvation and calculation of entropy term even more complex. Therefore, the application of MM-PBSA binding energy calculation was not intended to reproduce the exact binding free energy, but to estimate the energetic contribution of the key protein residues to the protein-ligand binding. The calculated binding free energies of the native co-crystallized ligands in two mPGES-1 crystal structures (4YL0 and 4YL3) as well as three of the selected VS compounds (compounds **6**, **7**, and **8**) are listed in Table S3, with the contributions from vdW, electrostatic interaction, polar desolvation, and apolar desolvation energy terms using MM-PBSA method.

The decomposition of the binding energy of the mPGES-1/VS-hits complexes showed a significant contribution of aforementioned key residues (S127, R126, H53, and R52) in establishing a hydrogen bonds’ network between the ligand and the protein. The most recognized hydrophobic residues in the decomposition analysis appeared to be P124, A123, F44, L39, and Y130. The list of key residues can be extended to T131, Q134, I32, and Y28. The binding of Compound **6**, as an example, appeared to be mostly driven by interactions with several hydrophobic residues such as P124, L39, and I32, and A123 while the electrostatic and polar interactions were contributed mainly by S127, R52, R126, and Q134. In the case of Compound **8**, the hydrophobic interactions were contributed mainly by Y130, P124, L39, and I32, while polar interactions were mainly made with H53, R38, and S127. Moreover, the analysis of MD snapshots with an explicit solvent model can also show water-mediated interactions between the studied ligands (from the VS selected compounds) and some key residues such as R53, H53, or S127. In both cases of Compounds **6** and **8**, we observed an optimal orientation of one aromatic ring close to the residues F44 and L39, while other aromatic rings interact with other hydrophobic residues, e.g., Y28, Y130, T131, and I32 (Figure 6).

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**Figure 6.** The suggested binding mode of the identified mPGES-1 inhibitors compounds **6** and **8**, as docked inside the putative binding pocket of mPGES-1 (PDB ID: 4YL3).

## *2.5. Biological evaluation and SAR*

The mPGES-1 inhibitory activity of the acquired compounds was assessed using a well-established cell-free assay based on incubation of microsomes from IL-1-activated human A549 cells, which strongly express mPGES-1, with 20 µM PGH2 as substrate [34]. The mPGES-1 inhibitor MK-886 was used as a reference compound (IC50 = 1.6 µM). Among the tested compounds (1 and 10 μM, final concentration), two compounds, **6** and **8**, effectively repressed the activity of mPGES-1 while others were found less efficient (Figure 7). More detailed concentration-response analysis revealed the IC50 values of 1.15 ± 0.15 µM for Compound **6** and 1.23 ± 0.37 µM for Compound **8** (Table 4). Because many mPGES-1 inhibitors tend to inhibit also COX-1 (including MK886) [5, 35], we investigated the two hit compounds for COX-1 inhibition. However, Compounds **6** and **8** displayed no inhibitory effectiveness against COX-1 activity in a cell-based assay using human platelets up to 10 µM (Figure S4).



**Figure 7**. Inhibitory effects of newly identified hit compounds **1**-**10** on PGE2 formation at concentrations of 1 µM (white bars) and 10 µM (grey bars) using the IL-1-activated A549 cells expressing mPGES-1 and 20 µM PGH2 as substrate. Data are given as means  SEM, n=3.

**Table 4.** In vitro inhibitory activities of newly identified mPGES-1 inhibitors under cell-free conditions

|  |  |
| --- | --- |
| **Compound** | **IC50 (µM)a** |
| **6** | 1.15 ± 0.15 |
| **8** | 1.23 ± 0.37 |
| **12** | 1.9 ± ? |
| **14** | 8.6 ± ? |
| **15** | 0.5 ± ? |
| **16** | 5.0 ± ? |
| **17** | >10 |
| **18** | 1.0 ± ? |
| **19** | 0.3 ± ? |
| **20** | 0.6 ± ? |

a The IC50 values are given as mean ± SEM of n = 3-4 determinations.

Based on the VS results obtained, we selected Compound **8** for further structural optimization to derive a preliminary structure-activity relationship in a small series of synthesized (**12**, **14**-**16**,see supporting information for synthesis) or commercially available (**17**-**20**) compounds (Figure 8). Firstly, we focused on the heterocyclic ring occupying the deep hydrophobic binding cavity and obtained the closely related analogues having benzothizole (**12**) and benzoxazole (**14**) rings. Results from the biological assessment of mPGES-1 inhibition by **12** and **14** indicated that Compound **12** having a benzothiazole ring (IC50 = 1.9 µM) was superior to the compound having benzoxazole (**14**, IC50 = 8.6 µM) counterpart (Table 4). Next, we investigated the position of the 3-acetylamino function and obtained the 4-acetylamino congeners (Compounds **15**-**17**) with the hypothesis of the gain of further favorable contacts with amino acids R52, H53 and F44 at the upper part of the substrate binding cavity. As seen from Table 4, Compound **15** with benzothiazole ring as compared to **16** and **17** with benzoxazole and benzimidazole, respectively, resulted in significant inhibition of mPGES-1 activity (IC50 = 0.5, 5 and >10 µM, respectively). Removal of the acetylamino function in the benzothiazole series (Compound **18**) caused a small decrease in the inhibitory activity (IC50 = 1 µM). At this point, in particular, the presence of the benzothiazole ring guaranteed the formation of the most potent mPGES-1 inhibition, and additionally suggested that the presence of more appropriate polar aromatic functions at the upper end of the molecule might significantly contribute to the inhibitory activity by establishing additional polar and π-π interactions with amino acids at the cytosolic entrance (i.e., R52, H53, R126, F44). Therefore, we searched the MolPort database for closely related congeners for purchasing and obtained Compounds **19** and **20** having benzofurazan and benzoxazolone rings, respectively, as a replacement of the 4-acetylaminophenyl part in compound **15**. As expected, both compounds profoundly inhibited the mPGES-1 activity with IC50 values of 0.3 and 0.6 µM, thereby corroborating our hypothesis (Table 4).



**Figure 8.** Chemical structures of Compound **8** analogues

*2.6. Docking studies and molecular dynamic (MD) simulations*

To provide further insights into the interaction of **15**, **19**,and **20** with mPGES-1, we performed docking studies in combination with MD simulations (20 ns) using the mPGES-1 crystal structure (PDB code: 4YL3). According to the docking and MD simulations performed on the inhibitors/protein complexes inside lipid bilayer (POPC model), we clearly observe stable π-π interactions between the benzothiazole aromatic rings of all three compounds and the residue Y130 from mPGES-1 structure (Figure 9). Moreover, the enhanced protein-ligand interactions are mainly noticed inside the catalytic pocket above GSH where **15**, **19** and **20** interact with residues H53, D49, R126 or S127 by hydrogen bonds, while the aromatic rings of **19** and **20** (benzofurazan in **19** and benzoxazolone in **20**) establish additional π-π stacking or charge-π interactions with the residues F44 or R126, respectively. MD studies of both **19** and **20** offer an overview of stable hydrogen bond with H53 (for **19**) or S127 (for **20**), stabilized by one or two additional hydrogen bonds with R126 (for **19**) or D49 (for **20**). Moreover, both compounds were able to maintain stable π-π stacking with at least two of the aromatic residues of the binding pocket such as Y28 and Y130 (for **19**), or Y130 and F44 (for **20**).

|  |  |
| --- | --- |
| **A** | **B** |
|  |  |
| **C** | |
|  | |

**Figure 9.** Binding mode analysis of **15**, **19** and **20** during interaction with mPGES-1 (PDB code: 4YL3) considering membrane residues. Main interactions are represented schematically considering MD simulations in the time window 0-20 ns. Hydrogen bonds are represented by yellow dashes, while the π-π stacking interactions are represented by red dashes.

# 3. Conclusion

There is currently a strong interest in the development of mPGES-1 inhibitors because of their promising potential as effective therapeutics in inflammatory diseases and cancer with apparently fewer side-effects as compared to traditional NSAIDs. Here, we have implemented a rapid VS study on a large number of compounds from vendor libraries for identification of novel mPGES-1 inhibitor chemotypes using the recent mPGES-1 structures co-crystallized with some distinct and potent mPGES-1 inhibitors. The protocol starts with docking of a huge screening library comprising 5 million drug-like compounds, followed by fingerprints-based clustering and diversity-based selection. Subsequent steps were performed to ensure the protein-ligand interaction fingerprints as observed in the four crystal structures. Out of ten candidates that were tested, the sulfonamide-containing Compounds **6** and **8** were identified as potent suppressors of PGE2 biosynthesis without inhibiting COX-1 activity. Depending on the study of various crystal structures of mPGES-1, it appears that the binding to the region around glutathione is quite important in order to block the access of the substrate PGH2 in which the hit compounds **6** and **8** efficiently occupy. In addition, starting from **8**, we have performed a preliminary structure-activity study in a small series of closely related structures, and obtained **15**, **19** and **20** with an improved activity(IC50=0.3-0.6 µM). Computational data demonstrated that the more potent benzothiazole derivatives **15**, **19** and **20** were able to gain several interactions at the upper part of the substrate binding site without affecting the original binding mode of **8**. As a result, our protocol was successful in capturing two promising scaffolds for further chemical development as non-acidic mPGES-1 inhibitors.

# 4. Experimental methods

# *4.1. Computational studies*

## *4.1.1. Docking: Crystal structures preparation and enrichment studies*

Four scaffolds of active mPGES-1 inhibitors were presented in four recently published crystal structures (PDB ID: 4YK5, 4YL0, 4YL1 and 4YL3) in which 4YK5 and 4YL0 are in complex with two biaryl-indoles; 4YL0 is in complex with phenanthrene imidazole (MF63), while the inhibitor structure in 4YL3 is a biaryl-imidazole (Compd. 3 in Fig. S1B) [22]. The PDB coordinates of the x-ray complexes 4YK5, 4YL0, 4YL1 and 4YL3 were obtained from the protein data bank website as a biological assembly of three identical chains. The biological assembly files were imported into Maestro software and merged in order to obtain the homotrimer of mPGES-1 structures. Charges and bond orders were assigned, hydrogens were added to the heavy atoms, and all waters were deleted. Docking studies were carried out in one of the ligand binding sites of the crystal structures 4YK5, 4YL0, and 4YL3. Glide energy grids were calculated inside a box centered on the co-crystallized ligand’s centroid with dimensions of 15 Å [36].

The Schrödinger decoy set, which consists of 1000 drug-like compounds with an average molecular weight of 400 Daltons, was used to validate the docking protocol and the suitability of the used crystal structures [37]. The enrichment studies were carried out by docking Chembl compounds with reported bioactivity against mPGES-1 (from Chembl 21 database [38]) after seeding them with Schrodinger decoys set. We considered all reported structures with Ki or IC50 less than 1 μM as part of the mPGES-1 active inhibitors (303 structures), while the compounds with IC50 > 5 μM as a set of inactives (412 structures). The interactions between the protein with the ligand in the crystal structures were characterized using the open-source program ‘protein–ligand interaction profiler’ (PLIP) [28].

## *4.1.2. Virtual screening: Library preparation, docking and filtration*

Molport’s screening library is a well-maintained database of commercially available screening compounds integrated with ZINC library, and synchronized with the most warehouse databases of prominent chemical companies. The Molport screening library, which contains a collection of 6.5 million synthetic compounds from 21 chemical vendors (as downloaded in March 2016), has been imported into Canvas software [39] in order to build the 3D coordinates and calculate the physio-chemical properties. The imported structures have been filtered according to Lipinski’s rules of five (log P in the range −0.4 to +5.6, molecular weight in the range 150 to 500, polar surface area (PSA) ≤ 140, hydrogen bonds donors ≤ 5, hydrogen bonds acceptors ≤ 10) [27]. The drug-like selected 5 million chemical compounds have been prepared using LigPrep [40] in order to use for generation of all the possible isomers and tautomers, lowest energy ring conformations, and also all possible ionization states (protonation/deprotonation) at Ph = 7.0 ± 1 using the Epik software [41]. The generated isomers/tautomers were docked using GLIDE [42] in one of the binding pockets of the crystal 4YL0 inside the generated grid and Glide SP score as the fitness function. The top 40,000 docking solutions with highest Glide scores were later docked again inside the crystal structure of 4YL3 in order to perform ensemble docking. From both docking results, we have selected the top-scored 10,000 docking solutions in order to select a list of virtual screening hits. The selected 10,000 docking solution were imported in Canvas software [39] in order to generate two kinds of fingerprints such as structure-based and pharmacophore-based fingerprints. In the final step, a clustering and diversity-based selection was applied using Tanimoto similarity metric in order to choose 1,000 unique scaffolds from the list of the top ten thousand docking solutions, and later the protein-ligand interactions were applied on the chosen docking complexes also using the open-source ‘protein–ligand interaction profiler’ (PLIP) as a stand-alone program [28].

## *4.1.3. Molecular dynamics (MD) simulation*

In order to obtain the coordinates of the mPGES-1 crystal structures (PDB ID: 4YL0 and 4YL3) immersed in bi-layer of POPC (monounsaturated 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) membrane, the MD system preparation in SCHRODINGER suite 2016 was used [43], and the generated system was later compared to the membrane location in OPM database (4YL3.pdb is available in OPM) [44]. The MD simulation was performed using the open-source software Gromacs 5.1 [45] as the protein atoms were parameterized by the amber99sb force field, while the LIPID14 amber’s special force field for lipids was used for the lipid bi-layer membrane [46]. The docked ligand/inhibitor was parameterized by GAFF force field using amber14’s antechamber tool [47], and converted to Gromacs coordination and topology format using ACPYPE software [48]. For the preparation for the molecular dynamics system, the protein was centered inside a cubic box with dimensions 9.50, 9.50 and 8.50 nm. TIP3P model of water molecules has been used when the system was solvated on both sides of the membrane. The system was neutralized using a mixture of the chloride Cl- and sodium Na+ ions to provide 100 mM salt concentration. After energy minimization, a short NVT equilibrium was applied for 100 ps (50,000 steps with a 2 fs time-step) with position restrains applied on the protein’s backbone. Temperature coupling is performed on four groups of the simulated system: Protein, lipids (POPC), ligand, and water-ions using V-rescale algorithm (a modified Brendesen thermostat) in order to reach a temperature of 310 K. Leap-frog integrator was used with linear constraint solver (LINCS) algorithm applied to all bonds. The cutoff for short-range electrostatics and van der Waals interactions was 1.2-nm. The second phase of NPT equilibrium was performed for 500 ps (250,000 steps) using similar conditions like the previous NVT equilibrium (Position restrains and short-range interactions’ cutoff), except using Nose-Hoover thermostat for temperature coupling at 310 K. The temperature coupling was also applied to the exact four groups of the system. After the two equilibration phases, a production simulation is performed with the same parameters as NPT run but without position restrains.

## *4.1.4. MM-PBSA calculation*

For the calculation of MM-PBSA energy, 50 snapshots were extracted from the last nanosecond of 20 nanoseconds MD trajectory, which means using the interval of 20 ps from the production trajectory between 19 to 20 ns. The program g\_mmpbsa [30] was used for estimating the binding free energies, and later to perform the energy decomposition in order to obtain the contribution per residue to the binding energy. The energy components (∆EMM, ∆GP-SOLV and ∆GNP-SOLV) for individual atoms were calculated in the bound and the unbound form, and subsequently their contribution to the binding energy of residue x, abbreviated as ∆R\_BE(x)is calculated. ∆EMM is the average molecular mechanics potential energy in vacuum, while ∆GSOLV is the free energy of solvation and is divided into polar term ∆GP-SOLV non-polar term ∆GNP-SOLV. The calculation was performed depending on the solvent accessible surface area (SASA) model, which assumes linear correlation between SASA and the non-polar solvation energy. As the entropy contribution is not included in this protocol, the calculated energy is not expected to be comparable to the absolute binding energy, but to relative binding energy. In MM-PBSA, the binding energy is evaluated according to the equation below:

∆Gbind = ∆EMM + ∆GP-SOLV + ∆GNP-SOLV -T.∆S = ∆EVDW +∆EELE + ∆GP-SOLV + ∆GNP-SOLV -T.∆S

The Entropy term (-T.∆S) wasn’t calculated during this study. ∆GP-SOLV is the polar solvation contribution calculated by solving the non-linear Poisson-Boltzmann (PB) equation using the open-source program APBS [49]. The values for the solute and solvent dielectric constants were chosen to be 7 and 80, respectively [50, 51]. The non-polar solvation free energy, ∆GNP-SOLV was estimated by the solvent accessible surface area (SASA) using a water probe radius of 1.4 Å, according to the equation ∆GNP-SOLV = γ.SASA + b where the constants γ and b were set to 0.022 kJ/mol/Å2 and 3.84 kJ/mol, respectively [30].

## *4.2. Biological assays*

*4.2.1. Determination of microsomal PGE2 synthase activity*

For determination of mPGES-1 activity, microsomal preparations of A549 cells were prepared as previously described [34]. In brief, A549 cells were cultivated in DMEM medium containing FCS (2%) and IL-1β (2 ng/mL) for 72 h (37 °C, 5% CO2). Cells were harvested and resuspended in homogenization buffer consisting of potassium phosphate (0.1 M, pH 7.4), phenylmethanesulfonyl fluoride (1 mM), soybean trypsin inhibitor (60 µg/mL), leupeptin (1 µg/mL), glutathione (2.5 mM), and sucrose (250 mM). After shock-freezing the cells in liquid nitrogen, sonication (3 × 20 s), differential centrifugation at 10,000 g (10 min, 4 °C) and 174,000×g (60 min, 4 °C), the pellets were resuspended in homogenization buffer. Microsomes were diluted in potassium phosphate buffer (0.1 M, pH 7.4) with glutathione (2 mM) and pre-incubated with the test compounds or vehicle (0.1% DMSO) on ice for 15 min. After stimulation (1 min, 4 °C) with 20 µM PGH2 as substrate the reaction was terminated by addition of stop solution containing FeCl3 (40 mM), citric acid (80 mM), and 11β-PGE2 (10 µM as internal standard) and analyzed for PGE2 product formation by RP-HPLC as reported before [34].

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**Supporting Information Available**.

Additional computational data, chemical structures of x-ray ligands, COX-1 activity assays and synthetic procedures for compounds are given in Supporting Information.

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