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Mammary Carcinogen-Protein Binding Potentials: Novel and Biologically Relevant Structure-Activity Relationship Model Descriptors

A.R. Cunningham^{*,1,2,3}, S. Qamar¹, C.A. Carrasquer¹, P.A. Holt¹, J.M. Maguire¹, S.L. Cunningham¹, and J.O. Trent^{*,1,2}

¹James Graham Brown Cancer Center, University of Louisville, USA

²Department of Medicine, University of Louisville, USA

³Department of Pharmacology and Toxicology, University of Louisville, USA

Abstract

Previously, SAR models for carcinogenesis used descriptors that are essentially chemical descriptors. Herein we report the development of models with the cat-SAR expert system using biological descriptors (*i.e.*, ligand-receptor interactions) rat mammary carcinogens. These new descriptors are derived from the virtual screening for ligand-receptor interactions of carcinogens, non-carcinogens, and mammary carcinogens to a set of 5494 target proteins. Leave-one-out validations of the ligand mammary carcinogen non-carcinogen model had a concordance between experimental and predicted results of 71% and the mammary carcinogen non-mammary carcinogen model was 72% concordant. The development of a hybrid fragment-ligand model improved the concordances to 85 and 83%, respectively. In a separate external validation exercise, hybrid fragment-ligand models had concordances of 81 and 76%. Analyses of example rat mammary carcinogens including the food mutagen and estrogenic compound PhIP, the herbicide atrazine, and the drug indomethacin, the ligand model identified a number of proteins associated with each compound that had previously been referenced in Medline in conjunction with the test chemical and separately with association to breast cancer. This new modelling approach can enhance model predictivity and help bridge the gap between chemical structure and carcinogenic activity by descriptors that are related to biological targets.

1. Introduction

The advent of structure-activity relationship (SAR) and quantitative SAR (QSAR) paradigms have allowed for the prediction of toxicants and the rational design of therapeutic agents based on their similarity in chemical structure or property to previously tested compounds [1]. Moreover, the utility of QSAR approaches to investigate sets of similarly shaped chemicals with discrete mechanisms of action (*e.g.*, ligands to specific receptors) has been well demonstrated [2-4]. However, chemicals associated with adverse human health effects such as cancer are generally not amicable to traditional QSAR modelling for two reasons. First, there is a great structural diversity of chemicals being modelled for these endpoints. This is because, for the most part, the chemicals that are tested for potential carcinogenic effects are often in use or will be in use for a myriad of purposes (*e.g.*, industrial solvents, consumer products, pesticides, and drugs). Second, there is no

*Corresponding authors. al.cunningham@louisville.edu, john.trent@louisville.edu.

generalized *a priori* accepted mechanism of toxicity applicable to the entire set of compounds being modelled (*e.g.*, a specific receptor for carcinogenesis).

The Computer Automated Structure Evaluation program (CASE) was developed over 20 years ago by Rosenkranz and Klopman in order to address these difficulties [5,6]. This SAR expert system was one of the first developed to efficiently and rapidly analyse large numbers of structurally diverse compounds without the need for any *a priori* mechanism of action. The CASE program successfully used 2-dimensional (2D) structural features called biophores found among categorized active and inactive chemicals in the program's learning set that were associated with a particular biological, pharmacological, or toxicological activity. Other methods were also developed including John Ashby's "structural alerts" to potential carcinogenicity [7-9], TOPKAT [10], and DEREK [11]. SAR models are increasingly being used by regulatory agencies worldwide for both human health [12] and ecological endpoints [13] (*e.g.*, Oncologic by the U.S. Environmental Protection Agency [14] and CASE by the U.S. Food and Drug Administration's Centre for Drug Evaluation and Research [15]).

Previously, we reported SAR models based on data from the Carcinogenic Potency Database (CPDB) [16] analyses of mouse [17] and rat [18] cancer data using CASE/MultiCASE. In these studies, rat and mouse SAR models had a concordance between experimental and SAR-predicted values of 71 and 78%, respectively [17,18]. More recently, MCASE MC4PC and MDL-QSAR models developed by the FDA produced a concordance of 66 and 69%, respectively [15].

Our early CASE/MultiCASE models, while being predictive, also provided some insight into the structural underpinnings for carcinogenesis and were consistent with Ashby's "structural alerts" [7-9]. Recently, using the cat-SAR expert system, we developed models of rat mammary carcinogens [19] based on CPDB data [20,21]. One set of SAR models was based on a comparison of rat mammary carcinogens to rat non-carcinogens (MC-NC) and the second compared rat mammary carcinogens to rat non-mammary carcinogens (MC-NMC). While the MC-NC model was typical of carcinogen SAR models with comparisons of carcinogens to noncarcinogens (albeit for a specific tumour site), the MC-NMC model was unique since it was based on a learning set that contained carcinogens in both the active (*i.e.*, mammary carcinogens) and inactive (*i.e.*, carcinogens to sites other than the mammary gland) categories.

In that study, the rat MC-NC model achieved a concordance between experimental and predicted values of 84% and the rat MC-NMC model was 78% concordant. As such, both tissue-specific models were more concordant than previous models developed for whole animal carcinogenesis. More importantly, however, the MC-NMC model was able to distinguish between different types of carcinogens (*i.e.*, mammary carcinogens from all other carcinogens), not rather between carcinogens and non-carcinogens. Thus the MC-NMC model identified structural attributes that addressed the question of "why do some carcinogens induce mammary cancer."

However, even though these models are useful for predicting organ-specific carcinogenesis, they have limited applicability for mechanistic inquiry regarding organ-specific activity. One way to overcome this shortfall, as reported by Zhu et al., is to incorporate SAR descriptors that have direct biological relevance [22]. In this study, the authors described QSAR models for rodent carcinogenicity that were developed from chemical structural descriptors and high throughput screening cytotoxicity descriptors for the modelled compounds wherein the QSAR model's concordance went from 62.3% for the chemical descriptor only model to 72.7% when cell viability data was included [22].

Another way to include biologically-relevant descriptors in SAR model is to obtain potential chemical-protein interaction data by virtual screening techniques. As such, we developed and report herein a novel SAR modelling approach that uses descriptors not derived directly from chemical structure, but derived from whether or not the compounds are potential ligands for a wide variety of proteins. These biologically-based SAR descriptors are developed by virtual screening of the compounds in the model's learning set against a large and diverse set of proteins. The end result of this approach is a set of SAR descriptors associating chemical carcinogens and non-carcinogens to potential biological targets. This is in contrast to traditional SAR descriptors that associate chemical carcinogens to chemical structures. Hence this novel SAR modelling paradigm bridges the gap between chemical (sub)structure and observable toxicological phenomena by directly associating carcinogenic activity with biological targets. The activity part of the model is still empirical from some *in vitro* or *in vivo* experiment (*e.g.*, cancer bioassay). However, we demonstrate that the “structure” part of the SAR equation can be populated with biologically relevant descriptors (*i.e.*, ligand-receptor interactions) rather than the traditional chemical descriptors (*e.g.*, molecular weight, 2D fragments, or topological indices). This new SAR modelling paradigm could be referred to as (biologically relevant) structure – activity relationship modelling.

Cross validations of the ligand MC-NC model had a concordance between experimental and predicted results of 71% and the MC-NMC model was 72% concordant. Furthermore, the development of a hybrid fragment-ligand model improved the concordances to 85 and 83% for the two models, respectively. Therefore, the hybrid model, by bringing together chemical and biological descriptors, outperformed both models. As important, however, as the increased predictivity of the hybrid model is the observation in an example case that the ligand model provided a more complete SAR-based rationale for chemical carcinogenesis by identifying specific biological targets to which carcinogens may interact. The fragment and ligand models together therefore can provide a description of chemical features associated with carcinogenic activity as well as biological targets potentially affected by chemical carcinogens.

2. Materials and methods

2.1 Mammary gland carcinogen learning sets

Two distinct learning sets were used from data analysed in the CPDB which standardises the experimental results (whether positive or negative for carcinogenicity) and have been described previously [19]. The mammary carcinogens, non-mammary carcinogens and non-carcinogens were obtained from CPDB Summary Table by Target Sites [20,21].

Cat-SAR models are built through a comparison of descriptors found amongst two designated categories of compounds in the model's learning set. For these analyses, the categories and chemical composition for one learning set was MC-NC and the other set was MC-NMC. For the MC-NC model a random set of 104 noncarcinogens were selected from the CPDB and for the MC-NMC model a random set of 104 carcinogens to sites other than the mammary gland were selected as inactive compounds. (SDF files for both MC-NC and MC-NMC have been provided as Supplementary Material 2 and 3, respectively.)

2.2 SAR Model descriptors: Chemical fragments and ligand binding affinity

2.2.1 Fragment model—The cat-SAR program was developed to generate SAR models based on 2D chemical fragments. For these models, each chemical in the learning set is fragmented into all possible fragments, in this case between three and seven atoms in size. Atom type, bond type, and atomic connections are considered. A compounds-fragment matrix is then computed where the rows are intact chemicals and the columns are molecular

fragments. Thus for each chemical, a tabulation of all its fragments is recorded across the table row and for each fragment all chemicals that contain it are tabulated down the table column.

2.2.2 Ligand model—For the ligand model, the cat-SAR algorithm was adapted to use compound-protein interaction data as SAR model descriptors. Compounds from the MC-NC and MC-NMC learning sets were virtually screened for ligand-like character against a set of virtual screening targets developed by Kellenberger et al [23]. This set of targets consisted of 6415 ligand binding sites from x-ray crystallographic data obtained from the PDB used to develop a virtual screening version of the PDB (sc-PDB) [23]. For this set of ligand-protein crystal structures Kellenberger et al. extracted binding sites from PDB structures wherein a small molecule and protein cavity were identified. Solvents, detergents, and ions as well as the ligand were removed, leaving the binding pocket open for virtual screening [23]. For this exercise we used “scPDB 2007” downloaded on June 16, 2007, [24] that contained 5523 structures, of which 5494 were amicable to our analyses.

Each of the 208 chemicals in the two mammary carcinogen learning sets was virtually docked into each of the ligand binding cavities of the sc-PDB using Surflex-Dock 2.3 [25,26]. The standard scoring function for each compound was calculated to estimate its affinity to the binding site as $-\log(K_d)$ using hydrophobic, polar complementarity, entropic, and solvation terms.

For each compound, the sc-PDB structures were sorted according to their affinity scores with “high” scores suggesting that the compound was a ligand for that particular protein. Range-find experiments were conducted to determine an appropriate number of sc-PDB structures to be used as cat-SAR descriptors for each compound. For example, in the sc-PDB 20 structure model, affinity scores were ranked for each compound from highest affinity to lowest. Then for each compound the 250 top sc-PDB structures were selected as its descriptors. A compound-ligand matrix was then computed where the rows again were the 208 chemicals and in this instance, each column was for one of the 5494 proteins. Thus for each chemical, a tabulation of the proteins it interacted with was recorded across the table row and for each protein all chemicals that interacted with it were tabulated down the table column.

2.2.3 Hybrid model—LOO validation results from the individual fragment and ligand models were compared to create two hybrid models. A concordant prediction model considered the compounds in which both the fragment and ligand models made the same positive or negative prediction, whether correct or incorrect to determine the activity of the left-out compound. In addition, a Bayesian model used Bayes' Theorem to combine the results of individual fragment and ligand models to determine the activity of left-out compounds.

2.3 Cat-SAR modelling

To ascertain an association between chemical descriptors (*i.e.*, fragments or ligands) and a chemical's activity (or inactivity), a set of rules is used to choose “important” from “unimportant” descriptors. The first selection rule (the Number Rule) is the number of chemicals identified in the learning set that possesses each particular description: For the fragment model, it is the number of chemicals in the learning set that contains the fragment. For the ligand model, it is the number of chemicals determined to be a ligand for each protein. The second selection rule (the Proportion Rule) is the proportion of active or inactive chemicals that then possesses the particular description: For the fragment model it is the proportion of active or inactive chemicals that derived the fragment and for the ligand

model it is the proportion of active or inactive chemicals that were classified as ligands for the protein. For example, the first model in Table 1 had a Number Rule of 3 and Proportion Rules for active and inactive descriptors both set to 0.75. Thus for a descriptor to be included in that model, it had to be found in at least three chemicals and the proportion of active (or inactive) chemicals that possessed it had to be 0.75. However, since it is not practical to determine the values for the Number and Proportion Rules *a priori*, these values are estimated by the cat-SAR rule optimization routine. The optimization routine in this instance allowed the Number Rule to range between 1 and 8 and the Proportion Rule to range between 0.50 and 0.95. Based on LOO validations (see below), final values were selected that yielded both highly accurate (*i.e.*, had a high concordance between experimental and predicted values) and highly predictive models (*i.e.*, made predictions on most of the chemicals in the learning set). In all instances a Standard model with Number Rule of three and Proportion Rule of 0.75 was also developed in conjunction with a Rule Optimized model.

2.4 Model validation and application

The resulting list of important fragments or proteins can then be used to validate or test the predictivity of the model, for mechanistic analysis, and to predict the activity of an unknown compound. To predict the activity of an untested compound the cat-SAR program determines which, if any, descriptors from the model's pool of significant descriptors the untested compound contains. If none are present, no prediction of activity is made for the compound (*i.e.*, there are no default predictions of activity or inactivity). If one or more descriptors are present, the number of active and inactive compounds associated with each descriptor is determined. The probability of activity or inactivity is then calculated based on the total number of active and inactive compounds that went into deriving each of the descriptors.

The probability of activity is calculated by cat-SAR by two similar techniques. The summation method adds all the active and inactive compounds that go into deriving each fragment and divides the active compounds by the total to determine the probability of activity. For example, if a compound contains two fragments, one being found in 9/10 active compounds in the learning set (*i.e.*, 90% active) and the other being found in 3/3 inactive compounds (*i.e.*, 0% active), the unknown compound will be predicted to have a probability of activity of 69% (*i.e.*, $9/10 \text{ actives} + 0/3 \text{ actives} = 9/13 \text{ actives}$ or 69% chance of activity). The average method calculates the average probability of the active and inactive fragments contained in the chemical by averaging the probability of activity associated with each fragment. Using the above example, the two probabilities of activity, 90% and 0% are averaged for an activity value of 45%.

A self-fit and two cross-validations analyses, and an external validation (EXT) test were conducted for each model. For the self-fit analysis, after a model was developed, the model was used to predict the activity of the chemicals in its learning set in order to ascertain whether or not the model was capable of at least fitting its own data. A leave-one-out (LOO) validation was conducted wherein each chemical, one at a time, was removed from the model's learning set and an n-1 model was derived. Using the same criteria described above, the activity of the removed chemical was predicted using the n-1 model. With selected models, a leave-many-out (LMO) validation was conducted wherein 10,000 randomly selected sets of 2.5% of the chemicals (~5) were removed from the total descriptor set, and the n-2.5% model was derived. The activity of each of the removed chemicals was predicted using the n-2.5% model and the average sensitivity, specific, and concordance were calculated. For the EXT, 10 random sets of 10% of chemicals in the learning sets were removed with the remaining 90% of the compounds used to develop a model and predict the activity of those left out. This was repeated 10 times and the average sensitivity, specificity,

and concordance values were calculated. Furthermore, while all predictions were considered in the self-fit, LOO, and LMO validations, for the EXT, compounds with a predicted activity value within one unit of the model's cutoff point for separating active from inactive compounds (see below) were considered equivocal predictions and were not included for the final assessment of the model.

In order to consider concordance, sensitivity, and specificity in terms of carcinogenic activity (i.e., a carcinogen or not a carcinogen), each compound's probabilistic activity value is converted back to an active or inactive category value using a cut-off point derived from the LOO validations [18]. Depending on the application of the model, the cut-off point can be adjusted wherein a model with the best overall concordance can be selected (i.e., a most predictive model), one with near equal sensitivity and specificity (i.e., a balanced model) or one with high sensitivity (i.e., a risk averse model). For this exercise, the cut-off point was selected for models that had a high overall concordance in order to compare these models with previously published cancer SAR models. As noted above, for the EXT validation, compounds with predicted activity values within one unit of the models cut-off point were considered equivocal and not used for the sensitivity, specificity, and concordance calculations.

3. Results and discussion

3.1 Predictive performance of the fragment and ligand mammary carcinogen models

In order to judge how well the fragment and ligand models performed in general (aside from comparing them to previous SAR models discussed above), one can consider the “accuracy” or reproducibility of the cancer bioassay data themselves. For instance, Gold and colleagues found that based on “near-replicate” experiments in the CPDB, 11 out of 54 chemicals tested for their ability to induce cancer in mice were discordant (i.e., 80% reproducible) and 16 out of 104 chemicals tested for cancer in rats were discordant (i.e., 85% reproducible) [27]. Gottman *et al.*, using the CPDB found only a 57% concordance between 121 compounds tested by the NTP/NCI where literature values were also available [28]. Furthermore, in an assessment of the predictivity of QSAR models for discriminating between carcinogens and non-carcinogens, Benigni and Bossa report that while internal validation methods along the line of LOO may overestimate the accuracy of the models, true external validation correct prediction rates may be between 70 and 100% [29].

Leave-one-out (LOO) validation of the standard MC-NC fragment model returned a concordance of 75%, a sensitivity of 69%, and specificity of 81% and the ligand model returned a concordance of 67% with a sensitivity of 69% and a specificity of 64% (Table 1). The fragment model made predictions on 182 out of the 208 chemicals (88%) and was based on 1583 significant fragments (724 active and 859 inactive). The ligand model made predictions on all 208 chemicals (100%) and was based on 835 proteins (216 active and 619 inactive).

For the standard MC-NMC model, the LOO validation of the fragment model returned a concordance of 77%, a sensitivity of 74%, and a specificity of 81% and the ligand model returned a concordance of 69%, a sensitivity of 64%, and a specificity of 73% (Table 2). In this case, the fragment model made predictions on 179 out of the 208 chemicals (86%) and was based on 1779 fragments (885 active and 894 inactive) and the ligand model again made predictions on all 208 chemicals (100%) and was based on 829 proteins (209 active and 620 inactive).

With the establishment that the standard models could be developed, the cat-SAR Rule Optimizer was used to develop MC-NC and MC-NMC with optimal concordance and high

predictivity (*i.e.*, predicting 90% or more of the compounds in the learning set). The best MC-NC fragment model returned a concordance of 79%, a sensitivity of 72%, and specificity of 86% (Table 1). The best ligand model returned a concordance of 71%, a sensitivity of 66%, and a specificity of 76% (Table 1). The fragment model made predictions on 199 of the 208 chemicals (96%) and was based on 2021 fragments (632 active and 1389 inactive) and the ligand model made predictions on all 208 chemicals (100%) and was based on 835 proteins (216 active and 619 inactive).

The best MC-NMC fragment model returned a concordance of 77%, a sensitivity of 74%, and a specificity of 81% (Table 2). The best ligand model returned a concordance of 72%, a sensitivity of 60%, and a specificity of 84% (Table 2). The fragment model made predictions on 187 of the 208 chemicals (90%) and was based on 12190 fragments (6338 active and 5852 inactive) and the ligand model made predictions on all 208 chemicals (100%) and was based on 1213 proteins (691 active and 522 inactive).

In all instances the LOO validation results were confirmed with leave-many-out (LMO) cross-validations. The LMO cross-validations returned about the same sensitivity, specificity, and concordance values as the LOO did (Tables 1 and 2). Considering the self-fit, LOO, and LMO validation results of the standard and rule optimised models, it is apparent that both 2D fragment and ligand models independently can identify sufficient differences (*i.e.*, chemical substructure and ligand binding preferences, respectively) to produce predictive models that can distinguish between mammary carcinogens, non-mammary carcinogens, and non-carcinogens.

Thus differences exist between the classes of chemicals, whether they are 2D structure or protein affinity that has predictive value. Since the fragment and ligand models are both predictive and have been developed from very different perspectives, it is possible that they reflect different attributes of the chemicals being modelled as well as different facets of the toxicological phenomena under study. We therefore hypothesized from a predictive and mechanistic standpoint that they could be complimentary to each other. That is to say, a hybrid fragment and ligand model may be superior to either model individually.

To test this hypothesis we combined the standard MC-NC and MC-NMC ligand models and also the rule optimised models in two ways. The first, and simplest, was the consistent prediction hybrid model wherein the fragment and ligand models were required to yield identical predictions for each chemical. Second, a Bayesian hybrid model was developed that combined predictions from the fragment and ligand model (*i.e.*, consistent as well divergent predictions) with a final determination as to activity class based on Bayes' theorem [30-32].

Using the standard models, the MC-NC hybrid consistent prediction model had a concordance of 81% compared to 75 and 67% for the standard fragment and ligand models, respectively (Table 1). The MC-NMC hybrid consistent prediction model had a concordance of 82% compared to 77 and 72% for the standard fragment and ligand models, respectively. Hence for both the standard MC-NC and MC-NMC models, the hybrid consistent prediction models were more accurate than either model alone, albeit while making less predictions (*i.e.*, 125/208 and 121/208 respectively).

Using the rule optimised models, the MC-NC hybrid consistent prediction model had a concordance of 85% compared to 79 and 71% for the individual fragment and ligand models (Table 1). Likewise the MC-NMC hybrid consistent prediction model was better than either model alone with a concordance of 83% compared to 78 and 72% for the fragment and ligand models, respectively (Table 2). Again, the consistent hybrid models were better than either model alone, and at the cost of fewer predictions (*i.e.*, both at 142/208).

On the other hand, for both the standard and rule optimized models, the Bayesian hybrid models yielded the same concordances as the individual fragment model although the sensitivity and specificity for the Bayesian models were different than for the fragment models.

Lastly, the predictivity of the individual ligand and fragment based models were assessed by EXT validation sets. These EXT sets were also then combined to judge the predictivity of the hybrid models. In each case, the concordances of the individual and hybrid models were less than that observed by the LOO or LMO validations (Table 3). The concordance values for the fragment MC-NC and MC-NMC models were 71 and 72% respectively, while those for the ligand model were 65 and 61% with the low concordance values related to the low specificity for these models (57 and 45% for the MC-NC and MC-NMC models, respectively). However, similar to the observation in the LOO hybrid models, the combined consistent prediction MC-NC and MC-NMC models had concordance values of 81 and 76% and the Bayesian model had concordance values 75 and 73%, respectively.

Generally, for the MC-NC and MC-NMC standard and rule optimised models, the fragment models outperformed the ligand models (Tables 1 and 2) as far as concordance is concerned. However, by analysing the LOO predictions from both models jointly as in the case of the hybrid model, the MC-NC and MC-NMC models' concordances both increased allowing the two modelling methods to reinforce each other. The same increase in concordance values for the hybrid models was verified by the EXT validations (Table 3). This suggests that the fragment and ligand models provide complimentary sets of descriptors to the problem. In other words, while the fragment model describes the sub-structural characteristics of mammary carcinogens, the ligand model brings to bear ligand-like characteristics of mammary carcinogens (see examples below). As such, we speculate that these two modelling methods take into account different aspects of chemical carcinogenesis. As mentioned, data in the rat CPDB may be about 85% reproducible [27], hence concordances achieved for the MC-NC model of 85% and the MC-NMC model of 83% indicate that with the logical combination of chemical-based and biological-based SAR descriptors, cat-SAR predictions for rat carcinogens and rat mammary carcinogens have likely reached the reproducibility of the animal bioassays themselves.

3.2 Analyses of ligand mammary carcinogen models

3.2.1 Example: PhIP—PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) has been demonstrated to be a genotoxic carcinogen and an estrogen receptor ligand [33] and is reported in the CPDB as a *Salmonella* mutagen [34] and mammary carcinogen [20,21]. The International Agency for Research on Cancer (IARC) indicates that there is inadequate evidence to determine its carcinogenicity in humans and antiquated evidence for carcinogenicity in experimental animals [35]. During a previous fragment-based cat-SAR analysis of rat mammary carcinogens it was observed that 1) structural fragments were able to accurately classify PhIP as a mammary carcinogen and 2) some of the fragments that were used for this classification were related to genotoxicity and other fragments, while being related to carcinogenicity, were not apparently related to genotoxicity [19]. In other words, this latter set of fragments suggested a non-genotoxic mechanism to PhIP's carcinogenic potential. In the present study, analysis of the ligand MC-NC model demonstrated that PhIP was accurately predicted during the LOO validation to be a mammary carcinogen rather than a non-carcinogen due to its potential interaction with 60 proteins (Table S1) (activity value = 0.64, cutoff value = 0.61). Interestingly, of the 60 proteins identified several were related to “estrogenicity” including estrogen sulfotransferase PDB (Protein Data Bank) (PDB 1HY3), estrogen receptor alpha (PDB 1×7E), and estrogen receptor beta (PDB 1×78). Analysis of the ligand MC-NMC model again demonstrated that

PhIP was accurately predicted to be a mammary carcinogen as opposed this time to a carcinogen at all other sites based on its interactions with 21 proteins (Table S2) (activity value = 0.54, cutoff value = 0.53). Of the 21 identified proteins, estrogen sulfotransferase (PDB 1HY3) was again associated with the mammary carcinogenicity of PhIP. Interestingly, estrogen sulfotransferase has been shown to bioactivate PhIP into a DNA reactive form [36]. It has also been demonstrated that PhIP induced upregulation of ER beta and localized it to the nuclear membrane in rat mammary carcinomas which is positively correlated to cell proliferation and cell cycle progression [37].

Additionally, an automated Medline search wherein (generally) the exact PDB name (i.e., quoted) and the words breast cancer and PhIP identified several other protein targets that had previously been referenced with regards to PhIP or breast cancer in the MC-NC model including NAD(P)H dehydrogenase [quinone] 1, serum albumin, glutathione-S-transferase, and cell division protein kinase 2.

3.2.2 Example: Atrazine—Atrazine, a triazine herbicide, is reported in the CPDB as a *Salmonella* non-mutagen [34], and rat mammary carcinogen [20,21]. IARC indicates that while there is adequate evidence of carcinogenicity in experimental animals there is inadequate evidence to determine its carcinogenicity in humans [38]. Considering the LOO validation, atrazine was correctly predicted to be a rat mammary carcinogen by both the MC-NC (activity value = 0.66, cutoff value = 0.61) and MC-NMC (activity value = 0.57, cutoff value = 0.53) models (Tables S3 and S4, respectively). Of the 79 PDB structures used for the MC-NC prediction for mammary carcinogenicity, an automated Medline search identified six proteins that had references to both breast cancer and atrazine. These included aspartate aminotransferase (PDB 1AKA, 1ARG, 1CQ8), L-lactate dehydrogenase (PDB 1LLD), glycogen phosphorylase (PDB 1P4G), chitinase (PDB 1W1T), chloramphenicol aminotransferase 3 (PDB 1CLA), and glutathione S-transferase (PDB 4GST).

For example, in rats treated with atrazine, glutathione S-transferase has been shown to be inhibited, aspartate aminotransferase activity is decreased, and lactate dehydrogenase activity is increased [39]. Interestingly, aspartate aminotransferase has been considered as a novel target for breast cancer therapy [40]. Furthermore, an older publication has suggested that atrazine can increase glycogen phosphorylase activity and elevate c-AMP levels [41]. With regard to the LOO prediction for atrazine by the MC-NMC model, one of the heat shock protein 90s (PDB 1UY9) was shown to be depleted in female rats ovaries treated with a subacute amount of atrazine [42].

3.2.3 Example: Indomethacin—Indomethacin is a non-steroidal anti-inflammatory drug (NSAIDs) and is reported in the CPDB as a *Salmonella* non-mutagen [34] and rat mammary carcinogen with the female rat mammary gland being its sole target [20,21]. Although indomethacin was not predicted to be a mammary carcinogen by MC-NC model (activity value = 0.59, cutoff = 0.61), it was correctly predicted by the MC-NMC model (activity value = 0.61, cutoff value = 0.53). However, even though it was not correctly classified as a mammary carcinogen by the MC-NC model, of 74 PDB structures used in the prediction of indomethacin as a mammary carcinogen by the MC-NC model, (Table S5) 25 of them had Pubmed references relating to indomethacin and breast cancer. Similarly of the 24 PDB structures used in the prediction that indomethacin was a mammary carcinogen by the MC-NMC model (Table S6) eight had Pubmed references for indomethacin and breast cancer.

It can be expected that indomethacin, being a long prescribed drug, will have an abundance of Pubmed references so we will only list an exemplary few here relating to its potential interactions with molecular targets. These include several versions of aspartate aminotransferase (PDB 1AKB, 1AMA, 1CQ7, 1CQ8, 1MAQ), acetylcholinesterase (PDB

1E66), serum albumin (PDB 1HK1), peroxisome proliferator-activated receptor alpha (PDB 1KKQ), heat shock protein 90 (PDB 1UY9, 1UYD) thymidylate synthase (PDB 1ZPR), glutathione S-transferase (PDB 1YDK), and dihydrofolate reductase (PDB 1CD2). For example, indomethacin has been shown to diminish acetylcholinesterase activity in rats while altering intestine contractions [43], significantly decrease glutathione S-transferase [44], act as a competitive inhibitor of dihydrofolate reductase [45], alter thymidine kinase activity in rats during liver regeneration [46], act as an agonist for peroxisome proliferator-activated receptors alpha [47] and belong in a cluster of other NSAIDs [48].

Given these three examples of rat mammary carcinogens and the observation that some of the PDB structures used for their accurate assessment as a rat mammary carcinogen have already been shown to be associated with the agent in question and breast cancer, it is evident that the cat-SAR ligand model can be used to provide a degree of insight into biologically relevant descriptors of activity. In other words, if no mechanism-based explanation for the mammary carcinogenic activity of these agents had yet been discovered, the modelling process described herein would have pointed to some likely targets for the agent and its carcinogenic activity.

4. Conclusions

The present investigation demonstrated the utility of an innovative SAR modelling approach that used the propensity of chemicals to act as ligands to 5494 proteins as potentially biologically relevant SAR model descriptors. While both the ligand and fragment MC-NC- and MC-NMC learning sets separately produced models with good concordances and predictivity as determined by LOO and LMO cross-validation methods, combining predictions from both models yielded a hybrid model with higher overall accuracy than either model alone. Noting that internal measures of validation may overestimate the true accuracy of SAR models [29], we also tested the development of hybrid models with true external validation sets. Here, when 10 random samples of compounds were removed as EXT validation sets and were predicted by models derived from the remaining 90%, the overall accuracy of the individual models was lower than that estimated by LOO. However, the hybrid models from the combined ligand and fragment-based models (while also having lower concordance values than estimated by LOO) maintained correct prediction rates of 81 and 76% respectively for the MC-NC and MC-NMC models. These findings indicate that the fragment and ligand models are complimentary to each other.

These data further support our previous SAR analysis of mammary carcinogens where it was apparent that dual mechanisms existed in carcinogens, in which certain structural attributes defined a chemical as a carcinogen, and others then distinguished a carcinogen as a mammary carcinogen. In essence, we speculated that structural features related to genotoxicity separated carcinogens from non-carcinogens, while structural features that related to non-genotoxic activity such as receptor binding could separate mammary carcinogens from other carcinogens.

For example, when considering the specific role that genotoxicants play in carcinogenesis, results from the comet (*i.e.*, alkaline single cell gel electrophoresis) assay are interesting in that the method can detect tissue-specific *in vivo* chemical-induced genotoxicity. When applying this assay to a set of 208 chemicals previously tested for carcinogenicity, Sasaki *et al.* found that many of the tissues that displayed DNA damage were not necessarily targets for carcinogenicity but nearly all tissues displaying carcinogenicity were targets of genotoxicity [49]. They concluded that although genotoxicity is generally necessary for carcinogenicity, it is not a sufficient predictor of organ-specific carcinogenicity [49,50]. In other words, although genotoxicity is a mechanistic link to cancer, DNA adducts can be

found in similar levels between cancer target and non-target organs [51]. Hence in the specific example of PhIP, our previous fragment analysis indicated that the mammary carcinogenic activity of PhIP was related to both its genotoxic and non-genotoxic features and at that time we speculated that the non-genotoxic feature could be indicative of estrogenic binding potential [19]. This SAR-based speculation has now been substantiated in the present analysis since of the PDB descriptors used for the determination that PhIP was a mammary carcinogen, estrogen receptors alpha and beta and estrogen sulfotransferase were found to be important. Moreover, in the additional examples of the non-mutagenic herbicide atrazine and NSAID indomethacin, a number of PDB structures used as their SAR descriptors had been previously reported as being related to breast cancer and influenced by these agents. In total, this new SAR modelling approach, involving the use of biological targets as SAR descriptors, while alone not being as predictive as our fragment-based approach, appears to be able to help bridge the gap between chemical structure and toxicological activity by developing SAR descriptors that are related to biological targets.

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Table 1
Fragment summary, self-fit, and cross validation results for the mammary carcinogen – non-carcinogen (MC-NC) SAR models.

Model	Fragments or PDB				Self-Fit			Leave-One-Out			Leave-Many-Out		
	Total	Model	Active	Inactive	Sensitivity	Specificity	Concordance	Sensitivity	Specificity	Concordance	Sensitivity	Specificity	Concordance
<u>Standard Model</u>													
Fragment Model	18450	1583	724	859	0.73 (66/85)	0.95 (87/92)	0.84 (149/177)	0.69 (63/91)	0.81 (74/91)	0.75 (137/182)	0.68	0.80	0.73
Ligand Model	5494	835	216	619	0.98 (102/104)	0.93 (97/104)	0.95 (199/208)	0.69 (72/104)	0.64 (67/104)	0.67 (139/208)	0.69	0.64	0.65
<u>Rule Optimized Models</u>													
Fragment Based	18450	2021	632	1389	0.82 (80/98)	0.83 (82/99)	0.82 (162/197)	0.72 (72/100)	0.86 (85/99)	0.79 (157/199)	0.72	0.83	0.78
Ligand Based	5494	835	216	619	0.98 (102/104)	0.93 (97/104)	0.95 (199/208)	0.66 (69/104)	0.76 (79/104)	0.71 (148/208)	0.65	0.76	0.69
<u>Combined Models</u>													
<u>Standard Models</u>													
Consistent								0.86 (49/57)	0.76 (52/68)	0.81 (101/125)			
Bayes'								0.79 (63/80)	0.73 (74/102)	0.75 (137/182)			
<u>Rule Optimized Models</u>													
Consistent								0.92 (54/59)	0.81 (67/83)	0.85 (121/142)			
Bayes'								0.84 (72/86)	0.75 (85/113)	0.79 (157/199)			

Table 2

Fragment summary, self-fit, and cross validation results for the mammary carcinogen – non-carcinogen (MC-NMC) SAR models.

Model	Fragments or PDB				Self-Fit			Leave-One-Out			Leave-Many-Out		
	Total	Model	Active	Inactive	Sensitivity	Specificity	Concordance	Sensitivity	Specificity	Concordance	Sensitivity	Specificity	Concordance
<u>Standard Models</u>													
Fragment Based	13954	1779	885	894	0.85 (79/93)	0.82 (70/85)	0.84 (149/179)	0.74 (71/96)	0.77 (64/83)	0.75 (135/179)	0.73	0.78	0.75
Ligand Based	5494	829	209	620	0.99 (103/104)	0.99 (103/104)	0.97 (206/208)	0.64 (67/104)	0.73 (76/104)	0.69 (143/208)	0.65	0.75	0.69
<u>Rule Optimized Models</u>													
Fragment Based	13954	12190	6338	5852	1.0 (95/95)	0.94 (91/97)	0.97 (186/192)	0.74 (68/92)	0.81 (77/95)	0.78 (145/187)	0.74	0.81	0.77
Ligand Based	5494	1213	691	522	0.96 (100/104)	1.00 (104/104)	0.98 (204/208)	0.60 (63/104)	0.84 (87/104)	0.72 (150/208)	0.62	0.81	0.72
<u>Combined Models</u>													
<u>Standard Models</u>													
Consistent								0.86 (51/59)	0.77 (48/62)	0.82 (99/121)			
Bayes'								0.79 (71/90)	0.72 (64/89)	0.75 (135/179)			
<u>Rule Optimized Models</u>													
Consistent								0.86 (49/57)	0.81 (69/85)	0.83 (118/142)			
Bayes'								0.75 (76/102)	0.81 (69/85)	0.78 (145/187)			

Table 3

External validation summary for PDB, fragment, and hybrid models for the mammary carcinogen – non-carcinogen (MC-NC) and mammary carcinogen – non-mammary carcinogens (MC-NMC) SAR models. Activity values within one unit of the model's cutoff point for separating active from inactive compounds were considered equivocal and not used to calculate values.

Model	External validation		
	Sensitivity	Specificity	Concordance
<u>MC-NC</u>			
Fragment Based	0.71 (57/80)	0.72 (71/99)	0.71 (128/179)
Ligand Based	0.73 (53/73)	0.57 (39/69)	0.65 (92/142)
<u>Combined</u>			
Consistent	0.84 (38/45)	0.78 (28/36)	0.81 (66/81)
Bayes'	0.74 (45/61)	0.72 (44/61)	0.75 (92/122)
<u>MC-NMC</u>			
Fragment Based	0.74 (75/101)	0.70 (56/80)	0.72 (131/181)
Ligand Based	0.74 (57/77)	0.45 (30/66)	0.61 (87/143)
<u>Combined</u>			
Consistent	0.78 (40/51)	0.72 (21/29)	0.76 (61/80)
Bayes'	0.76 (50/66)	0.70 (42/60)	0.73 (92/126)