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Optimizing Classification of Drug-Drug Interaction Potential for CYP450 Iso-Enzymes Inhibition Assays in Early Drug Discovery

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Abstract

In drug discovery, the potential of cytochrome P450 inhibition of new chemical entities (NCEs) is frequently quantified in terms of *IC*50 values. In early drug discovery a risk-classification into low, medium or high potential inhibitors is often sufficient for ranking and prioritizing of compounds. While often six or more inhibitor concentrations are used to determine the *IC*50 value, the question arises whether it is possible to predict the *risk-class* based on fewer inhibitor concentrations with comparable reliability. In the present article we propose a new integrated two-point method with inhibitor concentrations chosen in accordance with the risk-classification. We analyse its predictive power and the feasibility to not only classify the compounds into different risk classes but also rank those compounds that have been binned into the middle risk class. The proposed integrated two point method is thus highly suitable for automation. Altogether it maintains the quality of the prediction while considerably reducing time and cost. The proposed method is applicable to other IC50 assays and risk classifications.

Keywords: Drug-Drug Interaction, Cytochrom P450, Drug Discovery, High Throughput, IC50

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1 Introduction

Cytochrome P450 (CYP) mediated metabolism accounts for the main pathway of drug elimination from the body for the 200 world best selling drugs [10]. Inhibition of these metabolic enzymes by a coadministered second drug can lead to a substantial increase of the parent drug concentration [6]. This effect is commonly known as 'CYP mediated drug-drug interaction', and may give rise to severe side effects up to the necessity of a drug withdrawal from the market [9]. Therefore, along with determination of target potency and selectivity of a new chemical entity (NCE), it is common practise in drug discovery to screen for the CYP inhibitory potential applying various types of assays [11]. In early project phases, it may be sufficient to qualitatively guide the progress of the project by the so-called Crespi assay [2], a flow chart of which is depicted in Fig. 1. In a broad sense, the obtained IC50 values are used for ranking purposes within one compound series and binned into classes exhibiting a high ($IC50<1\ \mu M$), a moderate ($1\ \mu M<IC50<10\ \mu M$) and a low ($IC50>10\ \mu M$) potential for drug-drug interaction (DDI). In later project phases, more time and cost intensive assays are used to characterise the compounds of interest more accurately [1].

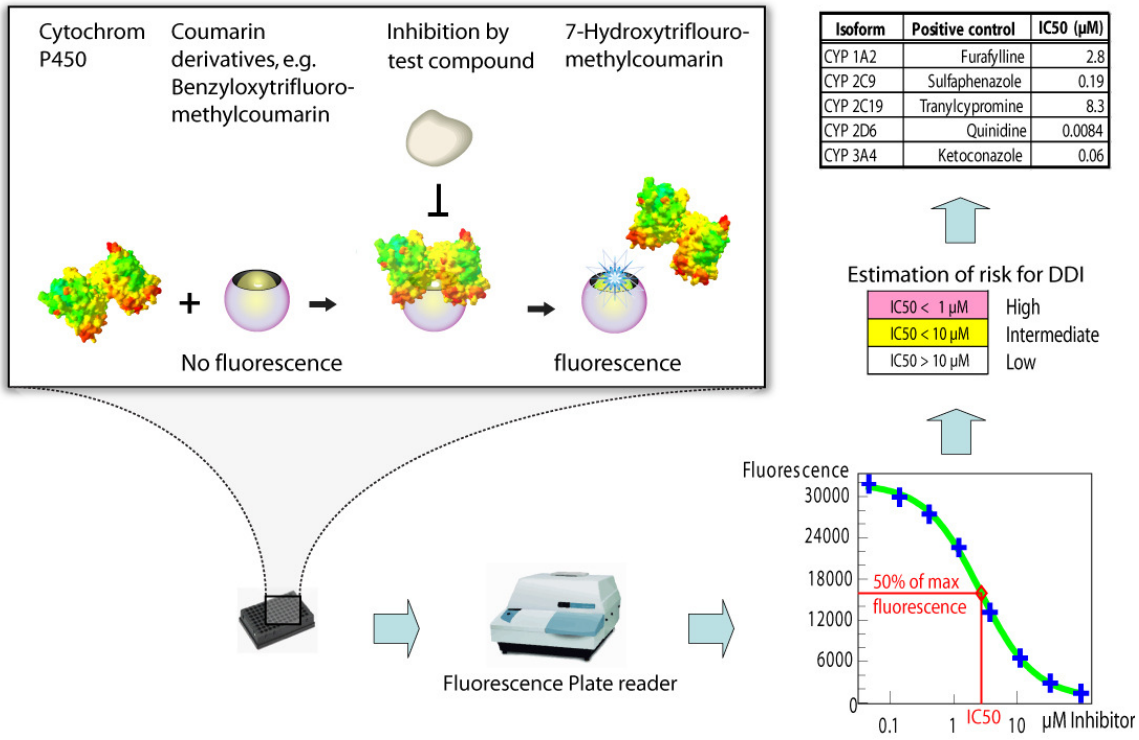

Figure 1: Principle of the Crespi assay: Fluorogenic substrates are metabolised by a recombinant human CYP iso-enzyme to its fluorescent metabolite. An IC50 value can be calculated from the

reduced fluorescence upon addition of increasing concentrations of a test compound. The IC50 is categorized into classes with a high (IC50 below 1 μM), medium (IC50 between 1 μM and 10 μM), and low (IC50 above 10 μM) risk potential.

To keep pace with the dynamics of drug discovery and further economize these routinely used *in vitro* assays, mathematical methods were exploited in order to predict IC50 values with as few as possible inhibitor concentrations while maintaining a high correlation to the reference IC50 assay with six inhibitor concentrations. As has been reported in [7, 8], the predictive power of methods based on one or two inhibitor concentrations varies with the chosen concentrations and suffers from increasing variability at IC50 values far away from the chosen inhibitor concentrations. To fix this drawback, either the statistical model is changed, as in [7], or different (*a priori* unknown) inhibitor concentrations are chosen for different compounds, as in [8]. Neither solution seems satisfactory from a theoretical nor a practical point of view. The present study focuses on the problem of how to reliably predict the risk-classes, in some sense a coarse-grained IC50 value, with as few as possible inhibitor concentrations and how to optimally choose the concentrations to obtain most reliable predictions. Applying the herein proposed approach, we obtain a high predictive quality (90% correctly predicted). Furthermore, the variability at the most critical regions is minimized by choosing inhibitor concentrations at the boundary of the risk-classes (1 μM and 10 μM for the chosen risk classification depicted in Fig. 1) and thereby avoiding the above mentioned drawbacks. The presented approach can easily be adapted to various assay types and different risk classifications.

2 Materials and Methods

2.1 Materials

For our analysis 290 compounds resulting from different drug-discovery projects have been arbitrarily chosen from the Schering compound library. Inhibitory controls (1A2: Furafyllin, 2C9: Sulfaphenazole, 2C19: Tranylcypromine, 2D6: Quinidine, 3A4: Ketokonazole) were purchased from Sigma Aldrich (St. Louis, MO). Other reagents were taken from the commercially available CYP High Throughput Inhibitor Screening Kits ® (Kat. Nos. 459100 (1A2), -200 (2C9), 300 (2C19), -400 (2D6) and -500 (3A4)) from BD-Gentest (Woburn, MA). 96-well plates were taken from Greiner Bio-One (Frickenhausen, Germany) using the black Greiner FIA plate model (Kat. No. 655076). Human recombinant cytochrome P450 enzymes were used as received in concentrations as listed in Table I. Fluorogenic substrates of the various iso enzymes were 3-Cyano-7-ethoxycoumarin (CEC) for CYP1A2 and 2C19, 7-Methoxy-4-trifluoromethylcoumarin (7-MFC) for CYP2C9, 3-[2-(NN-Diethyl-N-methylamino)-ethyl]-7-methoxy-4-methylcoumarin (AMMC) for CYP2D6 and 7-Benzyloxy-trifluoromethylcoumarin (7-BFC) for CYP3A4. Potassium phosphate buffer at pH 7.4 was used as incubation media, cofactors consisted of Glucose-6-phosphate (G6P), NADP+, $MgCl_2$, and G6P-dehydrogenase (G6PD) and acetonitrile / Tris Base at 0.5 M (80/20, (v/v)) served as stop reagent.

2.2 CYP Inhibition Crespi Assay

The assay was carried out according to the protocol published by Crespi and Stresser [1]. Incubations were performed by using a Heidolph Titramax 1000 incubator (Heidolph Instruments, Schwabach, Germany) at 37°C and 750 rpm for 15 min-45 min (see Table I). A Fluostar® Galaxy plate reader (BMG-Labtechnologies, Offenburg, Germany) was used for fluorometric data generation. Concentrations of the various components are listed in Tab 1. Test compounds were dissolved in acetonitrile to a stock solution at 3 mM yielding in a final solvent concentration of 2% in the assay. The compounds were investigated with regard to their DDI potential for the CYP iso-enzymes 1A2, 2C9, 2C19, 2D6, 3A4. IC50 determinations were based on six concentrations at 0.12, 0.37, 1.1, 3.3, 10, and 30 μM, and were performed at least in duplicates. Only compounds with an $IC50$ less than 30 μM were taken as a basis for the study since this group of compounds is the more critical and challenging one. For compounds showing inconsistent data, the $IC50$ has been corrected by excluding

misleading data points from the calculation. Compounds exhibiting auto-fluorescence were excluded from the data evaluation because for these compounds an IC50 determination cannot be reliably performed using the Crespi Assay.

2.3 Dose-Response Models

The commonly used model to determine the *IC*50 of a NCE from given *in vitro* data is to fit the *sigmoid dose-response*

$$\textit{percent inhibition at } x = \min + \frac{\max - \min}{1 + \left(\frac{IC50}{x}\right)^{h}} \qquad (1)$$

derived from the Hill equation [4, 5, 8] to the experimental data, where x denotes the inhibitor concentration, max the maximal *percent* inhibition, and min the minimal *percent* inhibition of the enzyme. Often, min and max are set to 0% respectively 100%, and we adopt this practise here. Moreover, h denotes the Hill coefficient that determines the slope of the sigmoidal curve and reflects the extent of cooperativity [7]. The *IC*50 value and the Hill coefficient are determined by solving a *non-linear least-squares problem* [3] in order to minimize the differences between the model and the experimental inhibition data.

Different number of experimental data (inhibitor concentrations) can be used as a basis for fitting the sigmoid dose-response model. In broad terms, the more data are used for the fit, the more accurately the *IC*50 can be determined, since outliers and variability in the experimental conditions will be averaged out. However, particularly in the early stages of drug discovery, a rough determination of the CYP inhibition potential of a NCE without many (costly) measurements is desired for ranking and prioritization purposes within one programme.

For our study, the reference scheme for determining the risk-class is based on the **six-point *IC*50**, i.e., fit of the sigmoid dose-response model to the six data points ranging from 0.12 μM to 30 μM. This method is taken as a reference to evaluate the predictive power of the **integrated two-point method**, i.e., fit of the sigmoid dose-response model

$$\textit{percent inhibition at } x = \frac{100}{1 + \left(\frac{IC50}{x}\right)^{h}} \qquad (2)$$

to two data points at 1.1 μM and 10 μM (chosen in accordance with the risk classification), with variable Hill coefficient or with h=1. In [4], Gao et al. suggested the use of a **one-point method**. In this case, the $IC50$ can be determined explicitly by solving for $IC50$:

$$IC50 = x \times \frac{100 - \textit{percent inhibition at } x}{\textit{percent inhibition at } x} \tag{3}$$

under the additional assumption that the Hill coefficient is fixed to one. In our analysis, the one-point method at 1.1 μM, 3.3 μM or 10 μM is included.

3 Results

In the following subsections, the different methods will be studied w.r.t. their ability to classify a compound into the same risk-class as the reference six-point *IC*50 method. This is done by plotting the predicted *IC*50 of the one-/two-point method versus the six-point *IC*50.

3.1 One-Point method at 1.1 μ*M*, 3.3 μ*M*, or 10 μ*M*

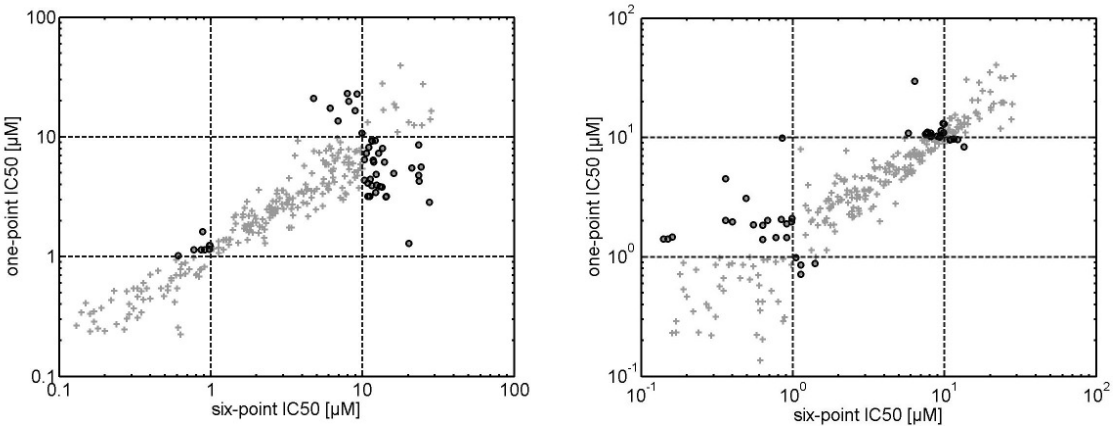

Figure 2: Comparison of the *IC*50 values on five major CYP Isoforms for 290 compounds obtained by the one-point method at 1.1 μ*M* (left) and 10 μ*M* (right) vs. the six-point *IC*50 values. Compounds for which the predicted risk-class differs from the six-point risk-class are marked with a black circle.

The one-point method at 1.1 μ*M*, 3.3 μ*M* or 10 μ*M* allows obtaining good predictions for compounds with an *IC*50 close to the corresponding inhibitor concentration at 1.1 μ*M*, 3.3 μ*M*, or 10 μ*M*, respectively (see Fig. 2 and Fig. 3, left). However, the larger the difference between the six-point *IC*50 and the chosen inhibitor concentration for the one point method, the larger are the variations of the predicted *IC*50 values. In other words, the one-point method at 1.1 μ*M* focuses on the range of *IC*50 values around 1.1 μ*M*, while the one-point method at 10 μ*M* focuses on the range of *IC*50 values around 10 μ*M* (and analogous for 3.3 μ*M*). In addition, due to the usage of just a single measurement, even negative *IC*50 values are predicted from eq. (3): there are eight negative IC50 values predicted by the one-point method at 1.1 μ*M,* six negative predictions by the one-point method at 10 μ*M*, and zero negative predictions by one-point method at 3.3 μ*M* (points not shown in Fig. 2 and 3).

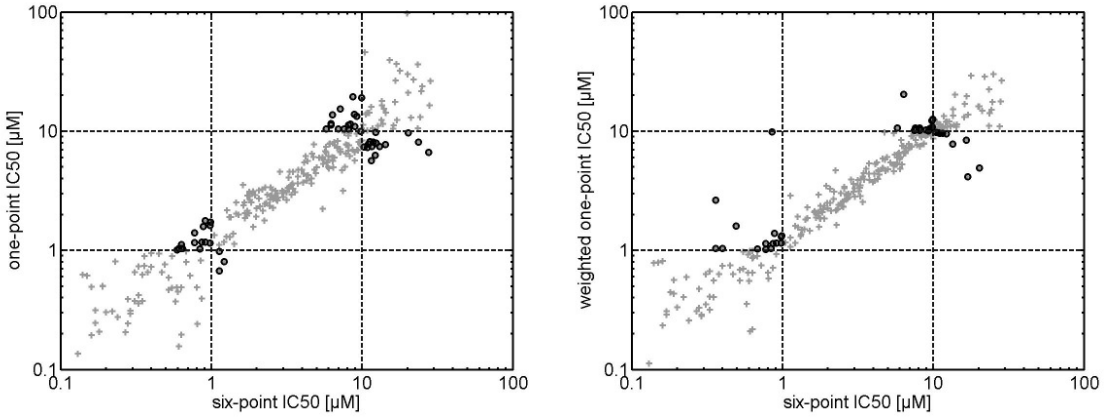

Figure 3: Comparison of the *IC*50 values for 290 compounds on five major CYP Isoforms obtained by the one-point method at 3.3 µ*M* (left) and the weighted one-point method at 1.1 and 10 µ*M* (right) vs. the six-point *IC*50 values. Compounds for which the predicted risk-class differs from the six-point risk-class are marked with a black circle.

One possibility of fixing the disadvantage of either one-point methods is to combine two *IC*50 values in a weighted sum. Denote by $IC50_x$ the *IC*50 value at x=1.1 µ*M* or x=10 µ*M*. Then we define the weighted sum by

$$IC50 = \frac{w_{10}}{w_{1.1} + w_{10}} IC50_{1.1} + \frac{w_{1.1}}{w_{1.1} + w_{10}} IC50_{10}$$

with w_x = |(percent inhibition at x µM) – 50)|. The above weighting scheme between the two *IC*50 values is designed in such a way that the inhibitor concentration with percent inhibition closer to 50% inhibition has more control of the overall *IC*50 value. As a result, the predictive power increases (see Fig. 3, right). However, still there is the possibility of predicting negative *IC*50 values (two negative predictions).

Another strategy to fix the disadvantage of the one-point methods could be to choose out of two *IC*50 values the one whose *IC*50 value is closest to its underlying inhibitor concentration. The results are comparable to the method based on the above weighting scheme (data not shown).

3.2 Integrated two-point method at 1.1 μ*M* and 10 μ*M*

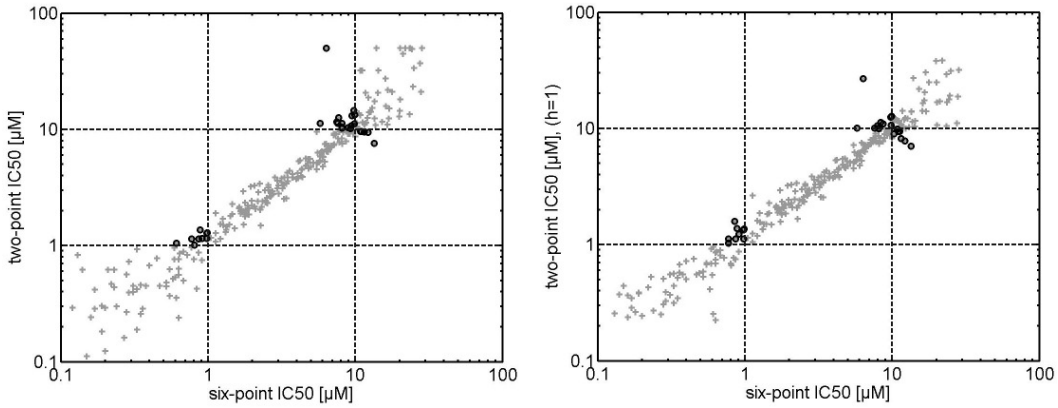

Figure 4: Comparison of the *IC*50 values for 290 compounds on five major CYP Isoforms obtained by the integrated two-point method with fixed Hill coefficient (*h*=1, right) and with variable Hill coefficient (left) vs. the six-points *IC*50 values. Compounds for which the predicted risk-class differs from the six-point risk-class are marked with a black circle.

Instead of combining two one-point *IC*50 values, the integrated two-point method directly fits the sigmoid dose-response model to two data points. The IC50 values resulting from the integrated two-point method are shown in Fig. 4 for variable Hill coefficient (left) and fixed Hill coefficient, h=1, (right), as in the case of the one-point methods. While the one-point methods at most allow to classify the compounds into the three different risk classes, the integrated two-point methods not only has a higher reliability to classify into the three risk classes, it also offers the possibility to *rank* compounds in the middle risk class. In order to quantify the correlation between the IC50 value of the six-point and the integrated two point method, Pearson correlation coefficients were computed based on all 290 compounds and based solely on those compounds that have been ranked into the middle risk-class (by the integrated two point method). As can be inferred from Table II, the overall correlation is less than 89% (for both methods), while the correlation in the middle risk class is as high as 95%.

3.3 Comparison of the different methods

In Table II the overall performance of the different methods is compared. For each method, the percentage of correctly predicted risk-classes, the number of data points in each risk-class, and the Pearson correlation coefficients based on all 290 compounds and based only the those compounds that have been ranked into the middle risk class are

reported. As a result, the integrated two-point method turns out to be the most reliable method.

4 Discussion

Streamlining *in vitro* assays to make them more cost-effective is of high importance when dealing with an increasing number of compounds in times of growing time, capacity und budget restrains. However, streamlining is only cost-effective, if it does not come at the cost of losing predictive power or accuracy, i.e. maintains the value of the assay for decision-making. We have therefore analysed mathematically how the six point CYP inhibition IC50 assay can be slimmed down without compromising its predictive power and hence its use for drug discovery.

Our analysis of the one-point method [4] at 1.1 μM, 3.3 μM and 10 μM clearly reveals some serious limitations. The one-point method has its *predictive focus* for $IC50$ values close to the inhibitor concentration used (1.1 μM, 3.3 μM or 10 μM, respectively). The larger the difference of the used inhibitor concentration to the predicted $IC50$ value, the larger the dispersion (see Fig. 2 and Fig. 3, left). In [4], Gao et al. analyzed the potential of using a single inhibitor concentration to estimate $IC50$ values. While their model well predicted the (logarithm of the) $IC50$ value for a small test set of compounds based on a single inhibitor concentration at 3 μM, they had to modify and increase the complexity of the model to regain a comparable predictive quality when analyzing additional 396 new compounds: 'With the larger set, it was found necessary to either fit a curve or fit a model with several slopes', [4]. From our analysis, this effect can easily be explained: The larger and more diverse the set of compounds the more diverse the range of $IC50$ values. As a consequence, the variability at values distant from the chosen inhibitor concentration increases and thereby decreasing the quality of the one-point method. In addition, the theoretical justification of their analysis is arguable since—based on the relation in eq. (1)—one would not expect a linear relation between the percent inhibition at x and the $\log(IC50)$ value, as done in [4]. Rather, one would expect a hyperbolic relation, which can nicely be seen in Fig. 4 of Gao et al. [4].

The insight in the one-point methods motivated the use of two-point methods. In [5], Moody et al. also analyzed the predictive power of a

two-point *IC*50 method. They found a high correlation between *IC*50 values computed by a seven-point method versus their two-point method. However, they used different inhibitor concentrations for their two-point method depending on the *IC*50 value predicted by the seven point method: 0.5 and 5 μ*M* were used for inhibitors with *IC*50 lower than 0.5 μ*M*; while 5 and 50 μ*M* were used for inhibitors with *IC*50 between 0.5 and 250 μ*M*; and 50 and 500 μ*M* were used for inhibitors with *IC*50 greater 250 μ*M* [5]. This way, they obtain a good correlation between their two-point *IC*50 and the seven-point *IC*50. However, by this method, a pre-*IC*50 determination needs to be done thereby not significantly lowering the time and costs to be invested.

Trying to overcome this practical limitation we advised a new integrated two-point method based on inhibitor concentrations chosen at the boundaries of the risk-classes, in our case 1.1 and 10 μ*M*. This way, we force the method to be as accurate as possible around the set risk boundaries. This results in a highly reliable classification into the three categories. The performance can be seen in Fig. 4: While the dispersion is moderate at 1.1 and 10 μ*M*, it increases at either end, where, however, it does not have any influence on the risk-class. In addition, the high correlation coefficient of the middle category permits to rank the compounds according to their IC50 value between 1 and 10 μ*M*. This has important implications for real life project support. For projects with a high scatter of IC50 values, binning may be of sufficient help to identify promising compounds. However, if many compounds would fall into the middle category, simple binning would be of no help to guide chemical optimisation. Since the integrated two-point method maintains the ranking power of the six-point method, it can fully be replaced without losing resolution for structure based support of medicinal chemistry and rational guidance of lead optimization.

When comparing the one/two-point methods to the reference six-point method, there are at least two possible explanations for deviations between either two methods: (i) When analyzing six points, it is obviously easier to spot experimental outliers or auto-florescence of a compound than by looking at just one or two-points. Thus, with fewer inhibitor concentrations used for the calculation, we loose the possibility of *a-posteriori* correcting the experimental data based on visual inspection. (ii) It is easier to cope with variability when analyzing six data points in contrast to just one or two, since errors will average out in the former case. As the analysis demonstrates the predictive power of the proposed integrated two-point method is 90% suggesting that by using this approach two measurements contain enough information for predicting a risk class of a NCE even without

the possibility of excluding misleading data points from the analysis like in the six-point method. In pharmaceutical practise the performance will even be higher compared to our analysis because only compounds with an *IC*50 less than 30 μ*M* were taken as a basis for the study, and NCEs showing very weak or no potential for CYP inhibition are determined easily.

The integrated two-point method is directly linked to the classification of the inhibitory potential into the three risk-classes high (*IC*50≤ 1 μ*M*), medium (1 μ*M*<*IC*50<10 μ*M*) and low (*IC*50≥ 10 μ*M*). In principle, the approach can easily be adapted to a different classification scheme. However, if the distance between the boundary concentrations chosen becomes too large (e.g. 2 orders of magnitude), the dispersion is likely to be increasing between these points, thereby compromising the resolution within this class.

5 Conclusion

In this article we show that the proposed integrated two point method allows reducing the number of concentrations from six to two while maintaining the resolution of the assay, both in terms of binning compounds into three risk classes and in reliably ranking compounds binned into the middle class.

The predictive power of the proposed integrated two-point method is 90% compared to the reference six-point IC50 method, which is well within the experimental error of the method. The method is attractive for automated assays which often run with few concentrations, and may allow to fully abandon the six point method from screening.

In conclusion, a validated, rapid and cost effective ranking and prioritization tool to estimate the drug-drug interaction potential for NCEs at an early stage of the drug discovery process is presented herein. The approach can easily be adapted to different assay protocols or borders of risk classification.

Acknowledgements

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CYP	1A2	2C9	2C19	2D6	3A4
NADP+	8.1 µ*M*	8.1 µ*M*	8.1 µ*M*	8.1 µ*M*	8.1 µ*M*
G6P	0.4 mM	0.4 mM	0.4 mM	0.4 mM	0.4 mM
G6PD	0.2 IU/ml	0.2 IU/ml	0.2 IU/ml	0.2 IU/ml	0.2 IU/ml
$MgCl_2$	0.41 mM	0.41 mM	0.41 mM	0.41 mM	0.41 mM
KPO_4	50 mM	50 mM	50 mM	50 mM	200 mM
Positiv controls	0.045-100µ*M* Furafylline	0.005-10µ*M* Sulfaphenazole	0.045-100 µ*M* Tranyl-cypromine	0.0002-0.5µ*M* Quinidine	0.00025-0.56µ*M* Ketoconazole
Enzyme amount	0.5 pmol/well	1 pmol/well	1.5 pmol/well	1.5 pmol/well	1.0 pmol/well
Substrate	5 µ*M* CEC	37.5 µ*M* 7-MFC	25 µ*M* CEC	1.5 µ*M* AMMC	50 µ*M* 7-BFC
Incub. time	15 min	45 min	30 min	30 min	30 min
Excitation/ Emmission	410/ 460 nm	410/ 530 nm	410/ 460 nm	390/ 460 nm	410/ 530 nm

Table I: Concentrations of used materials set up of the Crespi assay for different iso-enzymes.

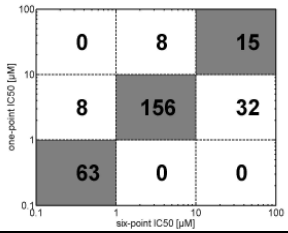
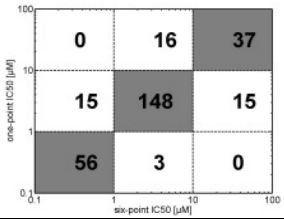
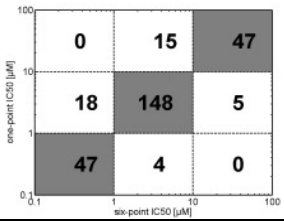
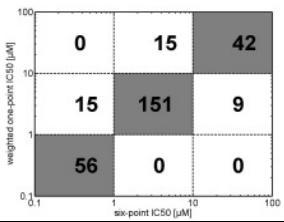
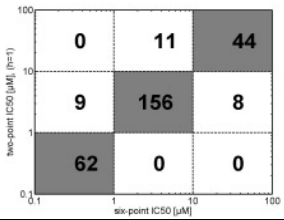
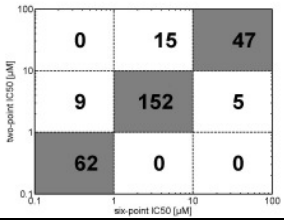
Method	Correctly predicted risk classes	Number of data points in each class	Pearson correlation coef. based on all data / for middle risk class
One-point method at 1.1µ*M*	81 %		0.2471/ 0.5279
One-point method at 3.3µ*M*	83 %		0.6967/ 0.7961
One-point method at 10µ*M*	83 %		0.8884/ 0.8631
Weighted one-point method	86 %		0.5549/ 0.7977
Integrated two-point method with Hill coef. =1	90 %		0.8867/ 0.9577
Integrated two-point method with variable Hill coef.	90 %		0.8401/ 0.9523

Table II: Overall evaluation of the methods for classifying compounds according to their inhibition potential on five CYP Isoenzymes.