Journal of Medicinal Chemistry

Article

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Experimental Confirmation of New Drug−Target Interactions Predicted by Drug Profile Matching

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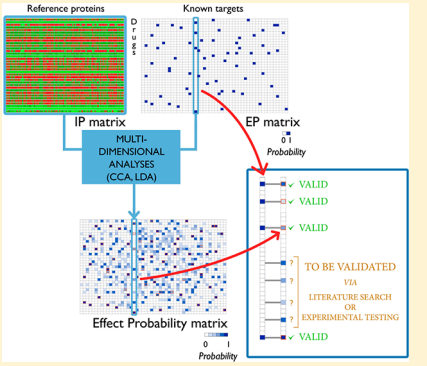
Supporting Information

ABSTRACT: We recently introduced drug profile matching (DPM), a novel affinity fingerprinting-based in silico drug repositioning approach. DPM is able to quantitatively predict the complete effect profiles of compounds via probability scores. In the present work, in order to investigate the predictive power of DPM, three effect categories, namely, angiotensin-converting enzyme inhibitor, cyclooxygenase inhibitor, and dopamine agent, were selected and predictions were verified by literature analysis as well as experimentally. A total of 72% of the newly predicted and tested dopaminergic compounds were confirmed by tests on D1 and D2 expressing cell cultures. 33% and 23% of the ACE and COX inhibitory predictions were confirmed by in vitro tests, respectively. Dose-dependent inhibition curves were measured for seven drugs, and their inhibitory constants (K_i) were determined. Our study overall demonstrates that DPM is an effective approach to reveal novel drug−target pairs that may result in repositioning of these drugs.



INTRODUCTION

The high failure rate of drug candidates due to unexpected adverse reactions and lack of expected clinical efficacy have become fundamental problems of drug development. Despite the increasing efforts and resources spent on biomedical research, the number of new molecular entities stagnates.[1]

One result of this trend was the development of alternative strategies in pharmaceutical research, such as a turn from the discovery of new chemical entities toward drug repositioning or repurposing. Drug repositioning seeks new therapeutic applications of existing drugs and requires on average approximately 5−8 years from discovery to the market.[2] While intellectually less novel, this process can also be considered a safer, cheaper, and faster way of drug development, given that compounds successfully passed clinical trials previously. However, drug repurposing also should not be oversimplified, since several aspects need to be considered before making decisions such as patent status, market characteristics, and whether the new indication represents an unmet medical need.[2] Nevertheless, the developmental risk can be said to be smaller,[2] compared to the discovery of new chemical entities. One of the well-known examples of drug repositioning is imatinib (Gleevec) that was first approved for chronic myeloid leukemia[3] but that was subsequently approved for gastrointestinal stromal tumors.[4] As another example, zidovudine was developed in the 1960s as a potential anticancer agent but failed to show efficacy. However, in 1985 it was found to be effective against AIDS as a reverse transcriptase inhibitor and became the first approved anti-HIV drug.[2] Finasteride was repositioned from the treatment of prostate enlargement to an antibaldness agent after the discovery that its target, 5α-reductase, is involved in these biologically distinct (though both hormonally driven) processes.[5] Thalidomide, which once caused severe fetal defects in pregnant women when used as an antiemetic agent, was successfully reintroduced as an antileprosy drug (with certain limitations on its usage).[6]

Aspirin was repositioned as a platelet aggregation inhibitor approximately 90 years after its introduction in 1899,[7,8] illustrating that the complete effect profiles, i.e., the whole therapeutic effect spectrum a compound exerts when administered to a human body, are often unknown even for the oldest drugs. Besides similarity in the molecular biology of diseases, drug repositioning is often driven by serendipity or SAR considerations. Therefore, there is a clear need for a systematic screening method that is able to predict the

Received: May 31, 2013

   dx.doi.org/10.1021/jm400813y | J. Med. Chem. XXXX, XXX, XXX−XXX

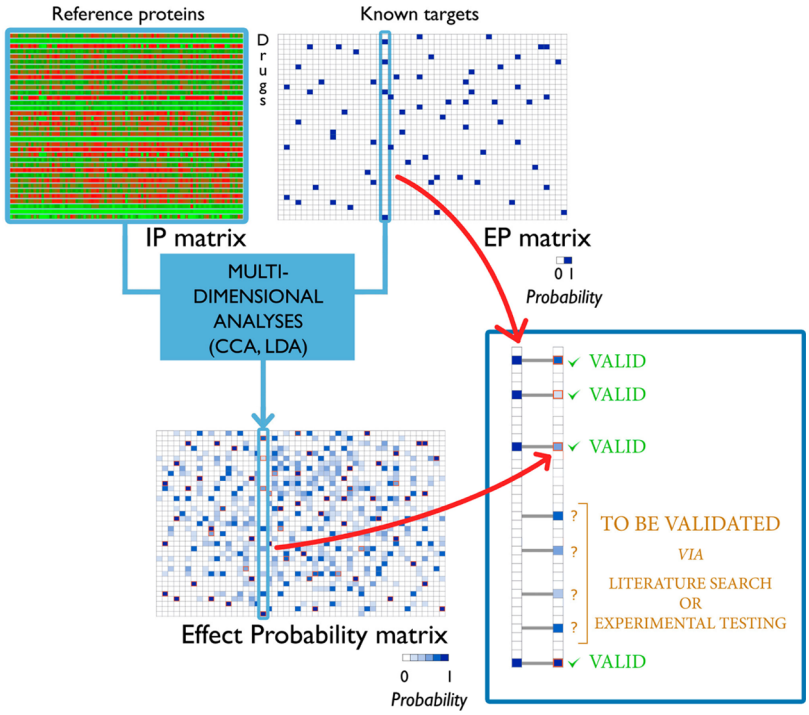


Figure 1. Graphical summary of the drug profile matching method. The interaction pattern (IP) matrix consists of the calculated binding free energies for the 1177 drugs studied on the reference panel of 149 nontarget proteins (i.e., proteins that are not known to be involved in the mechanism of action of the drugs). The effect profile (EP) matrix contains pharmacological effect information on the drug set in a binary form (blue and white cells represent the presence and the absence of a given effect from the 177 categories, respectively). A two-step multidimensional analysis (canonical correlation analysis, CCA, and linear discriminant analysis, LDA) was performed using the IP and EP matrices as inputs to calculate the effect probability matrix. This matrix displays the probability values for each drug−effect pair. The darker is a given cell, the higher is the predicted probability. Comparison of the same effect (i.e., same column) in both effect matrices reveals those predictions that are already known (marked as valid) and highlights the predictions that need to be validated via literature search or experimental testing.

complete effect profiles of compounds, quantitatively describing their probability of exhibiting a given effect.

Experimental pharmacological data can be a rich source of drug repositioning. For example, the BioPrint database by Cerep contains screening results of 2500 FDA drugs and reference molecules against 159 enzymes, receptors, ion channels, and transporters.[9] Part of this database was used to develop "biospectra analysis" that applies a series of in vitro percent inhibition values handled as a compound descriptor[10−12] that can then be used, for example, to retrieve compounds with a similar profile (and hence similar expected effects) from the database. Kauvar et al. developed affinity fingerprinting, a method for the characterization of compounds by their binding affinities to a set of proteins.[13] The vector of the binding affinity values is considered as a descriptor and is used to predict the activity of a given compound against a target not included in the protein set. Affinity fingerprinting was successfully applied to find new cyclooxygenase (COX) inhibitors among a set of druglike compounds.[14] When only 62 library compounds were tested, three structurally novel active compounds were discovered.

On the computational side, Koutsoukas et al. summarize several recently developed in silico pharmacology approaches that might offer candidates for off-target based drug repositioning.[15] These techniques are in line with current thinking of polypharmacology that states that drugs more often than not act on multiple targets.[16−19] In particular, each drug hits on average six known targets according to a recent analysis on interaction data.[20]

One of the first approaches in the field of in silico pharmacology was PASS (prediction of activity spectra for substances), which applies a set of 2D descriptors to compounds that are then correlated with a set of bioactivities.[21] Bender et al. developed a similarity approach called Bayes affinity fingerprint in which binding affinity information against a set of target proteins is used for virtual screening with retrieval rates higher than those of conventional fingerprints.[22,23] Keiser et al. used ligand chemical similarity to obtain biologically relevant clusters of 246 enzymes and receptors.[24] This method was used for biological activity prediction by calculating the chemical similarity values of the query set (one molecule or a set of compounds) to the 246 representative ligand sets for the studied activity classes.[25]

In order to expand on the previously published approaches, our group recently developed drug profile matching (DPM), a pattern-based in silico drug repositioning method.[26,27] This method enables the prediction of the effect profiles of small molecules on the basis of their docking scores against a panel of proteins and is therefore applicable in searching for drug repositioning candidates as well as in de novo drug development. Figure 1 represents the DPM method in graphical form. In DPM, each FDA-approved small molecule drug is docked to the ligand binding sites of 149 nontarget proteins that were selected by suitability for docking. On the basis of the docking results, interaction patterns (IPs), i.e., vectors containing the calculated best docking scores of the compounds on the 149 members of the protein set, were formed. Additionally, the effect profiles (EPs) of the drugs were also generated that are

dx.doi.org/10.1021/jm400813y | J. Med. Chem. XXXX, XXX, XXX−XXX

binary presence/absence indicators of 177 physiological effects. The effect database used in our study was extracted from DrugBank and was revised manually. Each effect entry contained at least 10 registered drugs (known actives) to provide sufficient amount of information for classification. Canonical correlation analysis (CCA) was applied between the IP matrix and a given effect to produce highly correlating factor pairs that were the inputs for linear discriminant analysis (LDA) that was used for separating the two classes (active and inactive molecules of a given effect). By use of this two-step multidimensional analysis, classification functions were created for each effect and probability values were assigned for each drug−effect pair in our data set, which is hence both reclassifying known drug−effect pairs and indicating novel hypothetic associations between both domains. The prediction accuracy of the DPM method was examined by receiver operating characteristics (ROC) analysis. Area under the curve (AUC) values were calculated for each effect to demonstrate the reclassification performance (which is also a measure of consistency within the effect class) of the method. To check the validity of the DPM predictions, the commonly used 10-fold cross-validation was performed and repeated 100 times.

The probabilities that were assigned for each drug−effect pair predicted several unregistered effects with high probability for many drugs. Besides the possibility that these "false positive" hits can refer to incorrect classification functions, they can also be considered as drug repositioning predictions ("putative true positives"). In order to test these findings, in the current work predictions by DPM were now verified by literature analysis as well as experimentally for three selected effect categories, namely, the in vitro inhibition of ACE (angiotensin-converting enzyme) and COX enzymes as well as cell-based activity tests on dopaminergic D1 and D2 receptors. For ACE and D1/D2 receptors, control sets containing compounds with low predicted probability for the given effect were also tested.

RESULTS AND DISCUSSION

The detailed description of DPM has been presented recently.[26] Our previous analysis showed that 84% of the 171 studied effects resulted in a reclassification AUC larger than 0.95, indicating sufficient performance on the data set used. Robustness was determined by 10-fold cross-validation producing the mean of the mean probability values (mean MPV) for each effect (see Experimental Section). Mean MPVs larger than 0.5 were calculated for 48.6% of the studied effects, while a random data set would result in a mean MPV of 0.027. Hence, we showed that significant differentiation of effects can be obtained by DPM, compared to random sampling.

The following criteria were considered in the selection of the experimentally tested effect categories: (1) robustness (mean MPV calculated from 10-fold cross-validation) of classification functions, (2) accuracy (reclassification AUC) of classification functions, (3) the potential importance of therapeutic effects, (4) availability of in vitro test kits or cell-based assays. The dopamine agent, ACE, and COX inhibitory effect categories are good representatives of the middle and the upper region of classification robustness (mean MPV values of 0.548, 0.420, and 0.693, respectively). All show high reclassification AUCs (0.922, 0.999, and 0.989, respectively), and hence, they were chosen in this study.

In Vitro Tests of ACE Inhibition. ACE inhibitors are widespread antihypertensive agents also used for the treatment of congestive heart failure and diabetic nephropathy.[28,29] Their blood pressure lowering effect is due to the inhibition of angiotensin-converting enzyme, which has a dual result. First, the conversion of angiotensin I to the vasoconstrictor angiotensin II is not performed, and second, the degradation of the vasodilator bradykinin by ACE is inhibited.

For ACE inhibitors, the DPM prediction acceptance threshold was set according to the level above which 14 out of the 15 originally registered ACE drugs were classified as positives. This threshold was exceeded by 15 drugs that are not registered as ACE inhibitors ("false positives" or predicted ACE inhibitors) (see Table 1 and Supporting Information Tables 1 and 4 for details on molecular structure and plasma concentrations). A retrospective literature analysis revealed that for three of the predicted compounds, i.e., candoxatril, carvedilol, and nebivolol, an effect on ACE inhibition was indeed described earlier and just not annotated in the data set because they are not FDA registered ACE inhibitors.[30−33] The remaining 12 compounds were tested for ACE inhibition experimentally, and for L-proline, tipranavir, dasatinib, novobiocin, nelfinavir, and telmisartan activity was confirmed. These compounds exerted 46−97% inhibition of ACE at 500 μM inhibitor concentrations except for tipranavir which was measured at 128 μM because of low solubility and produced 31% inhibition. In order to select reasonably active molecules, hit criterion was set to 90% inhibition at 500 μM. Screening at high concentration certainly carries the risk of detecting false positives because some molecules can aggregate and act promiscuously.[34] However, most drugs are not promiscuous, even at high screening concentrations.[35] 33% of the predictions were confirmed experimentally or by the literature, as two active molecules were discovered in the assay and three active molecules were confirmed by the literature out of the 15 predictions.

Dose−response ACE inhibition curves were determined for four molecules, namely, telmisartan, L-proline, novobiocin, and dasatinib (see Figure 2). The most pronounced ACE inhibition (with a K_i of 6 μM) was observed for telmisartan, which is

Table 1. Predicted ACE Inhibitors in Decreasing Order of Prediction Probability[a]

drug name	predicted probability	% inhibition at 500 μM
1. telmisartan	**1.000**	**+ (97 ± 0.5)**
2. paclitaxel	0.419	−
3. latamoxef	0.410	−
4. L-proline	**0.384**	**+ (93 ± 0.2)**
5. maraviroc	0.369	−
6. tipranavir[b]	*0.293*	*+ (31 ± 9.2)*
7. dasatinib	*0.175*	*+ (46 ± 1.6)*
8. novobiocin	*0.157*	*+ (75 ± 1.7)*
9. nelfinavir	*0.101*	*+ (48 ± 4.6)*
10. ambenonium	0.076	−
11. candoxatril[30]	**0.066**	**+**
12. carvedilol[31]	**0.060**	**+**
13. nitrofurantoin	0.049	−
14. clavulanate	0.036	−
15. nebivolol[32,33]	**0.029**	**+**

[a]Active compounds in the assay (inhibition of >90%) are in boldface along with predictions confirmed by the literature (references are included). Italic entries indicate those molecules that produced significant inhibition in the assay but failed to reach the limit of 90%. [b]Tipranavir was measured at 128 μM because of low solubility.

dx.doi.org/10.1021/jm400813y | J. Med. Chem. XXXX, XXX, XXX−XXX

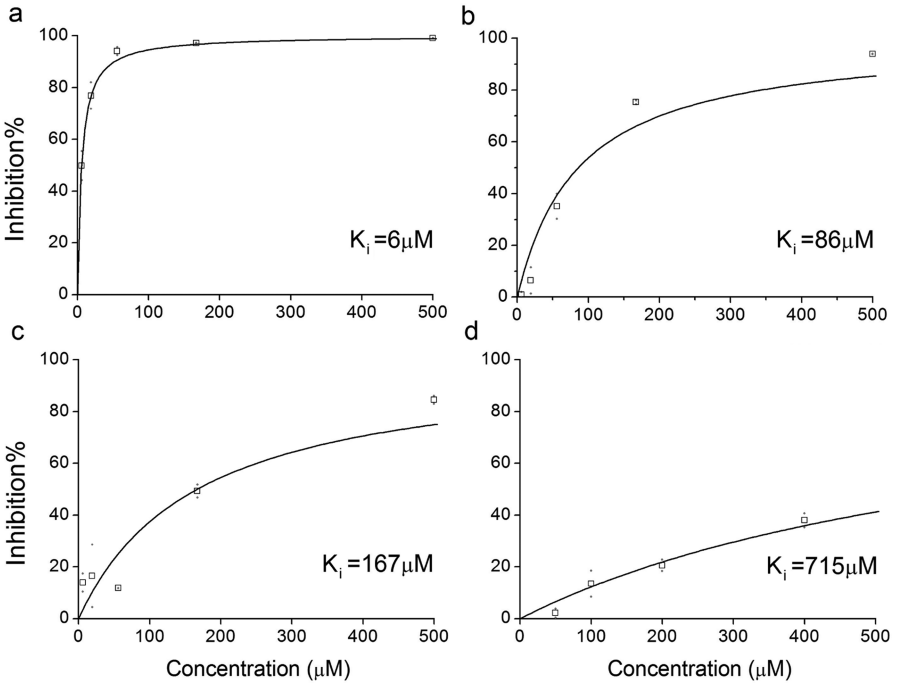


Figure 2. In vitro ACE inhibition curves for telmisartan (a) , L-proline (b) , novobiocin (c), and dasatinib (d). All compounds show dose dependent response with resulting K_i values ranging from 6 to 715 μM.

registered as an angiotensin II receptor antagonist, while its ACE inhibitory activities have not been previously reported. Interestingly, telmisartan is well tolerated in patients where ACE inhibitors are contraindicated.[36] Nevertheless, telmisartan could not substitute angiotensin-converting enzyme inhibitors in patients intolerant to ACE inhibitor therapy.[36] This finding clearly highlights the difficulty of transferring in vitro results to in vivo effects.

L-Proline also showed an inhibitory activity of ACE with a K_i of 86 μM. Visual inspection of the chemical structures of the common ACE inhibitors such as captopril reveals that they contain a proline moiety; however, there is no published evidence that would support the importance of this particular moiety in their pharmacologic actions (except for contributing to binding via nonspecific interactions achieved by being positioned in a lipophilic pocket). According to the measured ACE inhibitory activity of L-proline itself, we suspect that it is a key moiety in the mode of action of ACE inhibitors. Nevertheless, as expected, we measured a much stronger affinity for captopril (K_i = 2 nM), which was used as a positive control in our tests.

Novobiocin, an aminocoumarin antibiotic, possessed a moderate ACE inhibitory activity (with a K_i of 167 μM) that is commensurable to the plasma concentration of the drug reported in the literature.[37]

Low ACE inhibitory activity (with a K_i of 715 μM) was measured for dasatinib, an anticancer agent, which however does not appear to be relevant at the therapeutic plasma concentration of the drug.[38]

A random set of eight compounds that has very low predicted probabilities for ACE inhibition (below 0.003; see Supporting Information Table 8) was also selected for testing under the same conditions. The aim was to establish whether the percentage values calculated for the false positive compounds are meaningful. Although 15−67% inhibition effect was detected for them, the estimated K_i values for these compounds are considerably weaker than those of the false positives (Supporting Information Table 8). Known drugs do not typically possess K_i values above 50−100 μM, but higher K_i data may also be biologically relevant as indicated by the previously presented case of novobiocin. Thus, a limit of K_i = 200 mM was applied in the comparison studies of the low and high probability compounds throughout our work. If we apply this limit, three molecules remain that were all predicted with high probability (telmisartan, L-proline, novobiocin). On the basis of this analysis, we conclude that a definite enrichment can be observed on the top of the DPM prediction list regarding K_i data in the case of the ACE inhibitory effect.

In Vitro Tests of COX Inhibition. COX inhibitors are nonsteroidal anti-inflammatory drugs (NSAIDs) and are used worldwide.[8] Their mechanism of action is based on the inhibition of two COX isoforms (termed COX-1 and COX-2), thereby inhibiting the conversion of arachidonic acid into prostaglandin H2, which is part of an inflammation pathway relevant in a variety of diseases.[39] COX-1 is a constitutively expressed isoform in most cells, and its inhibition leads to positive anti-inflammatory effects; however, a number of side effects are also coupled with this action that include diarrhea, gastric ulcer, interstitial nephritis, and acute renal failure due to the high level of COX-1 expression in the gastric mucosa and the kidneys.[40] COX-2 is an inducible enzyme, expressed only in case of inflammation. Therefore, selective COX-2 inhibitors such as celecoxib, rofecoxib, and valdecoxib have reduced gastrointestinal and renal adverse effects. On the other hand, an increased probability of cardiovascular side effects including thrombosis and myocardial infarction was reported in connection with the administration of some COX-2 inhibitors, leading to the withdrawal of rofecoxib and valdecoxib.[41,42]

The DPM method was also applied for predicting COX inhibitors. The prediction threshold was set to a level above which 90% of the registered COX inhibitors appeared as positives (33 out of 37), giving rise to 54 putative "false

dx.doi.org/10.1021/jm400813y | J. Med. Chem. XXXX, XXX, XXX−XXX

Table 2. Predicted COX Inhibitors in Decreasing Order of Prediction Probability[a]

drug name	predicted probability	% inhibition at 500 μM	
		COX1	COX2
1. biotin	1.000	−	−
2. aminosalicylic acid	*1.000*	−	+ *(33 ± 8.9)*
3. flutamide	**0.999**	+ **(100 ± 0)**	+ **(100 ± 0)**
4. nitrofurazone	**0.999**	+ **(91 ± 8.9)**	+ **(98 ± 1.0)**
5. valproic acid[44]	**0.998**	−	+
6. lipoic acid	**0.998**	+ **(95 ± 5.1)**	+ **(76 ± 23)**
7. monobenzone	*0.997*	+ *(86 ± 1.2)*	+ *(54 ± 3.9)*
8. gemfibrozil	0.996	−	−
9. benzyl benzoate	*0.981*	−	+ *(41 ± 5.8)*
10. furosemide	*0.967*	+ *(36 ± 3.6)*	−
11. flucytosine	0.966	−	−
12. penicillin G	0.965	−	−
13. chlormezanone	0.947	−	−
14. furazolidone	0.940	−	−
15. ticarcillin	0.932	−	−
16.nitroxoline	**0.922**	+ **(97 ± 0.4)**	+ **(99 ± 0.8)**
17. tinidazole	0.900	−	−
18. lomustine	0.837	−	−
19. ticlopidine[49]	0.820	−	−
20. oxybenzone[45]	**0.792**	+	+
21. nilutamide	**0.771**	+ **(99 ± 0.5)**	+ **(98 ± 0.6)**
22. milrinone	0.744	−	−
23. ciclopirox[43]	**0.742**	+	+
24. α-linolenic acid[46,47]	**0.738**	+ **(100 ± 0)**	+ **(96 ± 2.3)**
25. chlorambucil	0.694	−	−
26. phenazopyridine	*0.646*	+ *(23 ± 3.5)*	+ *(67 ± 28)*
27. penicillin V	0.644	−	−
28. azithromycin	**0.582**	+ **(68 ± 5.4)**	+ **(99 ± 0.3)**
29. estrone sulfate	0.577	−	−
30. ethacrynic acid	0.567	−	−
31. carbenicillin	0.565	−	−
32. metronidazole	*0.558*	−	+ *(17 ± 1.2)*
33. nateglinide	0.541	−	−
34. L-carnitine	0.510	−	−
35. acitretin	0.508	−	−
36. nalidixic acid	*0.504*	+ *(34 ± 3.5)*	+ *(88 ± 6.9)*
37. L-proline	0.476	−	−
38. azathioprine[48]	0.441	−	−
39. pyridoxal phosphate	0.436	−	−
40. nitrofurantoin	*0.424*	+ *(66 ± 7.6)*	+ *(41 ± 7.1)*
41. captopril	*0.422*	+ *(48 ± 0.7)*	+ *(61 ± 12)*
42. chlorphenesin	*0.418*	−	+ *(47 ± 34)*
43. aspartame	0.402	−	−

[a]Compounds possessing >90% inhibition and predictions confirmed by the literature (references are provided) are in boldface. These molecules are considered active in the evaluation. Italic entries indicate molecules that produced significant inhibition in the assay but failed to achieve the limit of 90% inhibition.

positive" predictions. Eleven drugs were excluded from the analysis because of limited practical importance or lack of commercial availability (see Materials), resulting in 43 compounds that were studied further (see Table 2 and Supporting Information Tables 2 and 5 for details on molecular structure and plasma concentrations). Out of the compounds investigated the COX inhibitory properties for valproic acid, α-linolenic acid, oxybenzone, and ciclopirox were confirmed by the literature.[43−47] Two other compounds, ticlopidine and azathioprine, were tested for COX inhibition earlier, but activity could not be detected for them.[48,49]

The remaining 37 drugs, as well as α-linolenic acid, were tested experimentally for COX inhibition activity of COX-1 and COX-2, and COX-1 or COX-2 inhibitions were found in 17 cases. Seven compounds reached the applied criterion of 90% inhibitior concentration, resulting in a confirmation rate of 23% (10 actives including literature hits out of 43 compounds, Table 2). In this experiment, nitroxoline, α-linolenic acid, nitrofurazone, flutamide, and nilutamide were found to be the strongest inhibitors of COX.

Dose−response COX inhibition curves of captopril (COX-1 and COX-2), nitroxoline (COX-2), and α-linolenic acid (COX-1) were also determined, shown in Figure 3. The inhibitory

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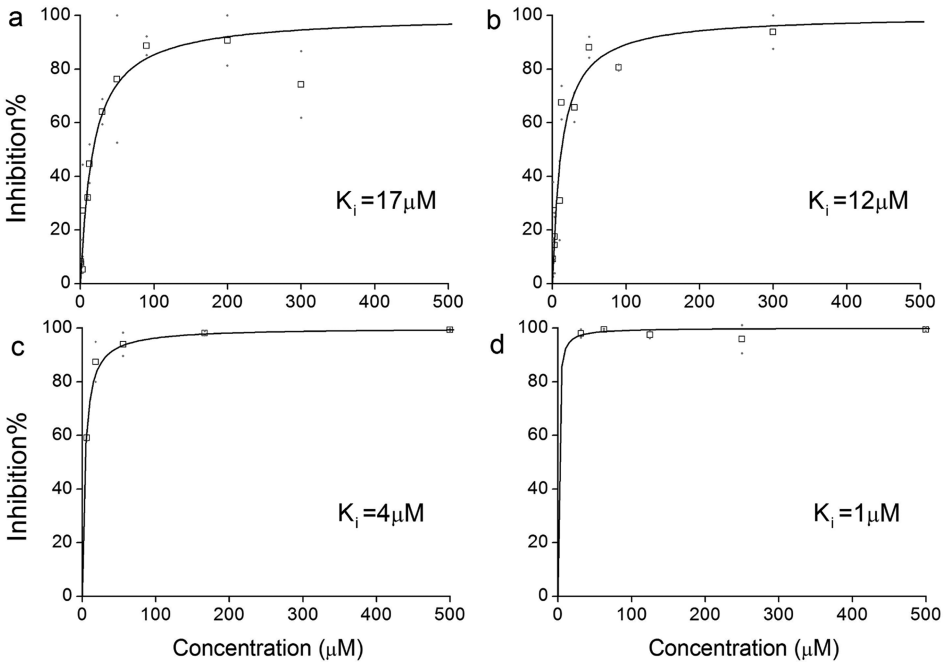

Figure 3. COX-1 and COX-2 inhibition curves for captopril (panels a and b, respectively), COX-1 inhibition curve for α-linolenic acid (c), and COX-2 inhibition curve for nitroxoline (d). All compounds show dose dependent response, and the determined K_i values range from 1 to 17 μM.

Table 3. Examined Dopaminergic Candidates in Decreasing Order of Predicted Probability[a]

		dopamine D1 receptor		dopamine D2$_{long}$ receptor	
drug name	predicted probability	agonist mode (%) (100 μM)	antagonist mode (%) (100 μM)	agonist mode (%) (100 μM)	antagonist mode (%) (50 μM)
celecoxib[b]	0.995	1.8 ± 5.7	**95 ± 2.8**	**175 ± 7.6**	**96 ± 0.11**†
doxazosin	0.991	−4.7 ± 1.7	**102 ± 4.5**	2.4 ± 1.9	−13 ± 18
cyclobenzaprine	0.977	−4.7 ± 2.1	**98 ± 4.8**	1 ± 1.7	**95 ± 1.5**
mitoxantrone	0.976	*66 ± 4.7*	29 ± 43	−0.4 ± 1.3	**81 ± 0.32**
flavoxate	0.971				
promethazine	0.966	−7.4 ± 3.2	**84 ± 7.4**	3.5 ± 0.67	**95 ± 1.3**
imipramine	0.952				
desipramine	0.951				
desogestrel	0.936	2.8 ± 3.1	*79 ± 0.26*	7.1 ± 3.3	**88 ± 2.6**†
epinastine	0.916	−1 ± 2.7	**96 ± 2.7**	−0.33 ± 0.7	5.5 ± 12
clomipramine	0.907	−4.5 ± 1.1	**99 ± 3.9**	13 ± 2.4	**92 ± 1.5**†
olopatadine	0.881	−1.7 ± 2	20 ± 2.7	−0.14 ± 1.5	*52 ± 2.5*
thioguanine	0.878	0.2 ± 3.1	2.3 ± 2.1	0.61 ± 0.88	21 ± 4.6
rimantadine	0.864	−0.8 ± 1.5	−0.4 ± 1.7	3.6 ± 7.1	*41 ± 5.6*
mefloquine	0.854	0.6 ± 2.1	**82 ± 1.1**	*45 ± 4*	**90 ± 2**†
etodolac	0.796	−1.9 ± 2.2	−1.4 ± 2.9	0.96 ± 1.9	13 ± 1.3
raloxifene	0.796	−7.2 ± 2.5	**97 ± 6.9**	4.7 ± 2.1	**88 ± 1.8**
fosfomycin	0.761	−1.2 ± 2.6	−1.3 ± 2.7	0.09 ± 2.3	29 ± 3.2

[a]Boldface font represents an activity value over 80%, while italic cells refer to activity between 40% and 80%. Celexocib, desogestrel, mefloquine, and clomipramine showed activation during preincubation for D2$_{long}$ antagonist tests; therefore, their respective values are overestimated (marked with †). Flavoxate, imipramine, and desipramine were not tested, but their dopaminergic effects were confirmed by the literature (see Results and Discussion for details). [b]Celecoxib was measured at 8 μM because of its low solubility.

effect of captopril for COX-1 increased gradually with increasing concentrations as expected; however, reduced effect was measured at 300 μM. Its K_i values were 17 and 12 μM for the COX-1 and COX-2 isoenzymes, respectively. These inhibitory constants are moderately stronger than that of acetylsalicylic acid, which is one of the most common COX inhibitors ($K_{i,COX-1}$ = 62 μM and $K_{i,COX-2}$ = 52 μM). However, the reported plasma level of captopril does not indicate the biological relevance of this finding.[50]

α-Linolenic acid showed a K_i of 4 μM for COX-1, and taking into account the inhibition results measured at 500 μM for COX-2, this compound appears to show a strong, nonselective COX inhibition that is in accordance with the results obtained by Ren and Chung.[46,47] Here, the authors demonstrated that this compound has an anti-inflammatory effect through different mechanisms, including COX-2 inhibition, while effects on COX-1 were not mentioned. Our results therefore extend

dx.doi.org/10.1021/jm400813y | J. Med. Chem. XXXX, XXX, XXX−XXX

the knowledge about the multitarget anti-inflammatory properties of α-linolenic acid.

The antibiotic nitroxoline showed a K_i of 1 μM for COX-2, and according to our results measured at 500 μM for COX-1 (97% inhibition), it also seems to be a reasonably potent nonselective COX inhibitor. This newly discovered COX inhibitory property of nitroxoline might have significance in its original antibiotic and recently described anticancer effects.[51,52]

Cell-Based Tests of Dopaminergic Activity. We also investigated the prediction power of DPM on the dopamine agent effect category. The experimental confirmations of the predictions were performed in an independent laboratory that has large experience in receptor tests. Dopamine receptors are G-protein-coupled receptors that have five subtypes. The D1-like family contains subtypes D1 and D5, while the D2-like family consists of three members: D2, D3, and D4. Dopamine receptors, associated with cognitive processes, learning, memory, motor control, and motivation, are important targets of a series of psychiatric drugs, e.g., antipsychotic agents.[53] Since the investigated category did not specify which receptor subtypes will be affected by the drugs and in order to gain insights for both families, the D1 and D2 receptors were selected for testing as representatives of both groups. Predicted compounds were analyzed by a team of medical doctors, and the most promising 18 compounds were selected for a comprehensive literature survey and experimental testing for agonist and antagonist effects on these receptor subtypes, based on their prediction probabilities, clinical importance, and commercial availability. Thus, in contrast to the ACE and COX measurements, compounds were subjected to an additional prescreening process. Table 3 shows the measured activity values and standard deviations, applying 100 μM compound concentration except for the measurements of D2 antagonism where 50 μM was used. Celecoxib was measured at 8 μM in all tests because of solubility issues. For details on molecular structure, plasma concentrations, and known activity of true positives, see Supporting Information Tables 3, 6, and 7. Measurements were not performed for three compounds because their dopaminergic effect was confirmed by the literature.

Celecoxib, a selective COX-2 inhibitor, was predicted to have dopaminergic effects with the highest probability (0.995), which has been confirmed experimentally for D1 receptor antagonism (95% activity at the concentration studied; note that agonist activity values are expressed as the percentage of the activity of the reference agonist at EC_{100} concentration while antagonist activity level is expressed as the percentage of decrease of the reference agonist activity at EC_{80} concentration). In the case of the D2 receptor, 96% antagonist activity was measured for celecoxib but the compound possessed activation during the preincubation, leading to the overestimation of antagonist activity. Interestingly, for the D2 receptor, very high agonist activity was measured: 175% of the activity of the reference agonist quinpirol. Considering the applied concentration of celecoxib (8 μM), we can conclude that the drug possesses a definite D1/D2 dopaminergic effect at a submicromolar K_i. The literature survey revealed that celecoxib was found to reduce the lipopolysaccharide induced dopamine transporter (DAT) expression.[54] Nevertheless, to our knowledge, exact interaction between DAT and celecoxib was not reported for this drug. However, it has been suggested for the treatment of psychiatric disorders, e.g., schizophrenia and major depression, in which the dysfunction of the dopaminergic system might be a key factor.[55,56] The reason for its effectiveness we are proposing here is the inhibition of COX-2, based on numerous observations that support an inflammation theory of schizophrenia.[57−60] Celecoxib proved to be efficient as an adjunctive therapy for schizophrenia in randomized controlled clinical trials when coadministered with risperidone[61,62] or amisulpride,[58] while other randomized controlled clinical trials confirmed the therapeutic efficacy of celecoxib as an add-on therapy in major depressive disorder.[55,63] In our study, we show that celecoxib might have a direct effect on the dopaminergic system, which could be at least partially responsible for its efficacy observed in the aforementioned clinical trials. However, the connection between the observed dopamine receptor agonist activity and the presence of the psychiatric benefits remains unclear.

We measured strong antagonistic effects of mefloquine on D1 and D2 (82% and 90%, respectively), while only a moderate agonistic effect was observed on the D2 receptor (45%) and no agonist effect was detected on the D1 receptor. Antimalarial drugs like mefloquine are known to induce psychosis.[64,65] The underlying mechanism is unknown; however, it is hypothesized that several neurotransmitter systems might be involved, including the dopaminergic system which would be in agreement with our findings.[66]

The selective estrogen receptor modulator raloxifene produced strong antagonistic effect on both dopamine receptor subtypes (of 97% and 88% on the D1 and D2 receptors, respectively), and the effect of steroids possessing estrogen-like activity on the dopamine system has been widely discussed in the literature. Gender differences in the epidemiology and course of schizophrenia (e.g., illness onset, symptom severity during the reproductive versus postmenopausal age, etc.) are hypothesized to be at least partially attributed to the influence of estrogens on the dopaminergic system. Randomized controlled clinical trials using estrogen as an add-on therapy to antipsychotics resulted in a significantly rapid reduction of symptom severity in patients receiving combined therapy compared to the reference group in both male and female persons with schizophrenia,[67−71] an observation not directly linked to the dopamine receptorial effect. Recent data also show that raloxifene had a good therapeutic effect as an adjunctive therapy to antipsychotics in postmenopausal women.[72] According to our in vitro measurements and the presented literature data, we may rationalize these effects by a direct dopaminergic effect of raloxifene.

The antispasmatic agent cyclobenzaprine possessed high antagonist activities on D1 and D2 receptors (over 90%). Literature data supported the interaction of cyclobenzaprine with the D2 receptor in vitro.[73] However, no indication was found that this effect might be relevant for efficacy or side effects.

The antihistamine epinastine proved to be a selective D1 antagonist (96% and 5.5% activities on D1 and D2, respectively). Roeder et al. found that epinastine has a strong antagonist effect on insect neuronal octopamine receptors with high affinity and specificity, thus influencing insect behavior[74] and visual learning.[75−77] We found that the α adrenergic antagonist doxazosin is even more selective for the D1 receptor than epinastine (with 102% and 0% inhibition on D1 and D2, respectively). On the other hand, desogestrel, an estrogen-type contraceptive, has a slightly higher affinity for D2 than D1 (88% and 79% on D2 and D1, respectively). Relevant literature was

dx.doi.org/10.1021/jm400813y | J. Med. Chem. XXXX, XXX, XXX−XXX

not found for these drugs that would support a phenotypically relevant effect on the dopaminergic system.

Other compounds were found to have lower activities on the dopamine receptors, of likely insignificant effect in physiological systems. Olopatadine, an antihistamine agent, and the antiviral rimantadine produced moderate antagonistic effect on D2 (52% and 41%, respectively) while not affecting D1. Similarly, the NSAID etodolac, the antimicrobial fosfomycin, and the antimetabolite thioguanine produced low antagonistic activity on D2 (13%, 29%, and 21%, respectively). Etodolac was proposed as an anti-inflammatory adjuvant to investigate its neuroprotective effect in a model of Parkinson's disease, but no effect was observed.[78]

Only three compounds possessed dopamine agonist activity in the assays, namely, mitoxantrone, celecoxib, and mefloquine. The last two compounds were mentioned before; they act selectively on D2 receptor (175% and 45%, respectively), while mitoxantrone acts on the D1 receptor (with an activity of 66%). Moreover, mitoxantrone produced antagonistic effect on the D2 receptor as well (81% inhibition).

The predictions for three tricyclic antidepressants, i.e., imipramine, desipramine, and the experimentally tested clomipramine, were confirmed by the literature. Imipramine and desipramine have very similar receptor profiles; they produce dopamine supersensitivity, a phenomenon related to strong D2 antagonism.[79−82] In our study, high dopamine antagonist activities were measured on both subtypes (over 90%) for clomipramine. A similar effect was determined for the first-generation antihistamine promethazine and confirmed by the literature.[83]

From a different structural class, flavoxate is a spasmolytic agent with a suggested anticholinergic mechanism of action. Oka et al. demonstrated the D2 affinity of this compound at micromolar concentrations, determined by radioligand assay.[84] Flavoxate obtained high prediction probability by DPM in accordance with the literature.

In summary, 67% of the tested dopaminergic predictions proved to be highly active (10 out of 15; activity of >80%) while 81% showed at least 40% activity (12 out of 15). 72% of the 18 predictions were confirmed experimentally on the receptor level (10 compounds) or by the literature (3 compounds).

Similarly as it was presented for ACE inhibition, a random set of 10 compounds was also selected for testing that has very low predicted probabilities for dopaminergic effect (below 0.015; see Supporting Information Tables 9 and 10). Molecules were tested for D1 and D2 antagonism, applying 100 and 50 μM concentrations, respectively. From the results obtained on the single concentrations, K_i values were estimated (Supporting Information Table 9 and 10.). In the case of D1 antagonism, applying the previously introduced limit of K_i < 200 μM resulted in nine molecules that were predicted with high probability while only one compound, natamycin, could fulfill this criterion from the low-probability molecules. Thus, we conclude that the results presented for the top of the list are valid here. In the case of D2 antagonism, there are 18 molecules below the limit of K_i = 200 μM, from which 12 molecules possess high probability value while 6 compounds have low probability. Among the strongest 9 molecules (K_i < 10 μM), only 2 have low probability; therefore, an enrichment can be observed here as well, but the experimental results point to the weakness of the classification function applied to calculate probability values for dopaminergic effects.

CONCLUSIONS

In this study, we prospectively validated the drug profile matching algorithm for three selected effect categories by systematically testing the highest-ranked predictions, i.e., those compounds that gained highest probability for exerting the studied effect. In the case of the inhibition of ACE and COX enzymes, 33% and 23% confirmation rates were obtained, respectively. DPM predictions for dopaminergic effect were confirmed by cell-based tests, and 67% of the tested compounds proved to be active. Several interesting bioactivities were discovered such as the ACE inhibition property of the angiotensin II receptor antagonist telmisartan and the interaction of the selective COX-2 inhibitor celecoxib with the dopaminergic system. The latter could be linked to clinical observations. On the basis of the presented tests, the performance of DPM is comparable to that of other state-of-the-art ligand−target prediction methods.[25] Our results demonstrate the applicability of DPM in identifying unknown bioactivities of already approved drugs and hence its possible use in drug repositioning.

EXPERIMENTAL SECTION

Drug repositioning predictions were created using drug profile matching as outlined in detail in previous work.[26] Three effect categories were prospectively validated in the current study, namely, angiotensin-converting enzyme inhibition, cyclooxygenase inhibition, and dopaminergic agonistic and antagonistic activity. In vitro ACE and COX tests were carried out at Eötvös Loránd University (Hungary) using a laboratory robotic system and commercially available test kits. Dopaminergic predictions were performed at EuroScreen SA (Belgium) using recombinant cell lines expressing human recombinant dopaminergic receptors D1 and $D2_{long}$.

Drug Profile Matching. The drug profile matching (DPM) method was used as described earlier.[26,85] A total of 1226 FDA-approved drug molecules were extracted from DrugBank database[86] as of June 2009. The DOVIS 2.0 software (docking-based virtual screening),[87] AutoDock4 docking engine,[88] Lamarckian genetic algorithm, and X-SCORE[89] scoring function were applied for docking preparations and calculations. The docking box was centered at the geometrical center of the original ligand of the protein. Twenty-five docking runs were performed for each job. Each drug was docked to each protein (1226 × 149 = 182 674 dockings, repeated 25 times). The calculated best docking scores were imported to the IP data matrix.

Physiological effect information on the 1226 FDA-approved small-molecule drug set was extracted from the DrugBank database.[86] Effects containing at least 10 registered drugs were considered in this study. The presence or absence of the studied 177 effects for each drug is then stored in a binary matrix, i.e., the effect profile (EP) matrix.

Canonical correlation analyses were performed in order to match the IP and EP matrices and find highly correlated factor pairs that are the linear combinations of the variables of the starting data sets. Subsequently, linear discriminant analysis was applied to determine a classification function that calculates the probability value for each drug−effect pair. The prediction accuracy of the DPM method was examined by receiver operating characteristics (ROC) analysis, i.e., determining the true positive rate (TPR) and the false positive rate (FPR) for every effect using a sliding cutoff parameter for the predicted probabilities. TPR and FPR values for each possible cutoff are plotted on a two-dimensional graph called the ROC curve. The area under the ROC curve, i.e., the AUC value, can be used to characterize the reclassification accuracy. In order to check the validity of the DPM predictions, the commonly used 10-fold cross-validation was performed and repeated 100 times. Robustness was determined for each effect by a measure called "mean of the mean probability values (mean MPVs)", which is related to the robustness of the method against the information loss occurring when a portion of the

dx.doi.org/10.1021/jm400813y | J. Med. Chem. XXXX, XXX, XXX−XXX

input information is removed. The closer the mean MPV is to 1, the more cohesive the group of active molecules is based on their interaction patterns.

The Statistical Analysis System for Windows (version 9.2; SAS Institute, Cary, NC) was used for the implementation of all statistical analyses.

ACE Inhibition Assay. In vitro tests were performed on a Hamilton Starlet liquid handling workstation (Hamilton Robotics, Bonaduz, Switzerland). Spectroscopic measurements were carried out on BMG FluoStar Optima (Offenburg, Germany). The robot was programmed according to the manufacturer's instructions. The selected drugs were initially tested at 500 μM, and certain drugs were further tested to determine K_i values. Each data point is an average of two independent measurements.

ACE inhibition was tested using the ACE Kit-WST from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan, catalog no. A502-10). The ACE kit of Dojindo was presented in research papers.[90,91]

3-Hydroxybutyril-glycil-glycil-glycine is utilized as a substrate in this kit, and under the actions of ACE and aminoacylase it is converted into 3-hydroxybutyric acid. In the development step it is further oxidized into acetoacetate by the action of 3-hydroxybutyrate dehydrogenase. At the same time, the cofactor NAD^+ is converted into the reduced form NADH. During the oxidation of NADH to NAD^+ a water-soluble tetrazolium salt is reduced coupled with an electron mediator and generates a yellow formazan. Tested drugs were incubated at the given concentrations with enzyme working solution and the substrate for 60 min at 37 °C. In the next step indicator working solution was added to the reactions and the plate was incubated at room temperature for 10 min and read at 450 nm. Captopril was used as a positive control in this assay.

COX Inhibition Assay. Experiments were carried out using the same equipment described in the previous section. Screening was performed in duplicate at a final compound concentration of 500 μM. For certain compounds, K_i values were determined by applying decreasing concentrations.

COX inhibition was investigated using the COX inhibitor screening assay kit from Cayman Chemical Co. (Cayman Europe, Tallinn, Estonia; catalog no. 560131). The COX kit of Cayman Chemicals was used in several reserch projects published in scientific journals.[92,93]

This enzyme immunoassay kit quantifies the inhibition of COX-1 and COX-2 activities by measuring the formation of prostanoid products from the substrate arachidonic acid. Tested drugs were preincubated at the given concentrations with enzymes COX-1 and COX-2 for 10 min at 37 °C. Reactions were started by adding the substrate and then incubating the mixture for 2 min at 37 °C and were stopped by 1 M HCl. Prostaglandin screening was performed on a 96-well microplate coated with mouse anti-rabbit IgG. COX reaction samples were mixed with an AChE-linked tracer, and the antiserum was then incubated for 18 h at room temperature. The washed plate was developed by Ellman's reagent for 60 min and read at 400 nm. Acetylsalicylic acid was used as a positive control in the assay.

Dopaminergic Agonist and Antagonist Assays. Dopamine receptor D1 and $D2_{long}$ tests were carried out at Euroscreen SA, Brussels, Belgium. For more information on the company, see http://www.euroscreen.com/.

Compounds were dissolved at 20 mM in 90% DMSO and sent to EuroScreen SA where they were stored at room temperature prior to the test. In the cases of celecoxib, desogestrel, mitoxantrone, raloxifene, and doxazosin precipitation occurred. 400 μM stock solutions were prepared for testing.

For D1 agonist and antagonist tests, cAMP-HTRF functional assays were used (CHO-K1 recombinant cell line, human recombinant dopamine receptor D1, catalog no. FAST-0100C). Reference compounds were SKF81297 and SCH23390 in agonist and antagonist modes, respectively. Compounds were screened in triplicate at a final concentration of 100 μM. CHO-K1 cells expressing human D1 recombinant receptor grown in antibiotic-free media were detached by gentle flushing with PBS-EDTA (5 mM EDTA), centrifugated, and resuspended in assay buffer containing 5 mM KCl, 1.25 mM $MgSO_4$, 1.24 mM NaCl, 25 mM HEPES, 13.3 mM glucose, 1.25 mM KH_2PO_4, 1.45 mM $CaCl_2$, and 0.5 g/L BSA. In agonist tests, 12 μL of cells was mixed with 6 μL of assay buffer and 6 μL of test compound solution, respectively. After 30 min of incubation at room temperature, lysis buffer was added. After 1 h of incubation, cAMP concentration was measured with the HTRF kit according to the manufacturer's specification. In antagonist tests, 12 μL of cells was mixed with 6 μL of test compound and incubated for 10 min. After that, 6 μL of reference agonist solution was added at a final concentration corresponding to EC_{80}. After 30 min of incubation, lysis buffer was added. The concentration of cAMP was measured after 1 h of incubation in the same way as described before.

For the long isoform of D2 receptor, an aequorin-based functional assay was used (CHO-K1 recombinant cell line, human recombinant dopamine receptor $D2_{long}$, FAST-0101A) with reference compounds quinpirol and haloperidol for agonist and antagonist tests, respectively. Compound screening was performed in triplicate at a final concentration of 100 and 50 μM in agonist and antagonist modes, respectively. Cells coexpressing mitochondrial apoaequorin and recombinant human $D2_{long}$ receptor were grown in antibiotic-free culture media, detached with PDB−EDTA, centrifuged, and resuspended in assay buffer at a concentration of 1×10^6 cells/mL. Prior to the tests, cells were incubated at room temperature with coelenterazine for at least 4 h. In agonist tests, 50 μL of cell suspension was mixed with 50 μL of test compound solution and the resulting light emission was detected using a functional drug screening system model 6000 luminometer (Hamamatsu). In antagonist tests, 100 μL of the reference agonist was added to the mix of cells and test compound, at a final concentration corresponding to EC_{80}, 15 min after the first injection. Signal detection was performed as described before. We note that the antagonist activity level can be overestimated because of the nature of the aquorin-based tests if the tested compound activates the system during the preincubation period. Such activation was observed in the cases of celexocib, desogestrel, mefloquine, and clomipramine.

For both tests (i.e., D1 and $D2_{long}$), agonist activity of the tested compounds is expressed as the percentage of the activity of the reference agonist at EC_{100} concentration. Antagonist activity is expressed as the percentage of the inhibition of the reference agonist activity, applying EC_{80} concentration.

Materials. Aminosalicylic acid, furosemide, monobenzone, nitrofurazone, and nitroxoline were purchased from Aldrich. Maraviroc was from AvaChem. Chlorambucil, clavulanate, ethacrynic acid, flucytosine, furazolidone, latamoxef (moxalactam), lipoic acid, nitrofurantoin, novobiocin, paclitaxel, penicillin V, phenazopyridine, and tinidazole were from Fluka. Carbenicillin was from Merck. Chlormezanone and chlorphenesin were from MP Biomedicals, dasatinib and tipranavir from Santa Cruz Biotechnology, acetylsalicylic acid, acitretin, adefovir dipivoxil, adenine, α-linolenic acid, amlexanox, aspartame, atovaquone, azithromycin, captopril, cefuroxime, chloramphenicol, cimetidine, creatine, estrone-sulfate, fluocinonide, flutamide, gemfibrozil, lamivudine, lamotrigine, L-carnitine, lomustine, L-proline, metronidazole, milrinone, nalidixic acid, natamycin, nateglinide, nelfinavir, nilutamide, penicillin G, pentoxyfiline, pyridoxal phosphate, rosiglitazone, sulpiride, salsalate, telmisartan, ticarcillin, and valproic acid from Sigma, and benzyl benzoate and biotin from Sigma-Aldrich. Ambenonium was from Tocris Bioscience. All tested dopaminergic candidates were purchased from Sigma-Aldrich.

Predicted ACE inhibitors pentosan polysulfate, polystyrene sulfonate, and udenafil were commercially not available at the time of testing. Astemizole was omitted from testing because it was withdrawn from the market in most countries.

Predicted COX inhibitors aminohippurate, amlexanox, bexarotene, phenprocoumon, procarbazine, rosoxacin, stepronin, tolcapone, and valrubicin were commercially not available at the time of testing. Gentian violet and sodium lauryl sulfate were excluded from testing because of their limited clinical applicability.

dx.doi.org/10.1021/jm400813y | J. Med. Chem. XXXX, XXX, XXX−XXX

■ ASSOCIATED CONTENT

Supporting Information

Chemical structures and plasma concentrations of the studied drugs and activity and inhibition data for compounds predicted with low DPM probability. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work has been supported by the National Development Agency (Grant NTP TECH_08_A1/2-2008-0106). The work has also been supported by the European Union and the European Social Fund under the Grant TÁMOP 4.2.1./B-09/KMR-2010-0003. The European Research Council has provided financial support under the European Community's Seventh Framework Programme (FP7/2007-2013)/ERC Grant 208319. A.B. thanks Unilever for funding. The authors thank Ildikó Brandhuber for her help in collecting plasma concentration data for several compounds.

■ ABBREVIATIONS USED

ACE, angiotensin-converting enzyme; ADMET, absorption, distribution, metabolism, elimination, and toxicity; AUC, area under the curve; COX, cyclooxygenase; DPM, drug profile matching; EP, effect profile; FDA, Food and Drug Administration; FPR, false positive rate; IP, interaction pattern; ROC, receiver operating characteristic; SAR, structure−activity relationship; TPR, true positive rate

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