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Development and Validation of Chemical Features-Based Proton-Coupled Folate Transporter/Activity and Reduced Folate Carrier/Activity Models (Pharmacophores)

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Abstract

All clinically used antifolates lack transport selectivity for tumors over normal cells resulting in dose-limiting toxicities. There is growing interest in developing novel tumor-targeted cytotoxic antifolates with selective transport into tumors over normal cells via the proton-coupled folate transporter (PCFT) over the ubiquitously expressed reduced folate carrier (RFC). A lack of X-ray crystal structures or predictive models for PCFT or RFC has hindered structure-aided drug design for PCFT-selective therapeutics. Four-point validated models (pharmacophore) were generated for PCFT/Activity (HBA, NI, RA, RA) and RFC/Activity (HBD, NI, HBA, HBA) based on inhibition (IC₅₀) of proliferation of isogenic Chinese hamster ovary (CHO) cells engineered to express only human PCFT or only RFC. Our results revealed substantial differences in structural features required for transport of novel molecules by these transporters which can be utilized for developing transporter-selective antifolates.

Graphical abstract

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Supporting Information

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Introduction

Reduced folates are essential cofactors for biosynthesis of purines and pyrimidines.¹ Mammals obtain these cofactors from dietary sources as they cannot synthesize folates *de novo*. In mammalian cells, three major transport systems mediate cellular uptake of folates and related molecules from the extracellular milieu across biological membranes. These include the reduced folate carrier (RFC),² folate receptors (FRs) α and β ,³ and the proton-coupled folate transporter (PCFT).⁴ RFC and PCFT are facilitative folate transporters and members of the Major Facilitator Superfamily of solute carriers,^{2, 4–7} whereas FRs are glycosyl-phosphatidylinositol-anchored proteins that mediate uptake of folates into cells by receptor-mediated endocytosis.^{3, 8} Transport by RFC is optimal at neutral pH and decreases dramatically below pH 7,² whereas PCFT is a proton-folate symporter that functions optimally at acidic pH by coupling the flow of protons down an electrochemical concentration gradient to the uptake of folates into cells.^{4, 5, 7, 9}

RFC is ubiquitously expressed in tissues and tumors and is the major systemic transporter for folate cofactors in mammals.² FR α and FR β , as well as PCFT, are more narrowly expressed in normal tissues, where they perform specialized transport functions.^{3–5, 9} For instance, FR α mediates reabsorption of folate from the urine in the proximal tubules of the kidney³ and FR β is expressed in tumor-associated macrophages.¹⁰ PCFT is expressed in the upper gastrointestinal tract and functions as the major transporter for dietary folates at acidic pH.^{4, 6, 9, 11} Although PCFT is expressed in other normal tissues, (e.g., liver, kidney),⁵ its acidic pH optimum (pH 5–5.5, with limited activity above pH 7)^{1, 4, 5} severely restricts transport of folates by this process in most normal tissues.

Several solid tumors (e.g., ovarian, non-small cell lung cancer, malignant pleural mesothelioma) express substantial PCFT with or without FRs.^{12–15} For PCFT, folate transport into tumors would be enhanced by the acidification of the tumor microenvironment, secondary to aerobic glycolysis (“Warburg effect”)^{16, 17} and increased expression of acid pumps [i.e., H⁺-ATPases,¹⁸ Na⁺/H⁺ exchanger (NHE1),¹⁹ monocarboxylate-H⁺ efflux symporters (MCT1 and MCT4),^{20, 21} carbonic anhydrases (CAIX and CAXII),²² Cl[−]/HCO₃[−] exchanger (CBE),²³ and the Na⁺/HCO₃[−] co-transporter (NBC)].²⁴

In addition to its important physiologic role in folate transport, RFC is the major transporter of clinically used classical antifolates for cancer, including methotrexate (MTX), pemetrexed (PMX), pralatrexate (PDX), and raltitrexed (RTX) (Figure 1).^{1, 2, 7, 25} These cytotoxic antifolates are also substrates, to various extents, for PCFT.^{5, 7, 25}

Clinically used antifolates (Figure 1) display dose-limiting cytotoxic side-effects, which reflect at least in part their uptake into normal cells through RFC.^{2, 7, 25} Thus, a promising new therapeutic strategy would be to develop tumor-targeted cytotoxic compounds with selective membrane transport for PCFT and little-to-none by RFC.^{5, 8} This increased specificity for PCFT uptake in cancer cells is envisaged to selectively kill tumor cells, while circumventing significant RFC-related side effects and toxicity toward normal cells,⁵ thus overcoming dose-limiting toxicity, one of the major causes of chemotherapy failure. Gangjee, Matherly and coworkers designed, synthesized and evaluated novel compounds that exhibit high selectivity for uptake by tumors via their uptake by PCFT over RFC, resulting in highly potent tumor cell inhibition.^{5, 14, 26–32, 13–15, 26, 29–37} These molecules contain a bicyclic 6,5-pyrimidine scaffold connected by multi-atom linkers to a terminal aryl *L*-glutamate moiety (Figure 2, Supplementary information). Their intracellular targets are β -glycinamide ribonucleotide formyltransferase (GARFTase) and/or 5-aminoimidazole-4-carboxamide (AICA) ribonucleotide (ZMP) formyltransferase (AICARFTase), which catalyze one-carbon transfers from 10-formyl tetrahydrofolate, leading to *de novo* synthesis of purine nucleotides.^{13–15, 26–37}

Of the three major folate transporters, only the X-ray crystal structures of FRs complexed with folic acid and/or antifolates have been reported.^{28, 32, 38, 39} No crystal structures for RFC or PCFT have yet been resolved, which makes drug design at best empiric and renders rational design of PCFT-selective agents exceedingly difficult. Thus, it was of interest to develop molecular models (pharmacophores) for RFC/Activity and PCFT/Activity to aid in the design of PCFT active-compounds with transport selectivity over RFC. We elected to use the terms models (pharmacophores) for PCFT/Activity and RFC/Activity since the IC₅₀ used for the “activity” is a composite of transport (by PCFT or RFC) and inhibition of target enzymes in purine biosynthesis, as well as metabolism (e.g., polyglutamylation). In the current study, we developed chemical feature-based models (pharmacophores) for both RFC/Activity and PCFT/Activity with high correlations. These are being used to identify leads.

Results and Discussion

A. PCFT/Activity pharmacophore model

A.1 Pharmacophore Generation for PCFT/Activity—The top 10 hypotheses (Hypo-A1-A10) for PCFT/Activity models (pharmacophores) were generated by the HypoGen algorithm using the HBA, HBD, RA and NI features of Discovery Studio 16.1.0 (DS)⁴⁰. The cost values, correlation, RMS, maximum FitValues and features of the 10 hypotheses are summarized in Table 1.

A.2 Pharmacophore Validation for PCFT/Activity

- (a) Cost analysis: All 10 generated hypotheses showed cost differences greater than 60, suggesting excellent correlation (Table 1).
- (b) Fisher’s randomization test: In the Fisher test, the activities of the training set of compounds are randomly scrambled. This is used to create a random pharmacophore, including the same parameters as described for the HypoGen hypotheses at a

confidence level of 95%. The resulting 19 columns showed high cost (SI, Table S3) and low correlation (SI, Table S4) values, compared to Hypo-A1-A10.

Since Hypo-A1-A6 displayed a confidence level of greater than 95%, they are statistically sound with a 95% chance of true correlation in the training set activity data. Hypo-A1 presented the highest correlation of 0.965, suggesting a linear dependence of the experimental and estimated activities. Hypo-A1 also displayed the lowest RMS, suggesting a highly accurate predictive model. Accordingly, we selected Hypo-A1 as the model for predicting PCFT activities.

(c) Test set prediction: To further validate Hypo-A1, 5 compounds (compounds 17–21) with reported differences in drug activity toward PCFT-expressing R2/PCFT4 cells (Table 2) were analyzed to check the predictive capability of Hypo-A1. The *Ligand Pharmacophore Mapping* protocol was implemented with *FAST* conformation generation to consider 300 conformations, using the maximum omitted features as –1 and flexible fitting method. Activities of all test compounds were accurately predicted with < 5% error (Table 2). On the basis of training and test set predictions, Fisher validation and cost analysis, Hypo-A1 clearly shows the ability to accurately classify active and inactive PCFT compounds.

Figure 3a shows the excellent correlation of Hypo-A1. The stereo view of the Hypo-A1 is displayed in Figure 3b, along with the optimum distances required between the features to obtain optimum PCFT binding. Compound **1**, a 6-substituted-4-atom bridged thiophene compound is the most active PCFT compound in the testing set.³¹ It shows a perfect alignment with the Hypo-A1 model (Figure 3c) with an excellent FitValue of 9.18. The 4-oxo group on **1** fits the HBA feature, the thiophene ring fused to pyrimidine fits the first RA feature and the thiophene ring in the linker fits the second RA feature. Finally, the γ -COOH fits the NI feature of Hypo-A1 model. The γ -COOH of **1** is extended into the plane of the scaffold. To study the alignment of inactive compounds in PCFT Hypo-A1, **16**, a 5-substituted-4-atom bridged phenyl compound was aligned with the Hypo-A1 model (Figure 3d). It showed no activity in PCFT assays³⁵ and had a low FitValue of 5.65 in the Hypo-A1 model. The distances between the features required are important, along with their directionality. Due to the 5-substitution, linker size and overall conformation, **16** fails to fit the HBA feature of the Hypo-A1. This suggests it might not have a perfect complementarity with the PCFT/Activity model. In order to fit the model, compound **16** adopts a more strained, curved conformation of the linker in the compound. Thus, the model suggests that PCFT/Activity binding can be modulated by varying the substitution position on the bicyclic scaffold and reflects conformational preferences for PCFT/Activity effects.

B. RFC/Activity pharmacophore model

B.1 Pharmacophore Generation for RFC/Activity—The top 10 hypotheses for RFC/Activity models (pharmacophores) were generated using the HypoGen algorithm in DS with HBA, HBD, RA and NI features. The cost values, correlation, RMS, maximum FitValue and features of Hypo B1-B10 are summarized in Table 3.

B.2 Pharmacophore Validation for RFC/Activity

(a) Cost analysis: Hypo-B1-B3 showed a difference in null and fixed costs of >60 with Hypo-B1 showing the maximum cost difference of 67.60. Since the cost difference is greater than 60, these three models have an excellent chance to represent a true correlation (>90% probability) (Table 3).

(b) Fisher's randomization test: A confidence level of 90% was set for the test, which resulted in 9 columns for cost and correlation values. Due to the small size of the set of training compounds (14 active and 2 inactive RFC compounds), only Hypo-B1-B5 showed acceptable confidence. All 9 columns showed cost values higher than Hypo-B1-B10 (Supplemental Information, Table S5) and correlation lower than Hypo-B1-B10 (Supplemental Information, Table S6). Hypo-B1 presented the highest correlation of 0.96, signifying true correlation of experimental and estimated RFC activities. Hypo-B1 also displayed the lowest RMS, suggesting a highly accurate predictive model. Thus Hypo-B1 was selected for predicting RFC activities.

(c) Test set prediction: To further validate Hypo-B1, 3 reported RFC compounds were analyzed to check the predictive capability, using methods described for the PCFT/Activity model above. Activities of all compounds in the test could be predicted with an error of < 4% (Table 4). These results suggest that Hypo-B1 is clearly able to classify active and inactive RFC compounds.

Figure 4a shows the excellent correlation of Hypo-B1. Compound **10**, a 5-substituted 4-atom bridged thiophene-containing compound is the most active RFC compound in testing set. It shows a perfect alignment with the RFC/Activity pharmacophore Hypo-B1 model (Figure 4b), with an excellent FitValue of 12.89. The 2-amino of **10** fits the HBD feature, the 4-oxo group fits the first HBA feature, the amide carbonyl fits the second HBA feature and the γ -COOH fits the NI feature of RFC pharmacophore Hypo-B1. The molecule adopts a curved conformation that fits all the features with the γ -COOH extending out of plane of the scaffold. To study the alignment in case of inactive RFC substrates, **4**, a 6-substituted 4-atom bridged thiophene compound was aligned with Hypo-B1. Compound **4** showed a low FitValue in RFC/Activity Hypo-B1 model (Figures 4c). It is unable to obtain the conformation required to fit all the four features of the RFC/Activity Hypo-B1 model. Thus, the model provides a basis for the differences in RFC transport observed with substitution patterns and presents avenues for future drug design.

Notably, the PCFT/Activity and RFC/Activity models show stark differences in pharmacophore elements for transport of the compounds. The RFC/Activity model consists of two HBAs spaced ~11 Å apart, a HBD and a NI feature. The PCFT/Activity model (pharmacophore) retains the NI feature, but has two RA features spaced ~8 Å apart, and a HBA feature. Interestingly, these pharmacophore features also reflect the different conformational requirements for binding of compounds to the RFC/Activity model (pharmacophore) compared to compounds that bind to the PCFT/Activity model. Both models show that the position of substitution on the bicyclic scaffold, and/or the conformation and length of the linker region dictates the bound conformation and hence the binding efficiencies in the two models. To visualize these differences in conformations required for binding to the PCFT/Activity and RFC/Activity pharmacophores, the most

active substrates, **1** and **10**, were aligned on the PCFT/Activity Hypo-A1 model and the RFC/Activity Hypo-B1 model, respectively. The aligned poses were extracted and superimposed using N1, N3 and N7 as tethers (Figure 5). Figure 5 further emphasizes that careful manipulation of the linker, scaffold and aryl group on the linker can lead to PCFT- or RFC-selective compounds.

The flexibility of the side chain of the ligands affords different conformations for the ligands. This suggests that ligands, such as, **1** (PCFT-selective) and **10** (RFC-selective) can align to accommodate both- the PCFT/Activity and RFC/Activity chemical feature-based models (pharmacophores). To evaluate the significance of the conformations on the alignment in the models, **1** was mapped in the PCFT/Activity chemical feature-based model (pharmacophore) (Figure 6a) and the RFC/Activity chemical feature-based model (pharmacophore) (Figure 6b). The flexibility of the three atom linker of **1** allowed a fit into both models PCFT and RFC.

However the conformation numbers for aligning to both the models differ significantly. A lower conformation number denotes a low energy conformation. The conformation number and absolute energy of **1** that aligns to the PCFT model was 98 and 85.4814, respectively; whereas conformation number and absolute energy of **1** that aligns to the RFC model was 166 and 91.9364, respectively. Thus, though **1** can adopt the conformation required to fit both models, it shows a preference for binding to PCFT over RFC. Similar results were obtained for **10**, where the conformation number and absolute energy of **10** that aligns to the PCFT model was 133 and 96.3839, respectively; whereas conformation number and absolute energy of **10** that aligns to the RFC model was 28 and 90.6178, respectively. This further corroborates the notion that **10** can adopt the conformation required to fit both models, but shows a preference for binding to RFC over PCFT. Thus, our models suggest that conformational restriction of the linker region can be used as a powerful design element in the development of novel transporter-selective antifolates.

In summary, we successfully developed and validated PCFT/Activity and RFC/Activity chemical feature-based models (pharmacophores). To our knowledge, only a few compounds have been synthesized and evaluated for activity against RFC and PCFT. The ligands utilized in the preparation of these models have distinct scaffolds- 2,4-diaminofuro[2,3-*d*]pyrimidine, 2-amino-4-oxohexahydropyrido[2,3-*d*]pyrimidine, 2-methyl-4-oxoquinazoline, and 2-amino-4-oxopyrrolo[2,3-*d*]pyrimidine, with linkers composed of various aliphatic side chains of different lengths with or without aryl groups. Though these do not represent an exhaustive set of ligands, in total absence of any structure-based drug design of compounds, these PCFT/Activity and RFC/Activity chemical feature-based models (pharmacophores) present an effective starting point to rationalize the design and synthesis of new analogs. They will be able to fill the void in the total absence of any pharmacophores. Further design, synthesis and evaluation of compounds designed using these models as PCFT-selective antifolates are in progress. These new analogs will aid to validate the PCFT/Activity and RFC/Activity chemical feature-based models (pharmacophores) and will create new data points, which will be used to modify the pharmacophores. Thus, the published pharmacophores will be continually reformed and will evolve with the addition of new scaffolds.

Experimental Section

Data Preparation:⁴⁰

All compounds used in the training and test sets contain a fused bicyclic pyrimidine scaffold linked with varying chain lengths and aromatic side chains. The 3D QSAR module in Discovery Studio 16.1.0 (DS)⁴⁰ was used for developing and validating PCFT/Activity and RFC/Activity models (pharmacophores). Compound structures were initially sketched in 2D using MOE 2016.09²⁸ and used to create a database in DS. Both PCFT and RFC targeted compounds were tested in novel isogenic Chinese hamster ovary (CHO) cell lines exclusively expressing human PCFT (R2/PCFT4) or RFC (PC43-10) such that inhibition of cell proliferation is a direct reflection of transport activity.^{33, 34, 41} The PCFT training set contained 16 compounds and were tested in R2/PCFT4 cells, with IC₅₀ values for inhibiting cell proliferation that ranged from 3.4 to 840 nM (Table 2). To stretch the IC₅₀ values across four orders of magnitude, *rescale* function was implemented during the ligand selection stage. The RFC training set contained 15 compounds tested in RFC-expressing PC43-10 CHO cells, with IC₅₀ values ranging from 38.3 to 641 nM (Table 4). As with the PCFT training set, *rescale* function was implemented during ligand selection stage for the generation of the RFC model. *Pharmacophore Model Generation*: The catalyst HypoGen algorithm was used to derive pharmacophore models from the PCFT and RFC training sets. The *BEST* method was used to generate 300 conformations with an energy threshold value of 20 kcal/mol. To create the pharmacophore hypotheses, the features selected were Hydrogen Bond Donor (HBD), Hydrogen Bond Acceptor (HBA), Ring Aromatic (RA) and Negative Ionizable (NI). HypoGen generates pharmacophores based on chemical features of active compounds in the training set. Ten pharmacophore hypotheses were returned using the default value of minimum spacing between feature points (2.97 Å). *Pharmacophore validation and selection*: The pharmacophore models were validated by cost analysis, Fisher's randomization test and test set prediction. *Cost Analysis*: The HypoGen algorithm selects the best pharmacophore hypotheses by cost analysis. The overall cost is a summation of weight cost, error cost and configuration cost. The weight component is a value that increases in a Gaussian form as the feature weight deviates from an ideal value (2.0). The error component increases as the root mean squared difference between the estimated and measured activities for the training set molecules increases. The configuration component is a constant cost, which depends on the complexity of the hypothesis space being optimized, and is equal to the entropy of the hypothesis space. The algorithm also calculates theoretical costs –null and fixed cost. The overall cost values of the optimized hypotheses should be between the fixed cost and null cost. The fixed cost represents the simplest model that fits the data perfectly. Null cost is calculated assuming no features. The closer the overall cost is to the fixed cost and the further it is away from the null cost, the more statistically significant the hypothesis is. If the difference between the costs is greater than 60, then the model has an excellent chance to represent a true correlation (>90% probability). If the difference between the null and fixed costs is between 40 and 60, then there is high probability (75–90%) that model shows true correlation. *Fisher's randomization test*: Fisher's randomization test was used to perform statistical validation to evaluate the significance of the pharmacophore models. The 95% and 90% confidence levels were selected to validate the study for the PCFT and RFC models, respectively. *Test set prediction*: A set of 5 compounds

with known PCFT activity and a set of 3 compounds with known RFC activity were used to create test sets for PCFT and RFC, respectively. These compounds were then scanned using the *ligand pharmacophore mapping* function in DS with the *BEST* conformation generation tool and *flexible* fitting method for the PCFT and RFC pharmacophore models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AICA	5-aminoimidazole-4-carboxamide
AICARFTase	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase
CA	carbonic anhydrase
CHO	Chinese hamster ovary
FR	folate receptor
GARFTase	β -glycinamide ribonucleotide formyltransferase
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
MCT	monocarboxylate-H ⁺ efflux symporter
MTX	methotrexate
NBC	Na ⁺ /HCO ₃ ⁻ co-transporter
NHE1	Na ⁺ /H ⁺ exchanger
NI	Negative Ionizable
PCFT	proton-coupled folate transporter
PDX	pralatrexate
PMX	pemetrexed
QSAR	Quantitative structure–activity relationship
RA	Ring Aromatic

RFC	reduced folate carrier
RTX	raltitrexed

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Figure 1.
Classical antifolates in the clinic.

Figure 2.
General structure of antifolates used in this study.

Figure 3.

(a) Plot of correlation between the experimental and the Hypo-A1 estimated PCFT activity values for the 16 training set compounds; (b) Chemical features of Hypo-A1 (stereo view). Green represents HBA, orange represents a RA and blue represents a NI feature; (c) Hypo-A1 aligned to the most active PCFT-targeted compound **1** (stereo view); (d) Hypo-A1 aligned to an inactive PCFT compound **16** (stereo view).

Figure 4.

(a) Plot of correlation between the experimental and the Hypo-B1 estimated RFC activity values of the 15 training set compounds; (b) Chemical features of Hypo-B1. Magenta represents HBD, green represents HBA and blue represents a NI feature; (c) Hypo-B1 aligned to an active RFC compound **22**; (d) Hypo-B1 aligned to an inactive RFC compound **4**.

Figure 5.
Stereo view of superimposition of aligned pose of **1** in PCFT/Activity model (pharmacophore) Hypo-A1 model over **10** in RFC/Activity model (pharmacophore) Hypo-B1 model.

Figure 6. Stereo view of aligned pose of PCFT-selective **1** to (a) PCFT/Activity chemical feature-based model (pharmacophore) and (b) RFC/Activity chemical feature-based model (pharmacophore).

Table 1

Statistical results of the generated for PCFT/Activity models (pharmacophores).

Hypo	Confidence level (%)	Total cost	Cost ^a	RMS ^b	Correlation ^c	Maximum Fit Value ^d	Features ^e
A1	95	73.909	70.421	0.923	0.965	9.53	HBA, NI, RA
A2	95	79.359	64.971	1.244	0.935	9.42	HBA, HBD, NI, RA
A3	95	79.630	64.700	1.189	0.942	10.45	HBA, NI, RA
A4	95	79.725	64.605	1.210	0.939	10.24	HBA, HBD, NI, RA
A5	95	79.990	64.341	1.167	0.945	10.86	HBA, NI, RA
A6	95	80.168	64.162	1.284	0.940	9.40	HBA, HBD, NI
A7	90	80.823	63.507	1.215	0.940	10.82	HBA, HBD, NI
A8	90	81.380	62.950	1.215	0.940	11.09	HBA, HBD, NI
A9	90	82.063	62.267	1.358	0.922	9.72	HBA, NI, RA
A10	85	83.218	61.112	1.453	0.910	8.27	HBA, HBD, NI, RA

Hypo, Hypothesis

Fixed cost = 66.295

Null cost = 144.33

^aCost difference = Null cost-Total cost

^bRMS is the deviation of log (estimated activity) from log (measured activity) normalized to log (uncertainty).

^cCorrelation is the coefficient based on linear regression derived from the geometric fit index.

^dMaximum Fit Value is the highest Fit Value obtained for the best compound in the PCFT/Activity model

^eHydrogen Bond Donor (HBD), Hydrogen Bond Acceptor (HBA), Ring Aromatic (RA) and Negative Ionizable (NI).

Table 2

Actual IC₅₀ values and estimated IC₅₀ values of 16 training set and 5 test set molecules through the model (pharmacophore) Hypo-A1 (A-active M-moderately active)

Proliferation assays were performed for the CHO subline engineered to express human PCFT (R2/PCFT4)³⁴. Results are presented as IC₅₀ values, corresponding to the concentrations that inhibit growth by 50% relative to cells incubated without drug.

Compound	Actual IC ₅₀ (nM)	Extended IC ₅₀ (nM)	Estimated IC ₅₀ (nM)	Error	Fit Value	Status	Reference
1	3.40	3.4	3.4	1.00	9.18	A	32
2	5.39	7.3	13	1.80	8.61	A	33
3	41.54	220	430	1.90	7.08	M	31
4	43.40	240	310	1.30	7.23	M	30
5	57.50	380	600	1.60	6.94	M	37
6	63.82	460	340	-1.30	7.18	M	31
7	87.40	770	690	-1.10	6.87	M	37
8	101.40	990	620	-1.60	6.92	M	30
9	134.80	1600	2500	1.60	6.31	M	32
10	141.00	1700	2600	1.50	6.30	M	35
11	238.5	4100	5900	1.40	5.94	M	29
12	267.00	5000	1000	-5.00	9.71	M	37
13	291.20	5800	9300	1.60	5.75	M	32
14	396.00	9700	5000	-2.00	6.02	M	35
15	444.60	12000	21000	1.80	5.39	M	35
16	840.00	34000	12000	-2.90	5.65	M	29
17*	109.7	—	126.45	1.20	7.61		37
18*	574.4	—	366.30	1.60	7.15		35
19*	81.70	—	78.03	-1.05	7.82		37
20*	>1000	—	52227.28	—	6.00		33
21*	>1000	—	2279.09	—	6.36		34

* Compounds in the test set.

Table 3

Statistical results of the generated for RFC/Activity models (pharmacophores).

Hypo	Confidence level (%)	Total cost	Cost ^a	RMS ^b	Correlation ^c	Maximum FitVa/lue ^d	Features ^e
B1	90	88.051	67.604	1.096	0.964	13.31	HBA, HBD, NI
B2	90	89.891	65.763	1.252	0.948	13.01	HBA, HBD, NI, RA
B3	90	92.266	63.389	1.398	0.932	12.79	HBA, NI, RA
B4	90	98.239	57.416	1.668	0.898	12.59	HBA, HBD, NI, RA
B5	90	102.816	52.839	1.746	0.892	13.31	HBA, NI, RA
B6	50	105.869	49.786	1.897	0.867	9.69	HBA, NI, RA
B7	50	105.935	49.720	1.967	0.851	12.26	HBA, HBD, NI
B8	40	109.138	46.517	2.008	0.848	12.87	HBA, HBD, NI
B9	40	109.380	46.275	1.998	0.851	13.03	HBA, NI, RA
B10	40	109.458	46.197	1.968	0.859	13.33	HBA, HBD, NI, RA

Hypo, Hypothesis

Fixed cost = 68.764

Null cost = 155.655

^aCost difference = Null cost- Total cost

^bRMS is the deviation of log (estimated activity) from log (measured activity) normalized to log (uncertainty).

^cCorrelation is the coefficient based on linear regression derived from the geometric fit index.

^dMaximum FitValue is the highest FitValue obtained for the best compound in the RFC/Activity model

^eHydrogen Bond Donor (HBD), Hydrogen Bond Acceptor (HBA), Ring Aromatic (RA) and Negative Ionizable (NI).

Table 4

Actual IC₅₀ values and estimated IC₅₀ values of 15 training set and 3 test set molecules through RFC/Activity pharmacophore Hypo-B1 (A-active, M-moderately active, I-inactive)

Proliferation assays were performed for the CHO sublines engineered to express human RFC (PC43-10)^{33, 41}. Results are presented as IC₅₀ values, corresponding to the concentrations that inhibit growth by 50% relative to cells incubated without drug.

Compound	Actual IC ₅₀ (nM)	Extended IC ₅₀ (nM)	Estimated IC ₅₀ (nM)	Error	Fit Value	Status	Reference
PMX	30.57	31	99	3.20	12.29	A	29
10	38.32	56	25	-2.20	12.89	A	35
2	50.70	120	230	2.00	11.93	A	33
22	54.00	140	290	2.10	11.82	A	33
16	56.60	160	380	2.50	11.71	A	29
23	59.88	180	270	1.50	11.86	A	35
11	68.80	260	440	1.70	11.64	M	29
1	101.00	720	750	1.00	11.41	M	32
14	110.00	900	820	-1.10	11.38	M	35
24	189.00	3800	4500	1.20	10.63	M	33
25	194.6	4100	2900	-1.40	10.83	M	29
18	243.20	7300	17000	2.30	10.07	M	35
7	510.00	52000	34000	-1.50	9.76	M	37
5	641.00	94000	12000	-7.80	10.21	M	37
6	>1000	310000	61000	-5.00	9.50	I	30
LMX*	12	–	39.17	3.26	13.12		30
RTX*	6.3	–	12.88	2.04	13.60		37
MTX*	12	–	10.66	-1.13	13.68		30

* are the compounds in the test set.