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Pragmatic Approaches to Using Computational Methods To Predict Xenobiotic Metabolism

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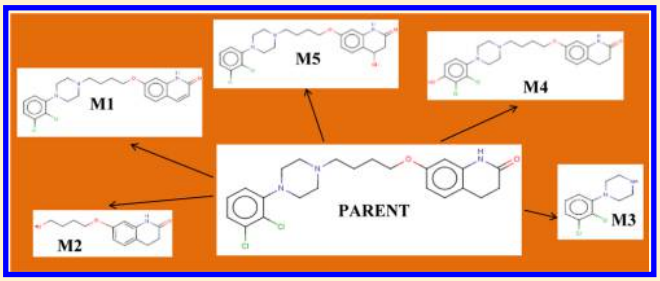
Supporting Information

ABSTRACT: In this study the performance of a selection of computational models for the prediction of metabolites and/or sites of metabolism was investigated. These included models incorporated in the MetaPrint2D-React, Meteor, and SMARTCyp software. The algorithms were assessed using two data sets: one a homogeneous data set of 28 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) and paracetamol (DS1) and the second a diverse data set of 30 top-selling drugs (DS2). The prediction of metabolites for the diverse data set (DS2) was better than for the more homogeneous DS1 for each model, indicating that some areas of chemical space may be better represented than others in the data used to develop and train the models. The study also identified compounds for which none of the packages could predict metabolites, again indicating areas of chemical space where more information is needed. Pragmatic approaches to using metabolism prediction software have also been proposed based on the results described here. These approaches include using cutoff values instead of restrictive reasoning settings in Meteor to reduce the output with little loss of sensitivity and for directing metabolite prediction by preselection based on likely sites of metabolism.



1. INTRODUCTION

Prediction of xenobiotic metabolism is a research priority in many areas including pharmaceutical, cosmetic, food safety, and environmental studies. The reason for this may be to help determine efficacy, for example certain pharmaceutical compounds require conversion from an inactive parent to an active metabolite. However, the predominant reason for predicting metabolism is concern for consumer or environmental safety as xenobiotics may be biotransformed to compounds that cause adverse effects. Poor absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties of drug candidates have resulted in high drug attrition rates in late stages of drug development and withdrawal of drugs from the market due to adverse drug reactions (ADRs), resulting in great financial and societal cost. Reactive metabolites formed by bioactivation may bind covalently to biological macromolecules such as proteins leading to idiosyncratic drug toxicity, otherwise known as type B ADRs.[1] Hence, the pharmaceutical industry has implemented the assessment of ADMET properties earlier in the drug development process which has already resulted in significantly reduced attrition rates.[2,3]

Other relevant factors for the prediction of metabolism include identifying which enzymes may be involved in the metabolism of specific drugs. For instance, one of the problems of an aging population is polypharmacy and the potential for drug−drug interactions where the presence of one drug affects the metabolism, and hence the *in vivo* concentration, of another. Drug-food or drug-herbal interactions can similarly alter *in vivo* concentrations, and knowledge of metabolic routes can help predict such interactions. Therefore, there is a great deal of interest in predicting potential metabolites and routes of metabolism for xenobiotics. This has resulted in a plethora of software and techniques available to predict metabolism. Different methods provide alternative solutions to the problem, and each is associated with advantages and disadvantages. Some methods are highly computationally expensive, and others may result in vast numbers of potential metabolites being produced, where the true metabolites become obscured by too many data or other packages where fewer of the true metabolites are predicted. Kirchmair et al.[4] provide an excellent review of the computational approaches to predicting metabolites and sites of metabolism. Table 1 lists some of the methods available. There are many other methods available particularly with respect to quantum chemical and docking approaches to predict likely sites of metabolism.

Kirchmair et al.[4] also list many methods for the prediction of enzyme interactions, such as binding affinity, inhibition, and induction of cytochromes incorporating docking studies and binding energy calculations. Such methods tend to be computationally expensive and were not considered in the present study which focuses only on software to predict metabolites or sites of metabolism.

In general, metabolism results in chemical modification of xenobiotics so that they become more polar and, therefore, more readily excreted via urine or bile. Phase I metabolism involves

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Table 1. *In Silico* Methods for the Prediction of Metabolites and Sites of Metabolism for Xenobiotics (Adapted from Kirchmair et al.[4])

Prediction of Metabolites	Summary of method	Web site or key citation
META	Uses a dictionary of biotransformations to predict the structure of likely metabolites. Analyses metabolite stability.	Klopman et al.[5]
Metabolexpert	Uses rule-based knowledge to predict likely metabolites in humans, animals, or plants.	http://www.compudrug.com/ ?q=node/36
Metabolizer, (ChemAxon, Budapest)	Includes libraries of biotransformations - enumerates all possible metabolites, predicts major metabolites, estimates metabolic stability, and supports species-specific predictions of likely metabolites.	http://www.chemaxon.com/products/online-tryouts/metabolizer/
Metaprint2D-React	Predicts structures of metabolites based on data-mining and statistical analysis − refer to Metaprint2D below.	http://www-metaprint2d.ch.cam.ac.uk/metaprint2d-react
MetaSite	Considers enzyme−substrate recognition and predicts metabolic transformations for cytochrome-mediated reactions in phase I metabolism. Provides the structure of metabolites and provides a ranking derived from estimated likelihood of metabolic reaction at a given position.	http://www.moldiscovery.com/soft_metasite.php
Meteor	Uses expert knowledge rules for metabolism to predict metabolites which are presented in metabolic trees.	https://www.lhasalimited.org/meteor/
OECD QSAR Toolbox	Contains both rat liver and skin metabolism simulators in addition to database of known biotransformations.	https://www.qsartoolbox.org
SyGMa	Predicts structures and probability of metabolites based on rules derived from Accelrys Metabolite Database.	Ridder et al.[6]
TIMES (TIssue MEtabolism Simulator)	A heuristic algorithm to generate plausible metabolic maps from a comprehensive library of biotransformations and abiotic reactions.	http://oasis-lmc.org/?section=software&swid=4

Prediction of Sites of Metabolism	Summary of method	Web site or key citation
ADMET Predictor − Metabolite Module	Derives likelihood of metabolic reactions occurring at specific atom positions; identifies substrates for five CYP isoforms.	http://www.simulations-plus.com/Products.aspx?pID=13&mID=15
Metaprint2D	Predicts sites of phase I xenobiotic metabolism in dog, human, and/or rat through data-mining and statistical analysis of known metabolic transformations reported in the literature.	http://www-metaprint2d.ch.cam.ac.uk/metaprint2d
MetaSite	See above - also predicts metabolites	http://www.moldiscovery.com/soft_metasite.php
SMARTCyp	Uses precalculated density functional theory activation energies with topological accessibility descriptors to identify sites liable to cytochrome P450 metabolism.	http://www.farma.ku.dk/smartcyp/index.php
StarDrop	Combines quantum chemical analysis and ligand-based models of CYP substrates to estimate potential sites of metabolism.	http://www.optibrium.com/stardrop/stardrop-p450-models.php
RS-WebPredictor	Uses topological and quantum chemical atom-specific predictors to identify sites of metabolism. Regioselectivity models for substrates have been developed for nine key CYP isozymes.	http://reccr.chem.rpi.edu/Software/RS-WebPredictor/

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oxidation, hydroxylation, deamination, and dealkylation reactions which are catalyzed by the cytochrome P450 superfamily of enzymes with CYP3A4, CYP2D6, and CYP2C9 isoforms being responsible for the majority of transformations for drugs. Phase II reactions yield hydrophilic conjugates, for example glucuronides may be formed via the action of glucuronyl transferases. Phase II metabolism may follow or be independent of phase I reactions. Hydrolysis and reduction reactions are also significant in terms of metabolism. Metabolism is a highly complex process involving a multitude of biotransformations that may occur consecutively, concurrently, or in competition with each other. Genetic and environmental factors can also affect the nature and abundance of metabolites making prediction a difficult task. Nevertheless, software packages for prediction of metabolism are widely used, particularly by the pharmaceutical industry, and especially during the screening phase of the drug development process. Predictions made by the software may aid the development of drugs with improved pharmacokinetic profiles and highlight potential metabolite induced toxicity.[7]

In 2002, the Metabolites in Safety Testing (MIST) committee defined how to approach metabolic data when assessing the toxicity of drug candidates;[8] these guidelines were subsequently modified.[9] As it is essential that the products of metabolic pathways are considered for their potential toxicity, *in silico* methods to predict metabolism are becoming increasingly important. Such methods need to be used with confidence, hence performance and best practice in using the methods needs to be assessed.

To assess any software used for metabolism prediction, specific criteria should be established so that the design of a particular package is taken into account. Due to differences between programs it is not always possible to apply the same criteria to each program when assessing performance. Some software (e.g., SMARTCYP Web Service) only provides sites of metabolism (SOM) rather than metabolite structures. However, predicting SOM is not equivalent to identifying the correct biotransformation that would take place at the particular atom. Prediction of SOM has been successful with studies indicating up to 90% correct identification of the three most likely SOM. However, prediction of the absolute likelihood of the formation of a certain metabolite is less accurate.[4]

Where predicted metabolite structures are provided in the output of an algorithm, direct comparison between different algorithms still may not be feasible. For example, some software may only predict the outcome of phase I, and not phase II, metabolism, or models may only consider specific cytochrome P450 enzymes. Other issues affecting direct comparison of software packages include selection of user-defined constraints such as species, probability level for a particular metabolite being formed, etc. Statistical analysis of predictions may provide a general indication of algorithm performance, but comparisons between packages must be assessed with caution. The underlying design and limitations of the software should be taken into consideration. Another important consideration is the training set on which the software was developed; this information may or may not be available. Output for a given package may reflect overall performance in identifying metabolites but not give a true indication of predictive power of the model when faced with unknown compounds. Therefore, a combination of the quantitative and qualitative assessment of performance may be more informative. For example, investigating not only statistical values for percentage metabolites correctly predicted but also considering which metabolites are not predicted, e.g., if there are specific metabolic routes that are not identified in the software; this gives scope for further improvements of algorithms. Clearly there are too many software routines of diverse functionality and output to evaluate in a fully consistent manner.

The aim of the present study was to investigate the performance of three well-known, representative, software packages for the prediction of metabolism. Performance was compared between two different data sets. The first data set (DS1) comprised 28 nonsteroidal anti-inflammatory drugs (NSAIDs) plus paracetamol, representing a homogeneous chemical space. The second data set (DS2) comprised the first 30 drugs listed in the Top 200 Drugs for 2010 by Sales in the United States, excluding drugs containing more than one active ingredient or peptides.[10] DS2 represented a more diverse chemical space than DS1 with a wider range of potential metabolic biotransformations.

Investigation into the influence of modifying user-defined constraints was also carried out, for appropriate packages, to enable recommendations to be made as to the most pragmatic software settings for a given query.

The three software packages selected from those listed in Table 1 were the following:

(i). Meteor. This is an industry-standard, knowledge-based expert system developed by the not-for-profit organization Lhasa Limited, Leeds, England. Meteor predicts metabolites from the structure of the parent compound, based on an extensive set of biotransformation rules which have been extracted from the literature or informed by confidential data from the pharmaceutical industry. It uses two types of reasoning: absolute reasoning describes the probability of a biotransformation taking place (in terms of probable, plausible, equivocal, doubted and improbable) and relative reasoning that allows further ordering of all possible metabolic outcomes in cases when there are competing biotransformations at a given SOM.[11]

(ii). Metaprint2D/Metaprint2D-React. Metaprint2D is a freely available algorithm that predicts the likelihood of a metabolic reaction occurring at a given position in the molecule using a circular fingerprints technique. This is applied to the data found in the Symyx(R) Metabolite database, which are a collection of 80,000 xenobiotic transformations from the literature (predominantly for pharmaceuticals). It identifies the environment of each atom in the molecule and then searches the database for similar environments to calculate the occurrence ratio. Metaprint2D-React can provide structures of potential metabolites associated with reaction at the identified positions.[12]

(iii). SMARTCyp. This software determines the sites in a molecule liable to metabolism using the 2D structure of the compound and calculations of energies required for oxidation. These are matched against precalculated activation energies for fragments represented as SMARTS patterns.[13]

2. METHODS

2.1. Data Sets. Data set 1 (DS1) and data set 2 (DS2) were chosen to represent homogeneous and diverse drugs respectively, in terms of action, structure, and metabolic transformation. DS1 comprised 28 commonly used NSAIDs and paracetamol; DS2 comprised 30 drugs taken from the Top 200 drugs in 2010 by sales in the United States. The top 30 drugs in the list (excluding peptides) were considered; if a drug comprised more than one active ingredient, then each of them were included providing they were subject to CYP450 metabolism.

Table 2. Common Names, CAS Registry Numbers, IUPAC Names, and Number of Phase I Metabolites Obtained from the Literature for Drugs Used in Data Sets 1 and 2 in This Study

Common name	CAS RN	IUPAC name	No. of metabolites
Data Set 1			
alclofenac	22131-79-9	[4-(allyloxy)-3-chlorophenyl]acetic acid	3
aspirin	50-78-2	2-acetoxybenzoic acid	3
azapropazone	13539-59-8	5-(dimethylamino)-9-methyl-2-propyl-1H-pyrazolo[1,2-a][1,2,4]benzotriazine-1,3(2H)-dione	2
bromfenac	91714-94-2	[2-amino-3-(4-bromobenzoyl)phenyl]acetic acid	3
carprofen	52263-47-5	2-(6-chloro-9H-carbazol-2-yl)propanoic acid	3
diclofenac	15307-86-5	{2-[(2,6-dichlorophenyl)amino]phenyl}acetic acid	6
diflunisal	22494-42-4	2′,4′-difluoro-4-hydroxy-3-biphenylcarboxylic acid	1
fenbufen	36330-85-5	4-(4-Biphenylyl)-4-oxobutanoic acid	4
fenclofenac	34645-84-6	[2-(2,4-dichlorophenoxy)phenyl]acetic acid	1
fenoprofen	31879-05-7	2-(3-phenoxyphenyl)propanoic acid	1
feprazone	30748-29-9	4-(3-methyl-2-buten-1-yl)-1,2-diphenyl-3,5-pyrazolidinedione	1
flufenamic acid	530-78-9	2-{[3-(trifluoromethyl)phenyl]amino}benzoic acid	3
flurbiprofen	5104-49-4	2-(2-fluoro-4-biphenylyl)propanoic acid	3
ibuprofen	15687-27-1	2-(4-isobutylphenyl)propanoic acid	4
indomethacin	53-86-1	[1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]acetic acid	3
ketoprofen	22071-15-4	2-(3-benzoylphenyl)propanoic acid	3
ketorolac	66635-83-4	5-benzoyl-2,3-dihydro-1H-pyrrolizine-1-carboxylic acid	1
mefenamic acid	61-68-7	2-[(2,3-dimethylphenyl)amino]benzoic acid	2
nabumetone	42924-53-8	4-(6-methoxy-2-naphthyl)-2-butanone	5
naproxen	22204-53-1	(2S)-2-(6-methoxy-2-naphthyl)propanoic acid	1
paracetamol	103-90-2	N-(4-hydroxyphenyl)acetamide	3
phenylbutazone	50-33-9	4-butyl-1,2-diphenyl-3,5-pyrazolidinedione	3
piroxicam	36322-90-4	4-hydroxy-2-methyl-N-(2-pyridinyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide	1
pirprofen	31793-07-4	2-[3-chloro-4-(2,5-dihydro-1H-pyrrol-1-yl)phenyl]propanoic acid	5
sulindac	38194-50-2	{(1Z)-5-fluoro-2-methyl-1-[4-(methylsulfinyl)benzylidene]-1H-inden-3-yl}acetic acid	2
suprofen	40828-46-4	2-[4-(2-thienylcarbonyl)phenyl]propanoic acid	5
tiaprofenic acid	33005-95-7	2-(5-benzoyl-2-thienyl)propanoic acid	2
tolmetin	26171-23-3	[1-methyl-5-(4-methylbenzoyl)-1H-pyrrol-2-yl]acetic acid	2
zomepirac	33369-31-2	[5-(4-chlorobenzoyl)-1,4-dimethyl-1H-pyrrol-2-yl]acetic acid	2
Data Set 2			
aripiprazole	129722-12-9	7-{4-[4-(2,3-dichlorophenyl)-1-piperazinyl]butoxy}-3,4-dihydro-2(1H)-quinolinone	5
atorvastatin	110862-48-1	(3R,5R)-7-[2-(4-fluorophenyl)-5-isopropyl-3-phenyl-4-(phenylcarbamoyl)-1H-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid	5
buprenorphine	52485-79-7	(5α,6β,14β,18R)-17-(cyclopropylmethyl)-18-[(2S)-2-hydroxy-3,3-dimethyl-2-butanyl]-6-methoxy-18,19-dihydro-4,5-epoxy-6,14-ethenomorphinan-3-ol	1
celecoxib	169590-42-5	4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide	2
clopidogrel	90055-48-4	methyl (2S)-(2-chlorophenyl)(6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)acetate	3
donepezil	120011-70-3	methyl (2S)-(2-chlorophenyl)(6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)acetate	4
duloxetine	116539-58-3	(3S)-N-methyl-3-(1-naphthyloxy)-3-(2-thienyl)-1-propanamine	11
efavirenz	154635-17-3	(4S)-6-chloro-4-(cyclopropylethynyl)-4-(trifluoromethyl)-1,4-dihydro-2H-3,1-benzoxazin-2-one	3
emtricitabine	143491-57-0	4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-2(1H)-pyrimidinone	1
escitalopram	59729-33-8	(1S)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carbonitrile	5
esomeprazole	73590-58-6	6-methoxy-2-{(S)-[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulfinyl}-1H-benzimidazole	5
ezetimibe	163222-33-1	(3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone	1
fenofibrate	49562-28-9	Isopropyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate	2
fluticasone propionate	80474-14-2	(6α,11β,16α,17α)-6,9-difluoro-17-{[(fluoromethyl)sulfanyl]carbonyl}-11-hydroxy-16-methyl-3-oxoandrosta-1,4-dien-17-yl propionate	1
levofloxacin	100986-85-4	(3S)-9-fluoro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-2,3-dihydro-7H-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxylic acid	2
methylphenidate	113-45-1	methyl phenyl(2-piperidinyl)acetate	6
modafinil	68693-11-8	2-[(diphenylmethyl)sulfinyl]acetamide	2
montelukast	158966-92-8	{1-[({(1R)-1-{3-[(E)-2-(7-chloro-2-quinolinyl)vinyl]phenyl}-3-[2-(2-hydroxy-2-propanyl)phenyl]propyl}sulfanyl)methyl]cyclopropyl}acetic acid	5
naloxone	465-65-6	(5α)-17-allyl-3,14-dihydroxy-4,5-epoxymorphinan-6-one	2
olanzapine	132539-06-1	(5α)-17-allyl-3,14-dihydroxy-4,5-epoxymorphinan-6-one	6
oxycodone	76-42-6	(5α)-14-hydroxy-3-methoxy-17-methyl-4,5-epoxymorphinan-6-one	3
pioglitazone	111025-46-8	5-{4-[2-(5-ethyl-2-pyridinyl)ethoxy]benzyl}-1,3-thiazolidine-2,4-dione	6
pregabalin	148553-50-8	(3S)-3-(aminomethyl)-5-methylhexanoic acid	1
quetiapine	111974-69-7	2-{2-[4-(dibenzo[b,f][1,4]thiazepin-11-yl)-1-piperazinyl]ethoxy}ethanol	5

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Table 2. continued

Common name	CAS RN	IUPAC name	No. of metabolites
Data Set 2			
rosuvastatin	287714-41-4	(3R,5S,6E)-7-{4-(4-fluorophenyl)-6-isopropyl-2-[methyl(methylsulfonyl)amino]-5-pyrimidinyl}-3,5-dihydroxy-6-heptenoic acid	2
salmeterol	89365-50-4	2-(hydroxymethyl)-4-(1-hydroxy-2-{[6-(4-phenylbutoxy)hexyl]amino}ethyl)phenol	2
sildenafil	139755-83-2	5-{2-ethoxy-5-[(4-methyl-1-piperazinyl)sulfonyl]phenyl}-1-methyl-3-propyl-1,4-dihydro-7H-pyrazolo[4,3-d] pyrimidin-7-one	4
sitagliptin	486460-32-6	(3R)-3-amino-1-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-4-(2,4,5-trifluorophenyl)-1-butanone	1
valsartan	137862-53-4	N-pentanoyl-N-{[2′-(2H-tetrazol-5-yl)-4-biphenylyl]methyl}-L-valine	1
venlafaxine	93413-69-5	1-[2-(dimethylamino)-1-(4-methoxyphenyl)ethyl]cyclohexanol	4

2.2. Collecting and Storing Metabolism Data. A thorough literature search was performed to obtain the known *in vivo* metabolites, in humans, for the 59 drugs considered in this study. Sources of information included original research papers, reviews, and online databases such as the following: Martindale: The Complete Drug Reference, and Clarke's Analysis of Drugs and Poisons. As some of the predictive software investigated here (such as SMARTCyp) was not designed to predict phase II metabolites, data collection focused on phase I metabolism only.

The complete list of known metabolites found in the literature and their associated references (117 references in total) are available as Supporting Information.

The structures of all parent drugs and metabolites were drawn in MarvinSketch, version 5.0.3 (Chemaxon) and stored in *.mol format. Information concerning the specific CYP450 isoform(s) involved in biotransformations for DS2 was also recorded and is also available as Supporting Information.

In total, 78 known metabolites for DS1 and 101 known metabolites for DS2 were obtained from the literature.

Table 2 lists the common names for the drugs used in this study along with their Chemical Abstracts Service Registration Number (CAS RN), International Union of Pure and Applied Chemistry (IUPAC) name, and the number of phase I metabolites retrieved from the literature for each drug. The Supporting Information also gives the SMILES strings for the parent drugs and their known phase I metabolites.

2.3. Use of Software. *(i). Meteor.* Meteor, version 13, Lhasa Limited, Leeds, England was used to predict metabolites for both data sets. The parent structures were submitted as *.mol format, and the resulting metabolites were stored as Meteor generic files (*.mtr). As stated previously, Meteor employs two types of reasoning - absolute and relative.

With regard to absolute reasoning, three levels were selected in this analysis: probable, plausible, and equivocal (doubted and improbable were not considered). For each of these three levels of absolute reasoning, three levels of relative reasoning were considered: levels 1, 2, and 3.

Table 3 shows the nine combinations of reasoning employed and the abbreviations for these that will be used throughout this paper.

Table 3. Combinations of Reasoning Used in Meteor and Abbreviations Utilized in This Paper

absolute reasoning	relative reasoning 1	relative reasoning 2	relative reasoning 3
probable	PRO1	PRO2	PRO3
plausible	PLA1	PLA2	PLA3
equivocal	EQU1	EQU2	EQU3

All the other parameters selected from the option "*Processing Constraints*" were kept constant for all combinations of reasoning. Processing constraints were the following: phase option = phase I only; species = human; enzyme = not specified; max number of metabolites = 400; max number of steps in a pathway = 4.

The presence of known metabolites for DS1 and DS2 was identified by means of the Meteor functionality that allows direct searching for structures in a metabolic tree (*.mol format). All the true positives (i.e., known metabolites from the literature that were correctly predicted by the software) along with their positions in the Meteor metabolic tree were recorded and saved in a Microsoft Excel spreadsheet. In the first part of the analysis described here, the percentage of known metabolites that were correctly predicted within each metabolic tree (of up to 400 metabolites) for each compound in DS1 and DS2 was calculated using reasoning level EQU3.

Further analysis was subsequently undertaken to establish the sensitivity for each of the nine reasoning settings given in Table 2 by taking into account the number of metabolites correctly predicted at different cutoff points. The cutoff points were 5, 10, 15, 20, 25, 30, 35, 40, and 400 and refer to the number of predicted metabolites appearing sequentially in the metabolic tree that were subsequently checked against structures of known metabolites. For example, using a cutoff value of 20 means that only the first 20 metabolites appearing in the Meteor tree of predicted metabolites were considered; a cutoff value of 400 means that all 400 predicted metabolites were checked against known metabolites to determine if the known metabolite had been predicted. The purpose of this was to investigate the most pragmatic use of the Meteor software. While it is possible to generate 400 metabolites, investigation into the structure of each of these (as may be performed for subsequent estimation of metabolite toxicity) is a very time-consuming process. It was considered useful to determine whether or not it was possible to identify the majority of known metabolites within the first 5, 10, or 20 etc. metabolites that appear in the highest positions of the metabolic tree(s), to increase the efficiency of using the program.

(ii). MetaPrint2D-React. The structures of the parent drugs were entered as SMILES strings into the MetaPrint2D-React web-service software at the following URL: http://www-metaprint2d.ch.cam.ac.uk/metaprint2d-react/. Fingerprint matching was set to a default value, and the human model was selected. The resulting predictions for metabolite structures were compared to known metabolites.

(iii). Combination of SMARTCyp and MetaPrint2D-React. A subset of metabolites from DS2 that are known to be products of CYP3A4 or CYP2D6 metabolism were identified from the literature. 39 of the metabolites from DS2 are known to be

products of CYP3A4, and 11 of the metabolites are known to be products of CYP2D6.

For each parent drug contained in this subset of data the following steps were followed. The parent structure was submitted as a SMILES string to the SMARTCyp Web Service version 2.0; URL: http://www.farma.ku.dk/smartcyp/. SMARTCyp was used to identify the top five ranking atoms (in terms of most likely site of metabolism) for both CYP3A4 and CYP2D6. The parent structure of the drug was then submitted as a SMILES string into the MetaPrint2D-React program (URL: http://www-metaprint2d.ch.cam.ac.uk/metaprint2d-react/); fingerprint matching was set to the default value, and the human model was selected. The output was analyzed in terms of biotransformations occurring at the atoms corresponding to the SOM previously predicted as being in the top five ranked sites by SMARTCyp. The metabolites predicted from biotransformations at these sites were compared to known metabolites. The procedure was repeated using only the top three ranked SOM from SMARTCyp. This process is outlined in Figure 1.

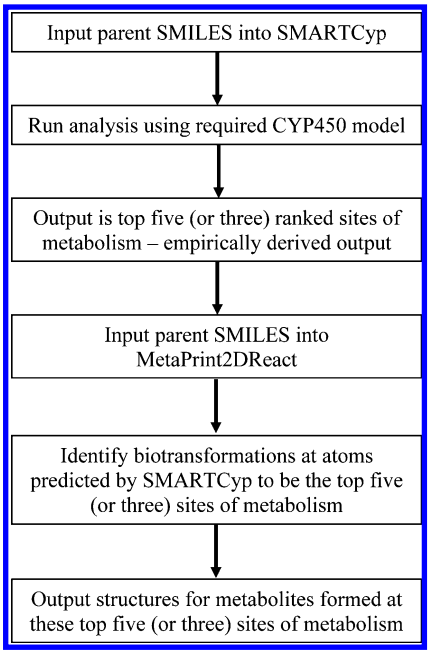

Figure 1. Flow diagram showing the method to predict metabolites using a combination of SMARTCyp (models for CYP3A4 and CYP2D6) and Metaprint2D-React.

2.4. Statistical Analysis. The performance of Meteor and MetaPrint2D-React was assessed in terms of the number of correctly predicted metabolites (sensitivity) and was calculated according to the following formula:

$$\text{Sensitivity (\%)} = ([\text{TP}]/\text{TOTAL}_{\text{invivo}})*100\%$$

Precision in the context used here was calculated as

$$\text{Precision (\%)} = ([\text{TP}]/[\text{TP}] + [\text{FP}])*100\%$$

where $\text{TOTAL}_{\text{invivo}}$ = total number of known metabolites observed (from *in vivo* literature data), TP = no. of correct predictions (predicted metabolites that are also observed *in vivo*), and FP = no. of incorrect predictions (predicted metabolites that are not observed *in vivo*).

3. RESULTS AND DISCUSSION

3.1. General Performance of the Algorithms. Two data sets were used to analyze performance of different software in predicting metabolites and sites of metabolism and to investigate pragmatic approaches to using the software. Data set 1 (DS1) was a more chemically homogeneous set of 28 NSAIDs and paracetamol for which a total of 78 known metabolites had been identified from a literature search. Data set 2 (DS2) was a more diverse set of 30 drugs for which 101 metabolites had been identified in the literature. Figure 2 shows an example of results for aripiprazole (and its five known metabolites) for illustrative purposes, along with the predictions from Meteor and MetaPrint2D. Note that M1, M4, and M5 were not predicted by Meteor when the probable (PRO) absolute reasoning engine was used.

Table 4 shows the overall performance of the software packages in predicting the known metabolites (as obtained from the literature) for DS1 and DS2.

Note that this analysis was carried out in order to obtain a general indication of performance and compare the number of correct predictions that are obtained when using two different data sets (one homogeneous, one diverse). The results are not a true measure of overall predictivity of the software used as it is likely that the drugs analyzed are present to a greater or lesser extent in the information used to develop the individual software packages.

Meteor and Metaprint2D-React both show good performance in predicting metabolites for data sets DS1 and DS2. What is interesting to note is that in all cases predictions were marginally better for the diverse data set (DS2) than for the more homogeneous data set (DS1). This suggests that areas of chemical space more predominant in one set of compounds may not be as well represented in the training sets for the software as other areas. For example, none of the software packages predicted the metabolites of the NSAID bromfenac. The metabolites of bromfenac are dependent on the formation of a cyclic amide metabolite which was not predicted by the software tested. Similarly, two metabolites of nabumetone and one metabolite of suprofen were not predicted by any of the software indicating a possible knowledge gap in metabolite prediction. Such information can be used to direct future improvements in software development. Aside from these examples, for DS1 72 of the metabolites (i.e., 92%) were predicted by at least one of the software packages (or combination of packages).

Cases where specific metabolites were not found by any of the packages studied also occurred for DS2. They include the following: three metabolites of clopidogrel, two metabolites of pioglitazone, and one metabolite of sitagliptin which gave a total of 6 out of 101 (refer to the Supporting Information). Thus, 96 metabolites (95%) were predicted by at least one of the packages used in the study.

3.2. Pragmatic Approach to Using Meteor. Table 5 shows the results of the predictions for the Meteor software where nine different reasoning levels were investigated for the prediction of metabolites of DS1. The cutoff values in row 1 show the number of metabolites that were checked against known metabolites, e.g., a cutoff value of five indicates the first five metabolites only in the metabolic tree *for each compound* were checked; for DS1 78 metabolites had been identified from the literature.

Trends in the data shown in Table 5 can be more clearly visualized graphically as shown in Figure 3.

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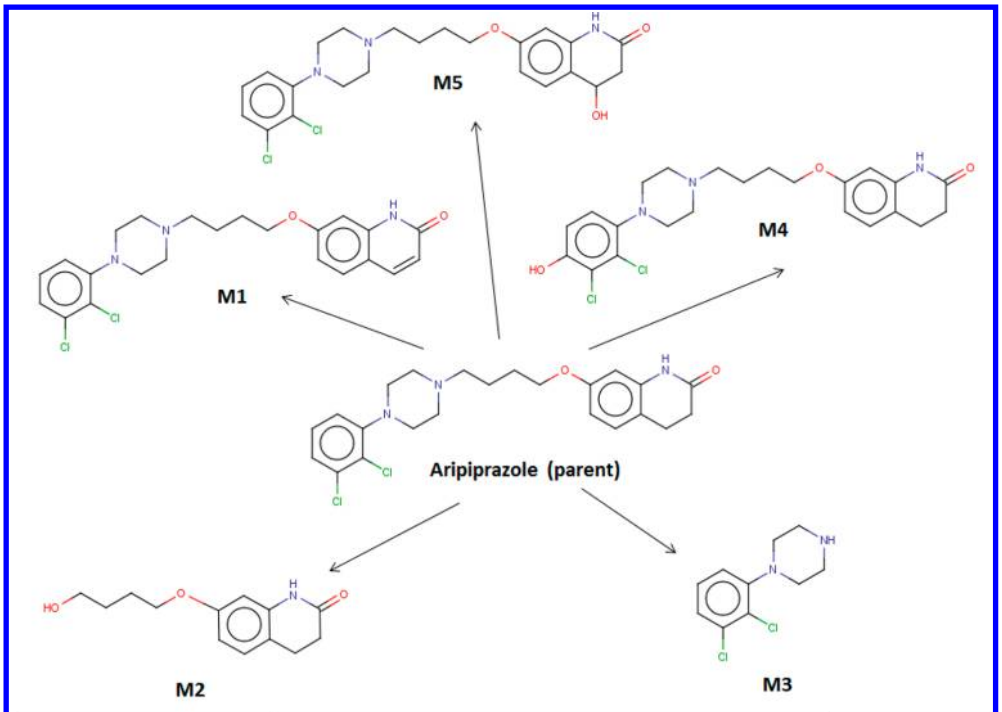

Software	Known Metabolites					Total Predictions
	M1	M2	M3	M4	M5	
Meteor	✓	✓	✓	✓	✓	5
MetaPrint2D-React	✓	✓	✓	✓	✓	5

Figure 2. Known metabolites of aripiprazole and the predictions obtained by Meteor and MetaPrint2D-React.

Table 4. Performance of Meteor and Metaprint2D-React in Predicting Metabolites of Data Sets 1 and 2

Software	No. metabolites correctly predicted for DS1	No. metabolites correctly predicted for DS2
Meteor (setting EQU3)	57 (73%)	86 (85%)
Metaprint2D-React	62 (80%)	90 (89%)

Table 6 shows the results of the Meteor predictions for DS2 using nine reasoning settings and nine cutoff values; for DS2 101 metabolites had been identified from the literature.

Similarly to data set 1 the results for the analysis can be visualized graphically as shown in Figure 4.

The above results show clear trends for both the homogeneous and diverse data sets. Setting absolute reasoning to probable is restrictive, and many known metabolites are missed; only 10% and 32% of known metabolites are correctly predicted for DS1 and DS2, respectively. When altering the absolute reasoning level to plausible many more metabolites are found. However, using the absolute reasoning level of equivocal results in the highest number of metabolites being found for DS1, for all levels of relative reasoning (1−3). For DS2 the combination of absolute reasoning of equivocal and relative reasoning 2 and 3 almost always gave the highest number of known metabolites.

One well-known drawback of using the least stringent reasoning settings (e.g., EQU2 or EQU3) is that these are associated with a high number of potential metabolites being generated. For example, the study of Tjollyn et al.,[14] which

Table 5. Predictions from Meteor for Data Set 1 Using Nine Settings for Reasoning (As Defined in Table 3) and Nine Cutoff Values[a]

	5		10		15		20		25		30		35		40		400	
Cutoff values	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
PRO1	6	8	7	9	7	9	7	9	7	9	7	9	7	9	7	9	7	9
PRO2	7	9	8	10	8	10	8	10	8	10	8	10	8	10	8	10	8	10
PRO3	7	9	8	10	8	10	8	10	8	10	8	10	8	10	8	10	8	10
PLA1	33	42	39	50	39	50	39	50	39	50	39	50	39	50	39	50	39	50
PLA2	37	47	45	58	46	59	46	59	46	59	46	59	46	59	46	59	46	59
PLA3	37	47	45	58	46	59	46	59	46	59	46	59	46	59	46	59	46	59
EQU1	37	47	46	59	47	60	48	62	48	62	48	62	48	62	48	62	48	62
EQU2	41	53	49	63	52	67	54	69	55	71	55	71	55	71	55	71	55	71
EQU3	41	53	48	62	51	65	54	69	56	72	57	73	57	73	57	73	57	73

[a] *N* = number of correctly predicted metabolites; % = percentage of correctly predicted metabolites.

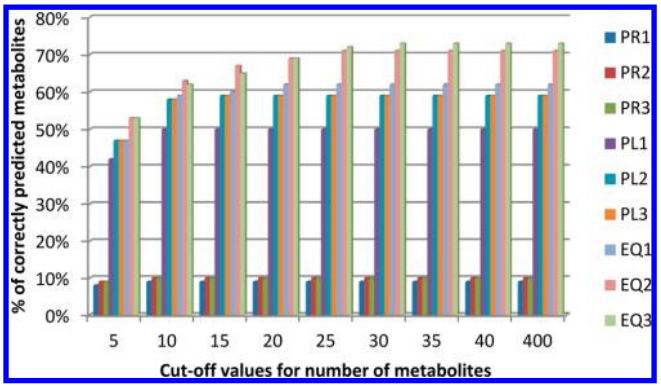

Figure 3. Meteor predictions for DS1 showing the variation in number of metabolites correctly predicted as a function of cutoff values and reasoning levels selected.

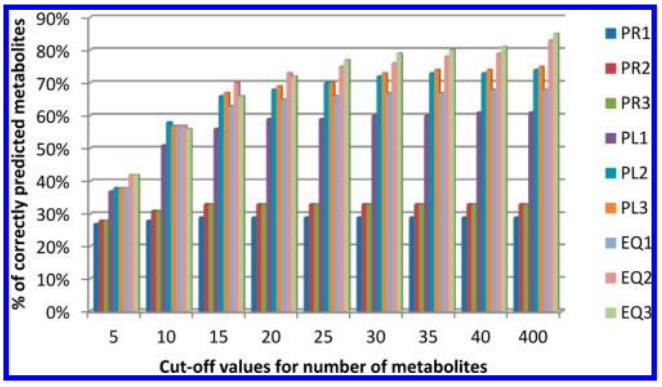

Figure 4. Meteor predictions for DS2 showing the variation in number of metabolites correctly predicted as a function of cutoff values and reasoning levels selected.

involved investigation of the predictive power of Meteor, showed that although the program is sensitive (i.e., performs well in correctly predicting known metabolites) it also has a tendency to overpredict biotransformations (for example, simple hydroxylations) giving the program low specificity. Hence the true metabolites may become "lost" in the plethora of possible metabolites. However, the analysis carried out here indicates that using cutoff values is a pragmatic solution to this problem. For DS1 using EQU3, but only investigating the structures of the top 30 metabolites gave the same results (in terms of number of metabolites correctly predicted) as investigating the top 35, 40, or 400 metabolites. Similarly for DS2 using a cutoff value of 30 returns 79% of true metabolites, with 400 metabolites needing to be investigated to obtain 85% of true metabolites.

It is evident that the sensitivity of Meteor can be controlled by selecting different absolute and relative reasoning levels. Due to the design of the program it is expected that changing the absolute reasoning from probable to plausible and then to equivocal is associated with an increase in sensitivity in each case. Similarly, more true positives can be identified using higher levels of the relative reasoning. However, this increased sensitivity is paired with a great reduction in precision. For instance, esomeprazole, a drug present in DS2, is known to have five metabolites *in vivo*. Three of these metabolites were correctly identified using reasoning PRO1, which produced a total of five predicted metabolites, hence precision can be calculated as 3/5. For all five metabolites to be correctly predicted required a reasoning setting of PLA1, which produced 77 predictions (precision = 5/77). This example illustrates how excellent sensitivity can be obtained but at the cost of a large number of false positives. On the other hand, it also indicates that by limiting the total number of predicted metabolites it would be possible to increase precision. Thus, instead of applying high filtering levels, and so reducing the variety of possible biotransformations, analysis of a subset of the output (as was performed here using cutoff values) may be more pragmatic. Cutoff points can be selected according to the level of sensitivity required by the user. The results here indicate that in terms of drug development a more pragmatic approach to predicting metabolites using Meteor may be to use EQU3 but to limit the number of predicted metabolites investigated to the top 25 or 30. This would offer significant savings in time and effort in predicting likely metabolites that can then be processed as necessary for toxicity assessment.

It should be stressed that the known metabolites, collated from the literature for both data sets, contained not only major metabolites but also those that were present in plasma or urine in trace amounts. Advances in analytical methods allow identification of less abundant metabolites so that the total number of metabolites can be relatively high. For almost 50% of the drugs in DS2, four or more metabolites have been identified *in vivo* (e.g., 11 known metabolites have been identified for duloxetine). Thresholds for identification of metabolites *in vivo* may vary from 5% to 10% (relative to the administered drug dose) according to different regulatory guidelines.[15−17] The software studied here was assessed for its ability to predict metabolites that had been identified *in vivo* irrespective of their relative abundance, i.e., it was tested without any quantitative assumptions. Meteor

Table 6. Predictions from Meteor for Data Set 2 Using Nine Settings for Reasoning (As Defined in Table 3) and Nine Cutoff Values[a]

	5		10		15		20		25		30		35		40		400	
Cutoff values	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
PRO1	27	27	28	28	29	29	29	29	29	29	29	29	29	29	29	29	29	29
PRO2	27	27	30	30	32	32	32	32	32	32	32	32	32	32	32	32	32	32
PRO3	27	27	30	30	32	32	32	32	32	32	32	32	32	32	32	32	32	32
PLA1	37	37	52	51	57	56	60	59	60	59	61	60	61	60	62	61	62	61
PLA2	37	37	58	57	67	66	69	68	71	70	73	72	74	73	74	73	75	74
PLA3	37	37	57	56	68	67	70	69	71	70	74	73	75	74	75	74	76	75
EQU1	39	39	58	57	64	63	67	66	68	67	69	68	69	68	70	69	70	69
EQU2	42	42	57	56	71	70	74	73	76	75	77	76	79	78	80	79	84	83
EQU3	42	42	56	55	67	66	73	72	78	77	80	79	81	80	82	81	86	85

[a] *N* = number of correctly predicted metabolites; % = a percentage of correctly predicted metabolites.

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assesses probabilities of metabolite formation according to its absolute and relative reasoning engines rules; it does not provide explicit information concerning the relative abundance of these metabolites in plasma or urine. Additionally, the method that Meteor uses to categorize metabolites in a tree may not always reflect an actual *in vivo* abundance.

It should be noted that the precision values were calculated relative to cutoff points. For instance, using the PRO1 setting gave 11 predicted metabolites for donepezil, and three of these (at positions 1, 2, and 3) were true positives; as the correct predictions were within the threshold of the cutoff point of 5 the precision was calculated as 3 out 5 (not 3 out of 11), and this gives a more favorable value for prediction. However, on some occasions, if the total number of predictions was lower than 5 and true positives were found, the value for precision appears lower. Limiting the number of metabolites in the output allows more permissive settings to be used (such as EQU2 or EQU3) where even hundreds of predictions can be provided. As most true positives concentrate within the first 15 or 20 predicted metabolites for the compounds considered here, it is reasonable to include equivocal reasoning as it improves sensitivity. For instance, when Meteor is used at EQU3 with the number of metabolites set at default value of 400, the sensitivity is 85.2%, while the precision equals 3.4%. If a cutoff point of 25 is selected using the same reasoning engine, the sensitivity drops to 78.2%, but the precision increases 3-fold to 10.7%. Although the obtained precision values are not very high in absolute terms, their significant increase with only a slight drop in sensitivity can still be valuable when computational time is considered. For example where identified metabolites are subsequently assessed for toxicity (e.g., using Derek Nexus (Lhasa Limited) or other predictive software), the significant reduction in metabolite numbers is beneficial not only in the time to generate the metabolite information but also in their subsequent processing. Figures 5 and 6 show the improvement in precision where lower cutoff values are used.

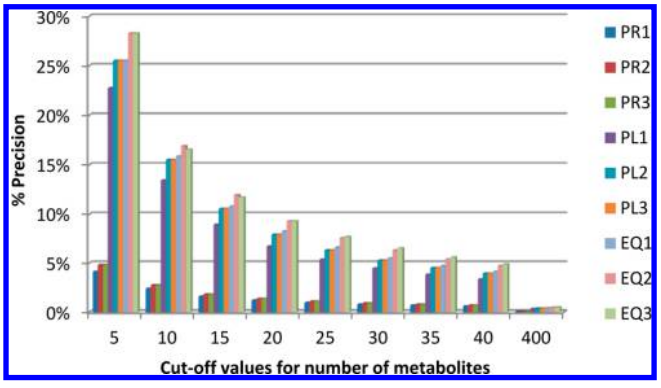

Figure 5. Percentage precision for DS1 as a function of cutoff values.

The results from this study show that, pragmatically, cutoff values in the region of 20−30 give a reasonable balance between sensitivity and specificity. The use of low filter settings with relatively low cutoff values is further supported by the fact that Meteor absolute reasoning categories are not directly correlated with the abundance of metabolites observed *in vivo*.[14] Meteor does not indicate which prediction is 'more important'; therefore, the selection of settings to enhance predictive power and adjustment of threshold points to levels that best suit the user is warranted.

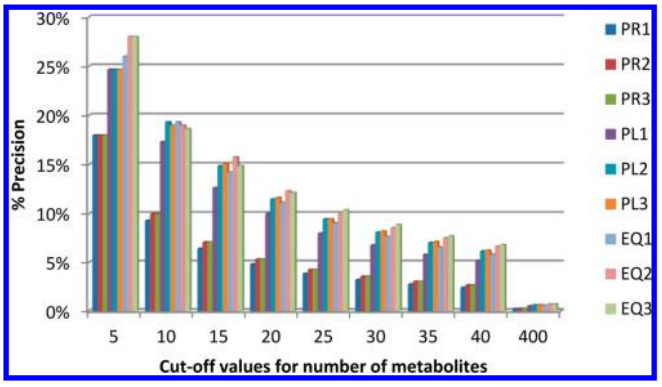

Figure 6. Percentage precision for DS2 as a function of cutoff values.

3.3. Combining SMARTCyp and MetaPrint2D-React.

SMARTCyp and MetaPrint2D can both be used to indicate SOM; however, in this study SMARTCyp (not MetaPrint2D) was combined with MetaPrint2DReact for the prediction of metabolites. The rationale behind selecting a combination of these two approaches is that the SMARTCyp method, in contrast to MetaPrint2D, does not depend on historical reaction data. SMARTCyp utilizes precalculated energies for a number of subfragments and as such can be described as an empirical approach. One of the benefits of such a method is that it could be applied with greater confidence to predict SOM for molecules that do not share substantial structural similarity with compounds found in historical databases. Therefore, such predictions would be less biased (towards already existing data) providing that the array of subfragments is large enough to cover a variety of structures. On the other hand, SMARTCyp does not provide metabolites' structures, which can be required by some users. Hence, MetaPrint2D-React was used to fill this gap. It should be noted that although accurate prediction of SOM presents a greater challenge than providing putative structures of the metabolites associated with a reaction as a given site, producing definitive structures is advantageous for further processing, e.g., predicting toxicity of identified metabolites. A combination of SMARTCyp and MetaPrint2D-React could be implemented to produce a form of potential metabolic tree. An example of such a tree for aripiprazole is presented in Figure 7.

Investigation of the literature had revealed that 39 of the primary metabolites for 17 drugs from DS2 were known to be a product of CYP3A4 metabolism. Similarly, CYP2D6 was responsible for the formation of 11 of the primary metabolites for six of the drugs in DS2. SMARTCyp was used to determine the top three and top five sites of metabolism for these drugs. These drugs were then entered into MetaPrint2D-React, and metabolites corresponding to the SOM identified using SMARTCyp were determined, according to the scheme shown in Figure 1, using the models for CYP3A4 and CYP2D6.

For aripiprazole and quetiapine, both CYP3A4 and CYP2D6 were involved in metabolism; in these cases both corresponding models were explored in SMARTCyp. Analysis of the predictions obtained using this combination of methods indicates that the CYP2D6 model performed better than the CYP3A4 model. The CYP2D6 model predicted 91% of metabolites correctly when the top five ranking SOM were considered and predicted 73% of metabolites when only the top three SOM were taken into account. For CYP3A4 the model predicted 56% of metabolites correctly when the top five SOM were selected and 44% correctly when the top three SOM were considered as shown in Figure 8.

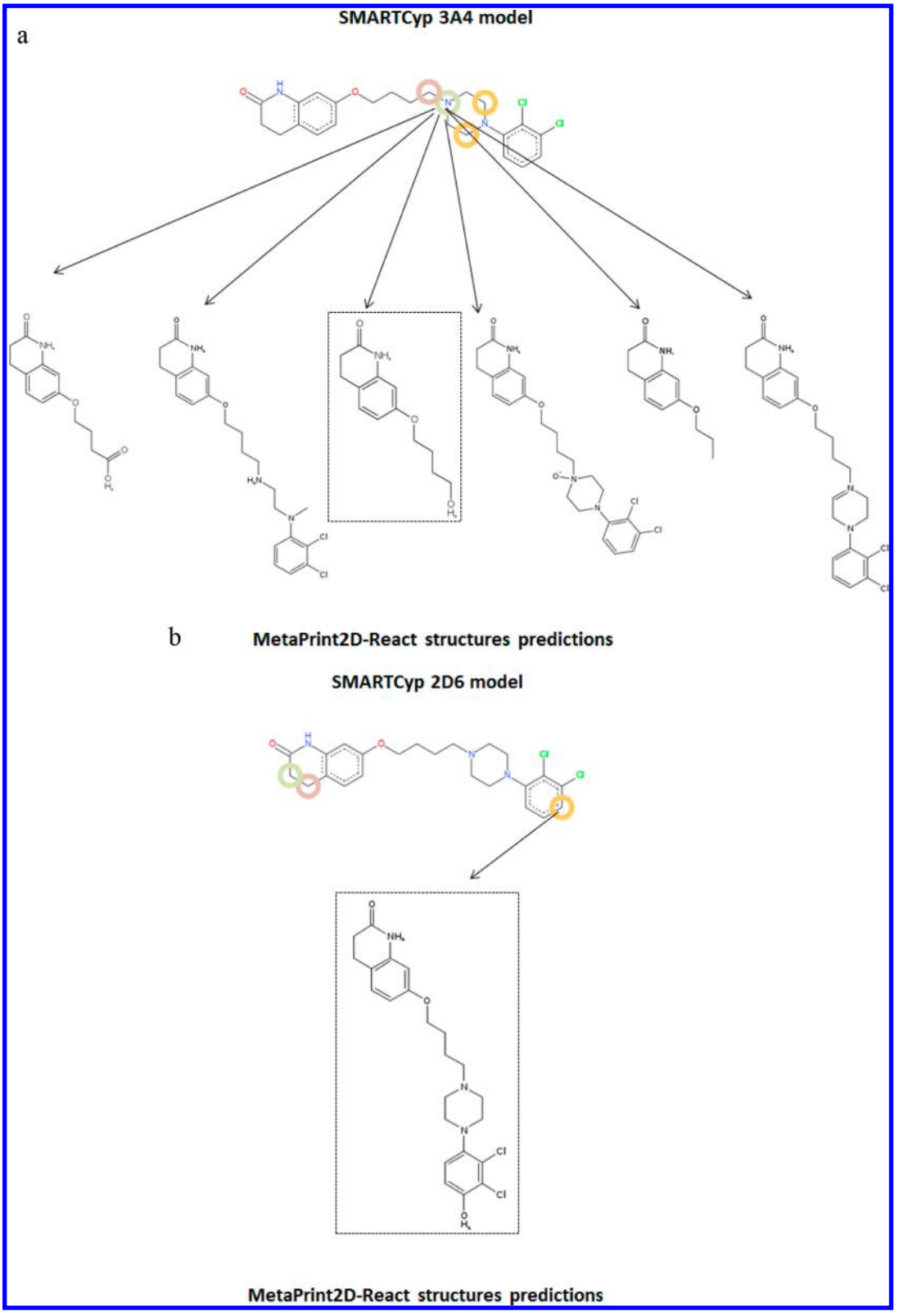

Figure 7. Simulations of metabolic trees using a combination of models from SMARTCyp (CYP3A4 (part a) and CYP2D6 (part b)) and MetaPrint2D-React. The circles indicate the top ranked SOM. One SOM for each model was selected to obtain possible structures from MetaPrint2D-React. The metabolites (shown in the boxes with dotted lines) are those that correspond to known *in vivo* metabolites.

It should be noted that these percentages were calculated by taking into account all of the experimentally observed metabolites. Rydberg et al.[13] used a different approach wherein they calculated the percentage of compounds for which SMARTCyp was able to correctly predict at least one metabolic site among the top-ranked atoms. They found that for a data set of 394 compounds, 81% of these had a correct SOM among the top three ranked SOM. Applying the method of Rydberg et al.[13]

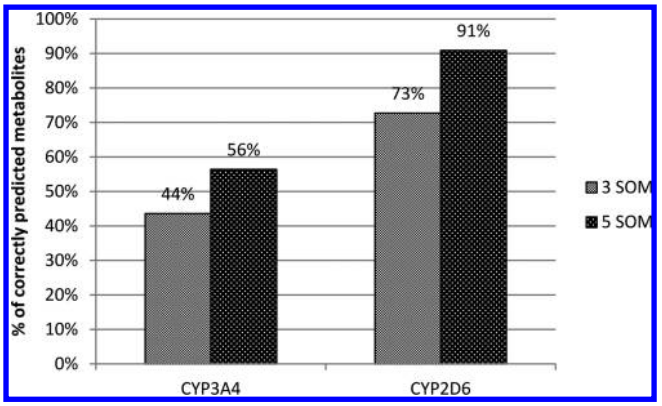

Figure 8. The percentage of known metabolites that were correctly predicted using a combination of SMARTCyp and MetaPrint2D-React using three (3 SOM) or five (5 SOM) sites of metabolism associated with CYP3A4 and CYP2D6. Note: The number of known metabolites in DS2 that were identified to be products of cytochrome CYP3A4 and CYP2D6 were 39 and 11, respectively.

to the data set of 17 drugs, known to produce 39 metabolites via CYP3A4, resulted in correct predictions for 76% of the compounds, i.e., for 13 of the 17 parent drugs SMARTCyp identified a correct SOM within the top three ranked SOM. In a similar analysis Rydberg and Olsen[18] showed that for a data set of 45 compounds, known to be metabolized by CYP2D6, 91% of the structures were found to have a metabolic site among the top-three atoms. In the data set studied here, six drugs are known to be metabolized by CYP2D6, and of these the metabolites associated with the top three ranked SOM were correctly identified in 83% of cases. Although smaller data sets were analyzed here than those studied by Rydberg et al.[13] and Rydberg and Olsen,[18] the results support the usefulness of the SMARTCyp tool.

SMARTCyp's use of precalculated energies for subfragments allows very fast processing in the prediction of SOM which is of great value when applied to large scale screening in drug discovery. Furthermore, the program has recently been enriched by including three additional cytochrome models, CYP2C9, CYP2C19, and CYP1A2, so that predictions can be made for five major cytochromes involved in drug metabolism. An evaluation of the latest three models[19] confirmed that the accuracy of SMARTCyp in terms of identifying metabolic hot spots is comparable or even better than that of the state-of-the-art software based on 3D structure-based methods such Metasite and StarDrop. The benefits of using SMARTCyp in conjunction with other programs to predict metabolism, as a filter to identify most likely SOM, and therefore reduce number of false positives is another pragmatic approach that warrants further research. Development of a method that allows discrimination between different models is in progress in collaboration with Lhasa Ltd.;[20] this directs a user in terms of identification of major contributors to metabolism for a query compound.

4. CONCLUSION

Within this study the overall performance of selected computational methods (Meteor, SMARTCyp, MetaPrint2D and MetaPrint2-React) in predicting metabolites or sites of metabolism has been assessed. The results indicate relative performance and highlight areas where improvements may be made in the software (or in use of the software); the results do not indicate true predictive performance as in many cases the compounds studied here may have been included within the training set for the programs.

The results show that both Meteor and MetaPrint2D-React both performed well in predicting metabolites for both homogeneous (DS1) and heterogeneous (DS2) data sets. Of particular interest is that all packages performed better in predicting metabolites of DS2 rather than DS1. This suggests that there may be specific areas of chemical space that are less populated within the data used to train the models. This study has identified structural types for which predictions could be improved if metabolite data for these compounds (such as bromfenac) were incorporated.

The combination of the empirically based SMARTCyp program, for the prediction of SOM, with software for predicting metabolites also offers a pragmatic approach to directing the results of metabolite prediction to those metabolites that are most likely to be formed.

A significant part of the research undertaken here was to determine a pragmatic approach to using the Meteor software. One problem identified with this package is its tendency to overpredict metabolites. The approach used here, i.e., using permissive settings such as EQU3, but incorporating cutoffs for the number of metabolites investigated shows that good precision can be attained with much reduced computational effort and simplification of output. As the importance of metabolites and their potential to elicit toxicity is becoming increasingly recognized, tools that can aid the rational prediction of such metabolites, such as the methods proposed here, will be of benefit.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

An EXCEL worksheet comprising the common names, CAS numbers, IUPAC names, and SMILES strings for all of the parent drugs and the SMILES strings for the known metabolites. The list of references from where the metabolite information was obtained. 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.

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