Current Topics in Medicinal Chemistry, 2016, *16*, 2078-2087

The Importance of Bioactivation in Computer-Guided Drug Repositioning. Why the Parent Drug is Not Always Enough

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Abstract: Although bioactivation is a well-documented process and the role of active metabolites in the drug discovery field has long been recognized, drug metabolites are usually ignored in virtual screening campaigns oriented to drug repositioning. The present article discusses different issues related to overlooking of the active metabolites in virtual screening campaigns, including an overview of the essential aspects of drug biotransformation and a summary of computational approaches that can provide solutions to those issues. Some valuable computational resources connected with this topic are also overviewed.

Keywords: Drug metabolome, Virtual Screening, In silico Screening, Drug repositioning, Computer-guided drug repositioning, Human metabolome database, Bioactivation.

1. INTRODUCTION

The term drug repurposing (also known as drug repositioning) refers to finding second or further medical uses of known drugs, including approved, discontinued, abandoned and experimental therapeutics. It is an innovative strategy which has raised intense interest in the last decade owing to both the abundance of successful repurposing stories and the possibility of introducing novel medications to the clinical practice with relatively little investment of time and resources. Recently, national institutions (e.g. the NIH's National Center for Advancing Translational Sciences or the UK's Medical Research Council) have launched rewarding (and expanding) programs to promote drug repositioning-oriented crowdsourcing partnerships between the academic sector and pharmaceutical companies [1-3]. While traditionally most of the repurposing cases have emerged from exploitation of a drug's known mechanism to a new therapeutic indication in which the same drug target is involved (on target repurposing) or from serendipitous observations of unexpected side-effects [4], computer-guided drug repositioning has recently been intensively explored to supply both efficiency and rationale to off-target, more innovative drug repurposing [5-7]. Briefly, this approach includes cheminformatics-, bioinformatics- and high-throughput literature analysis-based drug repurposing, along with synergistic combinations of the former (prominently, network-based approximations integrating different levels of experimental data and computational predictions) [7].

Virtual screening consists in the application of computational models and/or algorithms to rank digital collections of chemical compounds (chemical libraries) in order to decide which ones will move on to experimental testing [8]. Although within the vast realm of cheminformatics other approximations might be also used for drug repositioning purposes (the reader is referred, for instance, to the very interesting works related to the indication similarity ensemble approach [9-11]), virtual screening of specific chemical databases containing approved drugs such as DrugBank [12] or Sweetlead [13] is by far the most explored one [14-22].

While the opportunities linked to bioactivation and exploitation of bioactive metabolites have long been recognized within the drug discovery and development field [23-25], metabolite profiling is often neglected in virtual screening campaigns oriented to drug repurposing, with some very recent exceptions [26]. As clearly discussed in a very stimulating article from Oprea and Overington [27], the substances subjected to screening can often differ from the chemical species intrinsically responsible for the therapeutic effect, which should thus be also annotated in the screening drug library. This article overviews the role of bioactivation in pharmacology including discussion on which metabolites are more likely to be pharmacologically relevant. On the light of the importance of bioactivation, we will present some hints and considerations that should be taken into account when conducting virtual screening campaigns aimed at drug repurposing. Finally, a summary of some computational resources related to experimental and predictive information on potentially bioactive metabolites will be presented.

2. A GENERAL OVERVIEW OF DRUG METABOLISM

Drug metabolism involves a wide spectrum of biotransformations, enzyme-catalyzed reactions which, generally speaking, tend to produce biotransformation products (metabolites) that are more hydrophilic than the parent or origi-

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1873-5294/16 $58.00+.00

nal compound [28]. This increment in polarity facilitates the biliary and renal excretion, limiting gut or tubular reabsorption, respectively [28, 29]. Classically, drug metabolism was divided in Phase I and Phase II reactions. Such classification could be misleading: though many drugs undergo both Phase I and Phase II reactions, in that order, others undergo either one or the other [28, 30, 31]; thus, labeling Phase I biotransformations as functionalization or non-synthetic reactions and Phase II biotransformation as conjugation or synthetic ones is sometimes preferred nowadays.

Functionalization metabolism involves the creation of a functional group or the modification or exposure of an existing one, generally leading to a metabolite which is more chemically reactive than the parent compound. This type of biotransformations introduces a "chemical handle" or anchoring point in the substrate which predisposes the molecule to take part in a conjugation reaction [28, 32]. Essentially, they are either redox or hydrolysis reactions. It is estimated that oxidation reactions are involved in the metabolism of about 90% of the known drugs. The diversity in the chemical nature of the resulting functional groups (among them aliphatic and aromatic alcohols, aldehydes, ketones, carboxylic acids, primary and secondary amines, hydroxylamines, N-oxides, sulfides, sulfoxides, sulfones and epoxides) and the positional and stereochemical differences in the creation of a single type of functional group [33] show that functionalization reactions may result in an extraordinary diversity of metabolites. Furthermore, though the biotransformation products of functionalization reactions might be pharmacologically inactive, very often they are indeed active, and in some cases even more active than the original drug (in which case we will talk of bioactivation) [34]; this fact will be of utter importance for the discussion in the following sections. Some benzodiazepines (see, for instance, diazepam and some of its active biotransformation products in Fig. **1**) are a good example of such behavior: they are often metabolized to active products with long elimination half lives, leading to long lasting hypnotic effect.

Once a suitable anchoring point is available in the drug (whether it was originally present in it or added via a functionalization reaction), endogenous molecules or moieties such as phosphate, sulfate, glucuronic acid and glutathione may be transferred to it through a conjugation reaction; the resulting metabolite will have modestly to markedly higher molecular weight and usually much higher polarity that the parent compound, with both changes reducing drug tubular and/or intestinal reabsorption. Moreover, formation of polar conjugates is frequently coupled with their active excretion, favoring drug clearance [32, 35]. Though synthetic reactions mostly lead to inactive products, there are numerous significant examples of active conjugates [36], such as opioids glucuronides, N-acetylprocainamide and hydroxytriamterene sulphuric acid ester (Fig. **2**).

It should be highlighted that whether an active metabolite is or is not clinically relevant depends on a number of factors, including its intrinsic activity, rate and extent of its generation, its half life and whether it displays the same or different actions than the parent molecule [34, 37]. Metabolites can develop effects different from that of their parent compound. For example, vitamin A is metabolized to retinoic acid, which has anti-acne and anti-cancer effects [38, 39]. Aspirin and its metabolite, salicylate, share anti-inflammatory effects but, seemingly, they act through different mechanisms [40]. Doxepin is a potent antihistamine indicated for sedation; its metabolite, N-demethyldoxepin, binds to the norepinephrine transporter and exerts antidepressant effects [37]. Other examples of drugs that generate metabolites that have increased affinity at receptors different than the ones targeted by the parent drug can be found in the extensive review from Obach [37].

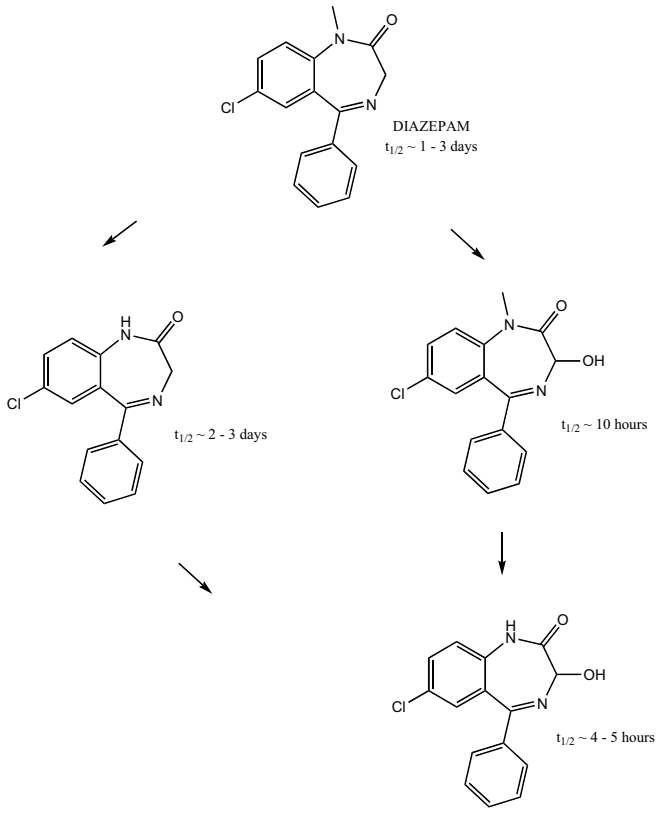

Fig. (1). Diazepam's Phase I active metabolites.

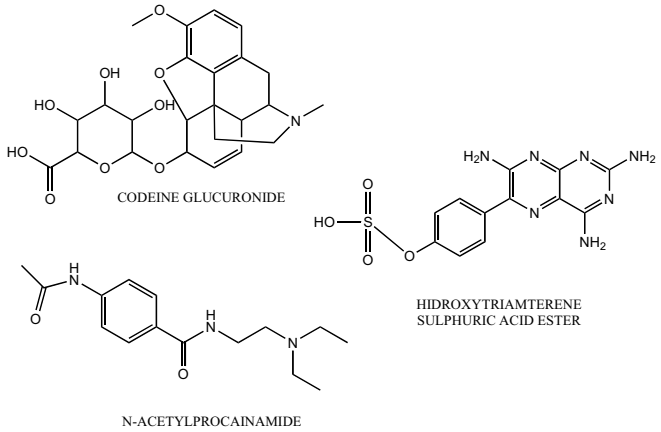

Fig. (2). Although Phase II metabolites are majorly inactive, a number of examples of active conjugates can be found.

Prodrugs constitute a particular case of bioactivation. They are bioreversible derivatives of drug molecules that undergo enzymatic or chemical transformation within the body to release the active parent drug [41]. Prodrugs should

have no or negligible biological activity per se, becoming active after biotransformation or *in vivo* chemical cleavage. Interestingly, around 10% of approved drugs worldwide are unintended or designed prodrugs [42-43], with a dramatic increase in the number of prodrug patents in the beginning in 2000s [44]: approximately one third of all the approved small molecule drugs in 2008 were prodrugs [45]. These figures should not be overlooked: chemical repositories oriented to drug repositioning such as DrugBank may include around 10% of prodrugs which will undergo rapid conversion into the active chemical species.

On the other hand, discovering drugs by mean of biological transformations might be a viable approach to drug design [46]. Note that while metabolites are chemically distinct from the parent drug, they are still chemically similar (specially the metabolic products of functionalization reactions), and a similar activity profile to the one of the precursor might be attained if the modification is introduced in a non-pharmacophoric group or whenever it leads to optimization in binding to the molecular target [23]. In a way, Phase I biotransformations might be considered as natural SAR studies in which functional groups are modified and/or new substituents are incorporated to different positions of an active scaffold. Occasionally, the metabolite might display enhanced target interactions, pharmacokinetics and/or safety. As a matter of fact, many metabolites of approved drugs have been later registered as novel therapeutics (see Fig. **3** for examples), a strategy that may prove rewarding from an intellectual property perspective.

From the previous discussion it is evident that considering the drug metabolome is of major importance when executing in silico screening campaigns directed to drug reprofiling, as will be discussed in detail in the next section.

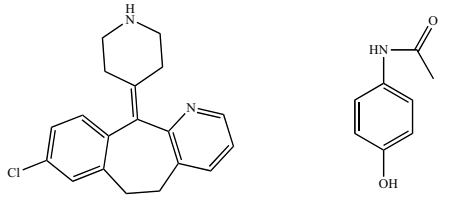

Fig. (3). Two examples of successfully marketed active metabolites.

3. HOW TO CONSIDER DRUG METABOLISM WHEN CONDUCTING CHEMINFORMATIC-BASED DRUG REPOSITIONING CAMPAIGNS

Docking, quantitative structure-activity relationships (QSAR) and pharmacophore searches can be mentioned among the most prominent cheminformatic approaches applied in the frame of computer-guided drug repositioning initiatives [7]. Either of these methodologies may be applied to prioritize the experimental testing of repositioned candidates emerging from chemical repositories or libraries containing approved, discontinued, shelved or investigational drugs such as DrugBank [47] or Sweetlead [48] repositories. Very briefly, docking refers to a target-based approximation which, from experimental knowledge on the drug target 3D structure and the binding site/s, aims to reproduce or predict the binding mode/s of a given ligand to a certain molecular target, by estimating the drug-target complex free energy and thus the binding affinity [49]. The QSAR approach consists in making a statistical, empirical inference (which will be termed *QSAR model*) on the correlation between the molecular structures of a set of chemicals (the *training or calibration set*) and some biological property of interest (e.g. the binding constant to a given molecular target) [50, 51]. The theoretical or experimental molecular features through which the statistical relationship is established are known as *molecular descriptors*. Finally, pharmacophore searches suppose obtaining, from one or multiple known active compounds (or occasionally, in a structure-based manner), a spatial arrangement of key chemical features which are fundamental for a compound to elicit the studied bioactivity (*pharmacophoric features*, e.g. H-bond donor or acceptor groups, aromatic rings, etc.) [52, 53]. Later, superimposition of the candidate molecules onto the pharmacophoric pattern will decide which candidates best accomplish the pharmacophore requisites.

It is clear that inherent to the formerly described approximations lays the assumption that the considered compounds are actually the chemical entities directly responsible for the biological activity under study. Provided that most drugs elicit their action after very specific ligand-target recognition events, no relationship between the structure and activity of a set of compounds can be imagined if those compounds do not share a common action mechanism and, even more, a common binding site. So how can we ensure that the compounds used to infer a QSAR of pharmacophore model and, later, the screened compounds (candidates for repositioning) are the chemical entities directly responsible for the biological activity? And how can we predict if the biological response to a given compound might be augmented through bioactivation? Practical hints to approach these issues will be discussed within the next sub-sections.

3.1. Practical Considerations to Build the Training Set

Very often it is stated in the specialized literature that when building a model (e.g. a QSAR model or a pharmacophoric hypothesis) from chemical and biological data the model can only be as good as the data itself [51, 54], thus requiring a very careful curation of such data to avoid unnecessary noise. In other words, *the quality of the model is training set dependent*. The modeler must then take all the possible cautions to assure that the training set compounds have been adequately represented and that they (and not their metabolites) are the chemical entities responsible for the biological property being modeled. It must be guaranteed that the training set instances do have intrinsic activity. In the case of docking, where the drug-target interaction is explicitly modeled, it is very clear that only if the chemical entity with intrinsic activity is docked in the correct binding mode/s one should expect highly scored complexes.

Assurance regarding the intrinsic activity of the training set instances can be easily achieved if binding or dissociation constants (or other binding parameters such as drug-target residence time) are chosen as the dependent variable of the model: in that case, by definition, only ligands with experimentally measured binding constants to the intended target

will be allowed in the training set. Fortunately, public online resources compiling experimental affinity data for a wide range of proteins, such as BindingDB [55], are today available, as will be discussed in the next section of the article.

Although binding parameters can be thus regarded as the gold standard choice for a model's dependent variable, many others such as ED_{50} [56, 57], IC_{50} [58, 59], carcinogenicity [60], or toxicity [61] are frequently used in modeling campaigns. When the modeled variable is measured in a phenotypic assay (e.g. effect of the drug on a microorganism culture, an animal model or a cell culture) the modeler cannot be sure regarding the specific action mechanism explaining the phenotypic observation or the number or identity of the pharmacologically active chemical species. In those cases, the complex nature of the biological response makes it impossible to describe, *a priori*, a well-defined action mechanism, or to discriminate the influence of other processes on the modeled activity apart from the studied drug-target interaction (e.g. transport processes, interaction with multiple targets, bioactivation). Succinctly: the more complex the *in vitro* (or *in vivo*) model used to measure the experimental variable from which the model's dependent variable is obtained, the noisier the *in silico* model and the less certainty regarding the identity of the chemical species with intrinsic activity.

From a statistical viewpoint the use of discriminant (classifier) models or even multiple-classifier systems (meta-algorithms) may help limiting the influence of noisy data on the quality of the model [62-64]. Noisy data points could behave as outliers: atypical training instances that cannot be fully explained (or explained at all) by the model. The choice of a discrete dependent variable (class or category label) instead of a continuous one may alleviate the incidence of noise (unknown molecular or cellular processes influencing the model's response, experimental variability in the biological data points). Some adaptive boosting algorithms are particularly suitable to deal with this issue. While most of such recursive algorithms focus on training examples that are repeatedly misclassified (i.e. misclassified instances gain weight previous to the addition of a new learner to the ensemble), others such as BrownBoost (an adaptive version of *boost by majority*) operate in the exactly opposite manner [65]: they decrease the weight of repeatedly misclassified examples, giving up on/downplaying those instances. The first type of boosting approaches might lead to overfitting, failing to obtain general hypothesis from noisy data; the second type could be a powerful tool to detect and lessen the influence of outliers. Several other statistical solutions to deal with the issue of outliers have been reported [66-69].

Alternatively or complementarily to pure statistical approximations to the problem of outlier detection, the modeler may perform knowledge-based prospective curation of the training data points previously to starting the model search procedure. Approved and investigational new drugs are much better characterized regarding their active metabolites and action mechanism/s, compared to drug candidates in early stage of development [70-71], thus being more reliable (less noisy) training examples. Inclusion of prodrugs in the training set should be avoided [72]. Eventually, models directed to predict metabolic stability or the sites and products of metabolism can provide insight into atypical training examples related to bioactivation processes [73-76] (the reader is particularly directed to the excellent review from Kirchmair *et al.* [75]).

The preceding considerations are synthetically presented in Table **1**.

3.2. Metabolites that Deserve Particular Attention

Since drug metabolism can result in active metabolites which may be even more active than the parent drug, drug repurposing *in silico* screening campaigns should not only explore known drugs but their metabolites as well [27]. A good example of the importance of considering drug me-

Table 1. Some practical considerations that can help compensating noisy biological data due to bioactivation and other unforeseen processes.

Issue	Solutions / Advices
The chemical structures used to simulate the ligand/target interaction (docking) or as training example (pharmacophore or QSAR models) should display *per se* the pursued activity	If possible, choose affinity constants as biological response Use outlier detection approaches to uncover atypical/noisy training examples. If outliers are found, remove them but try to find an explanation to the unexplained behavior
Biological responses obtained from complex model systems (e.g. *in vivo* preclinical tests) usually reflect the interaction of multiple molecular processes (e.g. transport processes, interaction with multiple targets, bioactivation).	Use classification QSAR approaches: class or category labels may mitigate the influence of noisy data. Use ensemble learning approaches (meta-algorithms). Prefer adaptive boosting algorithms that lessen the weight of misclassified examples. Exclude prodrugs from the training set. Prefer including approved drugs or drug candidates at late stage of development in the training set. They are usually extensively characterized regarding their metabolome. If not possible, consider the use of *in silico* tools to estimate metabolic stability and predict biotransformation site and/or products.

tabolome in virtual screening campaigns is our recent work related to the anticonvulsant effect of steviosides and their biotransformation products in rodents [77, 78]. Following previous *in silico* studies which helped us identifying the anticonvulsant effect of artificial sweeteners [79], we decided to explore the potential anticonvulsant effect of natural sweeteners from *Stevia rebaudiana*. For that purpose, we resorted to a combination of two different computational models (a linear discriminant function and a pharmacophore) previously reported, both capable of identifying anticonvulsants acting through voltage-operated sodium channels blockade. Interestingly, while steviosides were predicted as non-anticonvulsants by the models, the aglycone steviol and its phase I metabolites were predicted as active (Fig. **4** shows the superposition between steviol and several functionalization metabolites onto the pharmacophore). Later, both the aqueous infusion of *Stevia rebaudiana* and the isolated steviosides displayed anticonvulsant effect, presumably due to the aglycone and Phase I metabolites exposure. The results illustrate the importance of taking drug metabolites into consideration when conducting virtual screening campaigns oriented to drug repurposing: the anticonvulsant effects of *Stevia* would have not been identified by the models if only steviosides had been considered in the analysis.

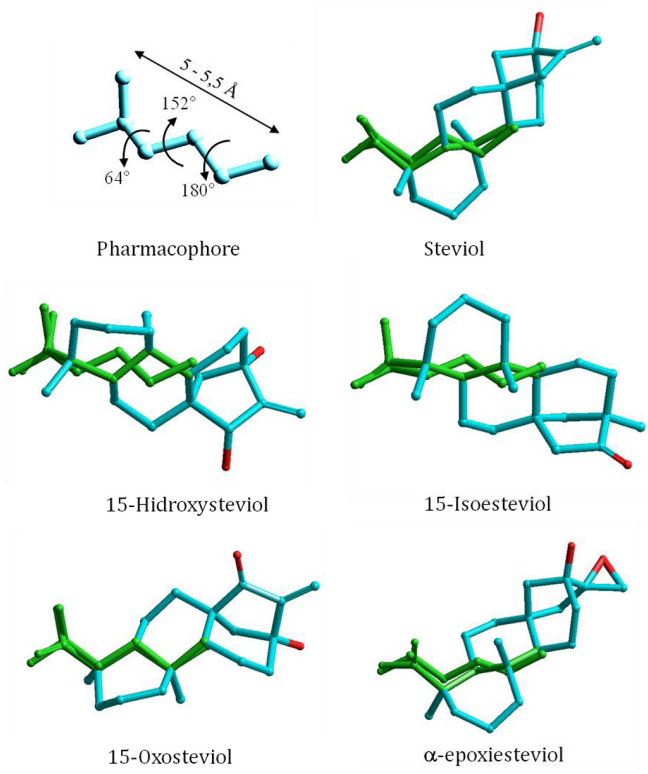

Fig. (4). Superposition of steviol and its Phase I metabolites onto the pharmacophore.

In 2014, Pan *et al.* introduced a computational framework to reveal putative off-targets of a set of analgesics (oxycodone, fentanyl, morphine, acetaminophen, liquicet and rofecoxib) in a high-throughput manner [80]. Recognizing the potential contribution of drug metabolites to off-target interactions, the authors also explored the putative off-targets of known phase I metabolites compiled from literature: acetimidoquinone (acetaminophen metabolite), oxymorphone (oxycodone metabolite), hydrocodone and hydromorphone (liquicet metabolites). Though the bioinformatics analysis carried out by the authors were intended to identify proteins and pathways linked to severe adverse drug reactions, the authors underlined that some of the predictions had therapeutic potential (e.g. as antihypertensives) and could be used to guide drug repositioning initiatives

Recently, Kigondu *et al.* explored the antimycobacterial activity of metabolites of the antipsychotic agent chlorpromazine [81]. To that purpose, they expose the parent drug to liver microsomes, obtaining six major phase I metabolites; two of them (7-hydroxichlorpromazine and norchlorpromazine) displayed weak inhibitory activity on the model organism *Mycobacterium smegmatis*; synergistic combinations with known anti-TB drugs were studied, with positive results.

In the light of previous discussions, do some metabolites deserve particular attention? Whether an active metabolite will or will not significantly contribute to the pharmacological response will depend, essentially, on two factors: a) its intrinsic activity/potency and; b) its exposure/pharmacokinetics. While small amounts of a highly active metabolite might have an impact on the pharmacological response, frequently those metabolites that achieve high concentrations in the vicinity of the molecular target (in general, the *major metabolites*) will be of more interest. The metabolite exposure depends on its rates of formation and elimination and its biodistribution profile. Since conjugation reactions often lead to inactivation, Phase I metabolites are, *a priori*, more relevant. Whenever the *in silico* model used in the screening study provides a continuous or categorical output, those metabolites predicted as more active will also be intuitively interesting. Finally, the more metabolites from the same drug that are labeled as active by the *in silico* models, the higher the probability than the parent drug elicit the pursued pharmacological response. It must be emphasized that the observed activity of a given metabolite (as in the case of any chemical) will depend on the balance between its potency and its exposure. A slightly active metabolite that achieves high levels in the biophase could be equally important to a potent metabolite that reaches the target in small quantities. Thus, resources compiling data on the biofluid and tissue concentrations of the metabolites are as important as the knowledge on their identity and their predicted pharmacological effect. Back in 2012, Patel *et al.* introduced the preparation of clusters of docking poses to identify preferences in the site of metabolism and correlate with the percentage of metabolites from antimalarial prodrugs proguanil and phenoxypropoxy biguanide derivatives found in previous *in vitro* studies [82]. Since the docking results displayed a nice correlation with the experimental ones, the authors postulate that their strategy could be applied in the future in the prediction of the quantity of desirable active triazine metabolites production over other less important or unwanted metabolites, the prediction of the role of specific CYP isoforms in the production of active metabolites and to assist the selection of optimal lead candidates.

The former (and some more) thoughts are synthetically presented in Table **2**.

Table 2. Some practical considerations that can help compensating noisy biological data due to bioactivation and other unforeseen processes.

Advice	Reasoning
Include drug metabolites in the screened library	The pharmacological response to many drugs is a function of the sum of effects of the parent drug and some of its metabolites
Focus on Phase I metabolites	Phase I metabolites are structurally similar to the parent drug, thus presenting better chances of interacting with the same molecular target. Phase II reactions entail a more radical change in the physicochemical properties (i.e. shape, molecular weight), and, with exceptions, lead to inactivation.
Focus on major metabolites	The intensity of the pharmacological response will not only depend on the intrinsic activity of the metabolite, but also on its level of exposure.
Focus on metabolites predicted as highly active	Even small amounts of a compound with high intrinsic activity may result in a significant pharmacological effect
The more biotransformation products predicted as active, the better	If a number of metabolites plus the parent drug are all predicted as active compounds by the models, there is better chance that an experimental activity be observed. Have in mind, however, that drugs with many active metabolites tend to present more complicated pharmacokinetics.
A drug might be repurposed even if it is not the chemical entity directly responsible for the new therapeutic indication	Many approved drugs are actually unintended prodrugs

4. SOME USEFUL COMPUTATIONAL TOOLS TO CONSIDER THE POTENTIAL CONTRIBUTION OF BIOACTIVATION TO CHEMINFORMATIC-BASED DRUG REPOSITIONING

Below these lines we will present a limited, non-exhaustive list of some interesting computational resources that may help addressing the preceding considerations related to the importance of potential or known active metabolites and bioactivation processes in computer-guided drug repurposing projects.

4.1. The Human Metabolome Database

The Human Metabolome Database (HMD, http://www.hmdb.ca) was released in 2007 [83] and it is currently in its 3.6 version. It is an impressive, manually curated, continuously expanding and publicly available database compiling detailed information of more than 40,000 detected or expected small molecule metabolites. Though most of the entries correspond to physiological compounds (e.g. food-derived compounds) the latest release contains >1500 drugs and drug metabolites. Detected metabolites are those that have been experimentally confirmed, while expected metabolites are those whose intake is frequent in human or for which molecular pathways are known in human, despite they have not been experimentally detected yet [84]. The complete structure library can be downloaded in *sdf* file format, thus making tit immediately available for virtual screening purposes. It should be highlighted that, as reflected in the resource name, the entries from the HMD exclusively refer to chemicals (either exogenous or endogenous) found or expected to be found in the human body, thus preventing any noise in the data owing to inter-species variability. Another highlight is that more than 5,000 entries contain information (if available) on biofluid (urine, plasma, CSF) and/or tissue concentration, though presently most of these data corresponds to endogenous compounds. Predicted biofluid concentration ranges have however been included in the 3.0 release for a significant number of drugs. Inclusion of dosing, pharmacokinetic and biofluid concentration data for indexed drugs and drug metabolites in future releases could strengthen the potential of this resource for virtual screening applications.

As already discussed, the effect of a parent drug can be prolonged when biotransformation products are also active on the same molecular target, or even enhanced through bioactivation. Therefore, if an *in silico* screening campaign indicates that both a drug and its metabolites could display a given pharmacological effect, such prediction would deserve special attention. Every predictive tool incurs into mistakes, but, in general, the more predictions pointing in the same direction, the less the probability of a misprediction (a false positive, for the example at hand). Furthermore, provided that all the predictions on a certain compounds and its metabolic products were true, one could be facing an indication of a long-acting drug (as in the case of benzodiazepines with many phase I active metabolites). Therefore, online repositories compiling information on known drug metabolites would be valuable to expand the screened library by including those biotransformation products; to the moment, the already determined drug metabolites must be in most cases compiled from literature, with the consequent time investment.

4.2. The Binding Database (BindingDB)

BindingDB (https://www.bindingdb.org) is a public database compiling experimentally determined binding affinities that focus on interactions between drug targets and drug-like

compounds [55]. At present it contains binding data for more than 7,000 proteins and about 500,000 small molecules, which makes it a valuable source of quality ("clean") training examples for QSAR modeling projects. It also includes a list of more than 5,000 compounds from the FDA Drugs database with their correspondent binding data to the target/s responsible of their therapeutic effects and/or other targets, a resource of great interest for all kind of *in silico* drug repositioning initiatives, including network-based approximations. This resource has been applied in the already discussed work from Pan et al [80]. The putative protein targets of the six analgesic drugs (and the correspondent metabolites) explored in that study were identified by simulation of ligand-receptor recognition using the reverse software INVDOCK; later, the proposed putative drug-target interactions were validated by looking for experimental evidences in BindingDB and ChEMBL. Adverse reactions associated pathways were then obtained by mapping the identified off-targets into the Kyoto Encyclopedia of Genes and Genomes pathway database.

4.3. MetaPrint2D

Freely available computational tools to predict metabolic stability, possible molecular sites undergoing metabolic reactions and metabolic products are still scarce [75]. Such resources, however, are valuable when no experimental data on a drug's metabolites identity are publicly available, to include putative metabolites in *in silico* screening campaigns.

University of Cambridge's MetaPrint2D and its extension, MetaPrint2D-React are remarkable exceptions (http://www-metaprint2d.ch.cam.ac.uk/) [85], since they are publicly available. It is a high-throughput data-mining tool that uses 2D circular fingerprints to identify sites of metabolism and possible metabolic products. The algorithm is based in the statistical analysis of the frequency of occurrence of certain atom environments in substrates and products of metabolic reactions. Two limitations of this application are that it is training set dependent (predictions cannot be made about atom environments which are not present in the training examples, i.e. outside the applicability domain of the algorithm) and that, since 2D substructures are employed, no discrimination between enantiomers can be expected. Opportunely, the application detects and acknowledges whenever novel sites outside of the applicability domain are found in the query molecule. The primary site of metabolism is found within the top three predictions in about 70-80% of the cases. In the frame of a QSAR or pharmacophore based VS campaign, this kind of application might be useful to: a) identify if a mispredicted case could be a result of bioactivation and; b) to predict the potential pharmacological activity of predicted metabolites.

4.4. MetaSite

MetaSite (http://www.moldiscovery.com/software/ metasite/) is a computational resource that predicts metabolic transformations related to cytochrome and flavin-containing monooxygenase mediated reactions [80]. The MetaSite algorithm is training set independent, and can be then used to make predictions for structurally novel pharmaceutical compounds. By applying a procedure that resembles flexible docking, MetaSite evaluates the substrate atoms accessibility to the correspondent catalytic domain and the substrate reactivity, which is computed from molecular orbital calculations using molecular fragments and *ab initio* methods, taking into consideration the inductive and mesomeric effects of adjacent groups. The primary site of metabolism is found within the top three MetaSite predictions in around 90% of the cases.

CONCLUSION

The role of bioactivation in the drug discovery and development field is well-documented. About 10 percent of marketed drugs are designed or accidental prodrugs: inactive precursors that are transformed to active species within the body. Furthermore, many drugs are biotransformed to metabolic products with intrinsic activity that impact on the pharmacological response.

However, to the moment, the role of active metabolites and bioactivation has been mostly ignored in virtual screening campaigns oriented to drug repositioning. Such neglect may impact on two instances of the virtual screening campaign: a) inclusion of noisy data as training examples of QSAR or pharmacophore models, owing to ignored or non-acknowledged active metabolites/bioactivation. Such training examples will not be fully explained by the model, increasing model's error and; b) ignoring that some known drugs might be repurposed not (or not only) because of their intrinsic activity but also due to the pharmacological activity of their metabolites.

Current state of the art of computational techniques and drug metabolism-related resources can help addressing the preceding issues. Such techniques include classifier QSAR models, ensemble learning approaches and outlier detection techniques. Useful tools to address this issue include metabolism-related resources include repositories compiling drug metabolome information such as the Human Metabolome Database and models and algorithms capable of predicting sites of metabolism and molecular structures of the resulting metabolites.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

The author is a member of CONICET. He thanks CONICET, UNLP and ANPCyT (PICT 2013-0520) for funding.

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Received: June 15, 2015 **Revised: September 13, 2015** **Accepted: November 06, 2015**