Target Identification

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# Revealing the Macromolecular Targets of Fragment-Like Natural Products**

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***Abstract:*** *Fragment-like natural products were identified as ligand-efficient chemical matter for hit-to-lead development and chemical-probe discovery. Relying on a computational method using a topological pharmacophore descriptor and a drug database, several macromolecular targets from distinct protein families were expeditiously retrieved for structurally unrelated chemotypes. The selected fragments feature structural dissimilarity to the reference compounds and suitable target affinity, and they offer opportunities for chemical optimization. Experimental confirmation of hitherto unknown macromolecular targets for the selected molecules corroborate the usefulness of the computational approach and suggests broad applicability to chemical biology and molecular medicine.*

Natural products (NPs) are the focus of strong interest as chemical matter for interrogating biological systems.[1] Acting as starting points for drug and chemical-probe discovery, they comprise biologically prevalidated architectures that allow the exploration of drug-relevant chemical space not covered by fully synthetic small molecules.[2] Consequently, computational tools have been deployed for enriching collections of screening compounds by gathering structural and physicochemical features from NPs.[3] However, their applicability is tightly bound to factual knowledge of the macromolecular counterparts (e.g., target receptors) of the parent NP. The on- and off-target interactions remain largely unknown for the majority of these molecules, thus hindering the widespread and efficient exploration of NP chemical motifs as leads for chemical biology.[4] Herein, we disclose the targets of the natural products goitrin, isomacroin, and graveolinine, which we discovered by using a ligand-based computational method for predicting the targets of (de-orphaning) NPs in the absence of apparent chemotype similarity to known macromolecular target effectors.

Chemocentric approaches for target prediction have already proven their applicability to drug repurposing,[5] identifying drug liabilities,[6] and driving innovative multi-objective drug design.[7] We have recently developed a software tool for predicting the macromolecular targets of structurally complex NPs.[4a] Conversely, small fragment-like chemical entities are generally recognized as better-suited starting points for hit-to-lead discovery programs.[8] In this study, we set out to identify the targets of fragment-like NPs (molecular weight $< 300\ g\,mol^{-1}$)[9] and probe the underlying molecular basis of macromolecular recognition.

By using molecular representation in terms of topological pharmacophore features (CATS2 descriptor),[10] we trained a self-organizing map (SOM)[11] on a set of 210 213 NPs (Dictionary of Natural Products, DNP), together with 12 661 small molecules with known targets (COBRA database).[12] This allowed us to span the combined pharmacophore feature space of both drugs and NPs. The software modeled qualitative ligand–receptor relationships by using statistically interpreted similarities between drugs and NPs that co-clustered in this comprehensive space.[5a] We derived a statistical significance estimate ($p$ value) for each individual target prediction (see the Supporting Information). In a previous study, we demonstrated that this method achieves receiver-operating characteristic area under the curve (ROC AUC) values of 0.88 based on an extensive retrospective study.[5a] ROC AUC values can be interpreted as probabilities for the algorithm to rank a correct ligand–target pair higher than an incorrect pair.

The SOM algorithm afforded at least one highly confident target prediction ($p < 1\,\%$) for 80 024 (38 %) of all of the NPs annotated in the DNP, with an average of 0.9 targets per chemical entity. From the 64 650 fragment-like NPs in the DNP, 34 239 (53 %) were confidently predicted to interact with at least one target (average of 1.4 targets per fragment-like NP). Importantly, the algorithm captured known ligand–target relationships for fragment-like NPs that were not part of the training data (see the Supporting Information). With these target-engagement predictions in hand, we selected goitrin, isomacroin, and graveolinine (Figure 1) for experimental validation, taking into account: 1) their fragment-like nature; 2) the confidence of the predictions ($p < 1\,\%$, i.e., top ranking); 3) their profound structural dissimilarity to the most similar reference compound from the drug database (structural Tanimoto similarity $\leq 0.2$; Table 1), thus ensuring that straightforward similarity searches would not have recognized these potential ligand–receptor associations; 4) synthetic tractability, and 5) a lack of known macromolecular targets. Importantly, other publically available target prediction tools, for example, SEA,[5e] PASS,[13] and SuperPred,[14] failed to confidently predict either these or any other targets for the selected NPs (see the Supporting Information), thus

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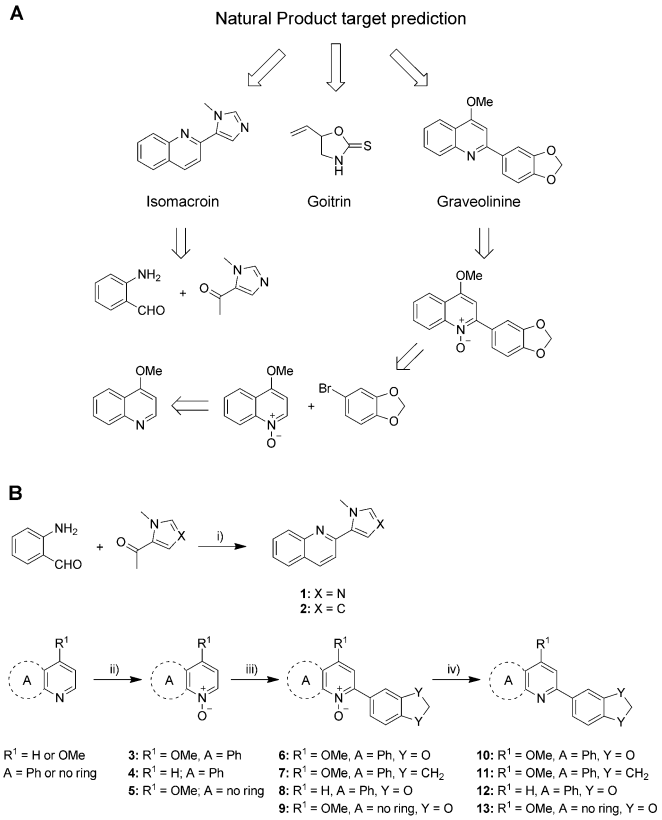



***Figure 1.*** A) Selected natural products and retrosynthetic analysis. B) Synthetic pathways. Reagents and conditions: i) KOH, EtOH, reflux; ii) *m*CPBA, $CH_2Cl_2$, RT, or $MeReO_3$, $H_2O_2$ 50%, $CH_2Cl_2$, 0°C →RT; iii) Bromoaryl, $K_2CO_3$, $P^tBu_3{\cdot}HBF_4$, $Pd(OAc)_2$, PhMe, reflux; iv) $HCO_2NH_4$, Pd/C 10%, MeOH, reflux.

highlighting the unprecedented domain of applicability of our SOM-based algorithm to extend the NP target space.

While racemic goitrin was purchased for screening, we devised concise synthetic approaches for the alkaloids isomacroin and graveolinine (Figure 1 A, B). Isomacroin (**1**) was obtained in one step via Friedländer chemistry from the required starting materials. Activation of 4-methoxyquinoline to its *N*-oxide counterpart **3** with either *m*CPBA or $MeReO_3$/$H_2O_2$ oxidation systems enabled subsequent regioselective direct Pd-catalyzed C–H arylation to intermediate **6**, as reported previously.[15] Catalytic hydrogen transfer with **6** gave access to graveolinine, **10**.

Racemic goitrin had been reported as a moderate inhibitor of dopamine β-hydroxylase.[16] According to our software, DL-goitrin was confidently predicted as a pregnane X receptor (PXR) ligand (Table 1) when predicting targets for the tautomer reported in the DNP. Interestingly, target inference was achieved from the PXR reference structure DL-sulphoraphane, which presents no substructure similarity to goitrin according to the Tanimoto similarity coefficient ($T_c = 0.02$). In a functional assay, goitrin presented modest antagonism against PXR (ca. 20% effect at 150 μM, Figure 2 A). Sulphoraphane had been previously reported as a potent PXR antagonist with a half maximal inhibitory concentration ($IC_{50}$) of approximately 12 μM,[17] but in our assay it exhibited only a similar functional effect to goitrin. Furthermore, from a constrained acetylcholine analogue, we also predicted and

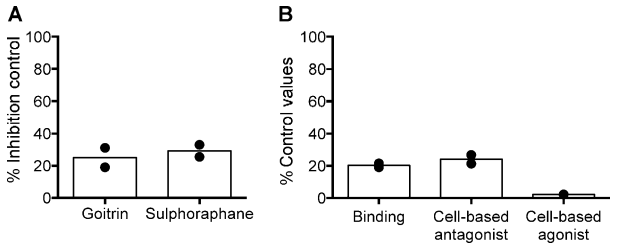
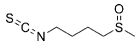
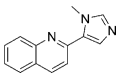
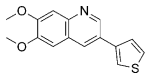
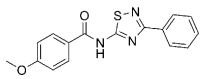
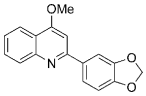
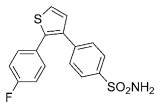
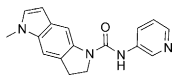



***Figure 2.*** A) Dot plot with median (bar) for the functional cell-based PXR assay. DL-goitrin and DL-sulphoraphane were assayed for antagonist effect at 150 μM; $N = 2$ (control: clotrimazole, $IC_{50} = 7.5$ μM). B) Dot plot with median (bar) for radioligand displacement and functional cell-based muscarinic $M_1$ receptor screening of DL-goitrin at 250 μM; $N = 2$ (controls: radioligand displacement: pirenzepine, $IC_{50} = 30$ nM; agonist: acetylcholine $EC_{50} = 1.8$ nM; antagonist: pirenzepine, $IC_{50} = 44$ nM).

***Table 1:*** Target predictions for fragment-like natural products and nearest neighbors from the drug reference data.

| Natural product | Structure | Target prediction (*p* value %) | Nearest neighbor structure | $T_c$[a] |
|---|---|---|---|---|
| DL-Goitrin |  | Pregnane X receptor (0.25) |  | 0.02 |
| | | Muscarinic $M_1$ receptor (0.36) |  | 0.04 |
| Isomacroin |  | Platelet-derived growth factor receptor (0.25) |  | 0.16 |
| | | Adenosine $A_3$ receptor (0.34) |  | 0.13 |
| Graveolinine |  | Cyclooxygenase-2 (0.72) |  | 0.08 |
| | | Serotonin 5-$HT_{2B}$ receptor (0.96) |  | 0.11 |

[a] Tanimoto similarity calculated from Morgan substructure fingerprints (radius = 2, nbits = 2048).

experimentally confirmed weak muscarinic $M_1$ receptor binding and engagement by goitrin at 250 μM (Figure 2B). Although the goitrin-inspired design of small molecules as PXR and $M_1$ modulators may require substantial medicinal chemistry effort, the results highlight the biophoric nature of this compound and the capabilities of the target-prediction tool in detecting subtle structural features of fragment-like NPs that are required for specific target recognition and modulation.

Isomacroin is of great pharmacological interest.[18] In accordance with the top-confidence target predictions, we screened it in a functional cell-based assay against the adenosine $A_3$ receptor with a confounding outcome at 150 μM (18% agonism and 13% antagonism). Despite the structural dissimilarity between isomacroin and its reference compound[19] ($T_c = 0.16$), we successfully revealed isomacroin to be a potent and ligand-efficient inhibitor ($IC_{50} = 25\ \mu M \pm 0.08$ log units; ligand efficiency $LE = 0.40$; Table 1 and Figure 3) of platelet-derived growth factor receptor alpha (PDGFRα). Similarly to the reference quinoline, no selectivity between the α and β isoforms was observed at 150 μM (35% inhibition of PDGFRβ). To the best of our knowledge, PDGFR kinase constitutes the first disclosed target of isomacroin. Kinase panel screening revealed more than 50% inhibition by isomacroin (25 μM) against only three (MNK2 > MAP4K4 > SIK) of 45 kinases assayed, which is in accordance with the predictions (see the Supporting Information). This outcome suggests the isomacroin scaffold as a candidate for the design of selective kinase inhibitors. We ruled out artifact and false-positive readout measurements by confirming the absence of colloidal aggregates at concentrations as high as 200 μM (see the Supporting Information). Notably, the nearest neighbor in ChEMBL[20] according to Morgan fingerprints, AG-1295 (CHEMBL7724), presents identical activity to isomacroin against PDGFRα (42% inhibition at 10 μM)[21] yet low substructure similarity ($T_c = 0.27$). These results support the feasibility of discovering innovative NP hits for the development of chemical probes and lead structures without significant loss of target modulation potential compared to structurally unmatched isofunctional molecules.

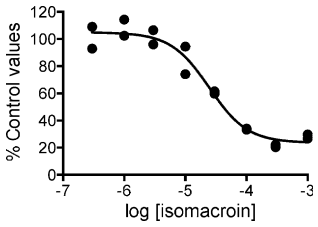



***Figure 3.*** Concentration–response curve for isomacroin against PDGFRα. $IC_{50} = 24.5\ \mu M \pm 0.08$ log units (ligand efficiency: $LE = 0.40$); $N = 2$ (control: staurosporine, $IC_{50} = 0.34$ nM).

We further probed the molecular basis of PDGFR kinase inhibition by synthesizing analogue **2**. From the sharply decreased inhibitory potency of **2** at 150 μM (3% and 10% inhibition of PDGFRα and β, respectively), our data suggest directed interactions of the imidazole moiety in isomacroin with the kinase hinge-binding motif of both PDGFR isoforms. Interestingly, isomacroin is a substructure of the recently reported single-digit nanomolar and micromolar inhibitors for PDGFRβ[22] and IKKβ,[23] respectively, thus further supporting the prevalidation of NP-derived molecular architectures, the suitability of NP fragments as leads for development and, in this specific case, the molecular binding hypothesis probed with **2**.

Finally, we selected graveolinine (**10**) from *Ruta graveolens*, which possesses antiangiogenic[24] and antitubercular[25] activities. Our software confidently predicted graveolinine to bind to the serotonin 5-$HT_{2B}$ receptor and cyclooxygenase-2 (COX-2), as inferred from the structurally unrelated small molecules pyrroloindole[26] and diphenylthiophene,[27] respectively. Efficient binding and potent serotonin 5-$HT_{2B}$ receptor antagonism were experimentally confirmed (binding: $K_i = 7\ \mu M \pm 0.14$ log units; $LE = 0.34$; antagonism: $IC_{50} = 12\ \mu M \pm 0.07$ log units; Figure 4A) together with an absence of colloidal aggregation at relevant bioactive concentrations. Analysis of known 5-$HT_{2B}$ ligands in ChEMBL revealed the equipotent alkaloid berbevine as its nearest neighbor (CHEMBL12089; $K_i = 2.2\ \mu M$; $T_c = 0.31$, Morgan fingerprints), thus endorsing the feasibility of using target prediction tools to identify potentially attrition-prone scaffolds, since the 5-$HT_{2B}$ receptor may be considered as an undesired off-target for drug development.[7b]

Graveolinine was also confirmed to engage COX-2 at 150 μM (79% effect inhibition; Figure 4B). Extracts of *Ruta graveolens* had previously shown antiplatelet aggregation

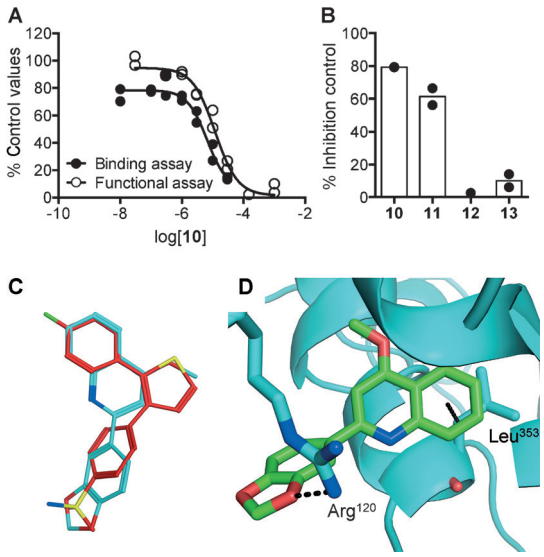



***Figure 4.*** A) Concentration-dependent binding and antagonism by graveolinine against the serotonin 5-$HT_{2B}$ receptor (binding: $K_i = 7\ \mu M \pm 0.14$ log units; Antagonism: $IC_{50} = 12\ \mu M \pm 0.07$ log units; $n = 2$). B) Dot plot with median (bar) for the inhibition of COX-2 by graveolinine (**10**) and its analogues **11**–**13** at 150 μM; $N = 2$. C) Flexible alignment superimposition of graveolinine (turquoise) onto its COX-2 COBRA reference[27] (red); Tanimoto similarity = 0.08. D) Docking pose of **10** in the active site of COX-2. Predicted key interactions are indicated with dashed lines. Image produced with PyMOL (Schrödinger LLC).

activity induced by 100 μM arachidonic acid,[28] which may now be explained by COX-2 inhibition by graveolinine. The result obtained pinpoints potential competence of the SOM algorithm in target deconvolution from observations of phenotype changes induced by complex compound matrices. In spite of negligible structural similarity to the reference compound, flexible ligand alignment (MOE v2014.9) with perfectly matched pharmacophore features corroborates the retrieval of COX-2 as a target of graveolinine (Figure 4B). The nearest neighbor of graveolinine in CHEMBL COX-2 inhibitor space (CHEMBL2023860)[29] also presents low structural similarity ($T_c = 0.42$). It is important to point out that graveolinine does not possess structural features of potential assay interference compounds, unlike its CHEMBL2023860 nearest neighbor, aminothiazole.[30]

We then set out to gain insight into the molecular basis of COX-2 recognition by graveolinine. Molecular docking into the active site of COX-2 was carried out with GOLD/GoldScore (v5.2.2)[31] (PDB 4PH9[32]) to afford a potential explanation for the observed functional effects. The binding model suggests key directed interactions with $Arg^{120}$ and $Leu^{353}$, as well as hydrophobic contacts stemming from the methoxy group (Figure 4D). Hence, we probed the mode of binding of graveolinine by synthesizing analogues **11**–**13**. In accordance with the binding model, compounds **11**–**13** showed reduced potency at 150 μM compared to graveolinine (Figure 4B), thereby corroborating the proposed basis for molecular recognition. Withdrawal of the ether hydrogen-bond acceptor that potentially interacts with $Arg^{120}$ (compound **11**) resulted in marginal effects on activity. In fact, aromatic ethers are weak hydrogen-bond acceptors,[33] which fully supports the obtained biochemical data. Truncation of the OMe group (compound **12)** revealed that this group significantly contributes to binding efficiency. Interaction with $Leu^{352}$ may also be key for enzyme inhibition since disruption of the suggested $CH_2 \cdots \pi$ interaction between $Leu^{353}$ and the quinolyl moiety resulted in a sharp loss of potency. Altogether, this small focused analogue library affords a rationale for future derivatization and the design of graveolinine-inspired COX-2 inhibitors.

With the biological prevalidation of NP architecture in mind, the results of our present study suggest an expeditious solution for rapid target identification for NPs. Together with fragment-based drug design, such platforms could be used for the efficient and economical design of NP-derived chemical tools and drug leads, as well as for the analysis of phenotypic screening hits. The results obtained support the use of target prediction tools as an enabling technology for the NP-inspired design of new chemical entities in chemical biology and molecular medicine.

**Keywords:** chemical biology · computer-assisted drug design · drug discovery · medicinal chemistry · target identification

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