Computational model for multiplex assay of drug immunotoxicity in macrophage-study of the anti-microbial G1 using flow cytometry

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ABSTRACT

The development of *in vitro* cytotoxicity assays has been driven by the need to rapidly evaluation of potential toxicity of large numbers of compounds, to reduce animal experimentation, and to save time and material resources. The large number of experimental results reported by different groups worldwide has lead to the accumulation of huge amounts of ontology-like data in large public databases as in ChEMBL. Conversely, many drugs have been assayed only for some selected tests. In this context, High-throughput multi-target Quantitative Structure-Activity (High-throughput mt-QSAR) techniques may become an important tool to rationalize drug discovery process. In this work, we train and validate by the first time mt-QSAR model using TOPS-MODE approach to calculate drug molecular descriptors and the software STATISTICA to seek a Linear Discriminant Analysis (LDA) function. This model correctly classifies 8,258 out of 9,000 (Accuracy = 91.76%) multiplexing assay endpoints of 7903 drugs (including both train and validation series). Each endpoint correspond to one out of 1418 assays, 36 molecular and cellular targets, 46 standard type measures, in two possible organisms (human and mouse). After that, we determined experimentally, by the first time, the values of EC_{50} = 21.58 μg/mL and Cytotoxicity = 23.6 % for the anti-microbial / anti-parasite drug G1 over Balb/C mouse peritoneal macrophages using flow cytometry. In addition, the model predicts for G1 only 7 positive endpoints out 1,251 cytotoxicity assays (0.56% of probability of cytotoxicity in multiple assays). Both experimental and theoretical results point to a low macrophage cytotoxicity of G1. The results obtained are very important because they complement the toxicological studies of this important drug. This work opens a new door for the "*in silico*" multiplexing screening of large libraries of compounds.

1. Introduction

Macrophages are phagocytic cells that recognize and kill microbial and tumor targets by cell-to-cell contactor through secretion of a wide array of products including reactive oxygen species , reactive nitrogen intermediates, cytokines, chemokines, *etc*. (Tripathi and Sodhi 2009). Macrophages are the heterogeneous

Abbreviations: QSAR/QSTR, Quantitative-Structure/Toxicity Relationship; LDA, Linear Discriminant Analysis; ChEMBL.

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grouping of cells that are derived from monocytes. They have a multitude of functions depending on their final differentiated state. These functions range from phagocytosis to antigen presentation to bone destruction, to name a few. Their importance in both the innate and acquired immune functions is undeniable. Xenobiotics that degrade their functional status can have grave consequences. Many published reports on the effect of xenobiotics on macrophage function make comparisons between treated versus untreated macrophages isolated in an identical manner to control for this problem. A commonly used source of mouse and rat macrophages is the peritoneal cavity. Two types of macrophages from the peritoneal cavity are used, resident and elicited (Barnett J. B. and Brundage Kathleen M. 2010). Often in the cytotoxicity assay to increase the number of macrophages, a sterile irritant, such as thioglycollate, is injected several days prior to harvesting the cells. The resulting peritoneal cells are referred to as elicited macrophages.

The process of cytotoxicity is the result of a sequence of stages and complex biological interactions that can be influenced by several factors, often contained in the same supernatant, have been identified that exhibit cytotoxic and/or growth inhibitory activities on a wide range of cells These factors include the interferons, lymphotoxins (LT) natural killer cytotoxic factor (NKCF) macrophage cytotoxins and tumor necrosis factor. The macrophages execute numerous functions such as antigen presentation, cytokine production, phagocytosis, migration, and the production of ROS (Cunnick Jess. 2006). The extent and duration of macrophage activation is critical to limit the detrimental effects associated with excessive inflammation. Many of the molecules generated during macrophage activation are toxic not only to microorganisms but also to the macrophages themselves. for this reason, mechanisms that account for macrophage deactivation play key roles in maintaining homeostasis and keeping the immune response under control (Valledor Annabel F. 2010). Numerous test development for toxicologist screening example the cytotoxicity test is a screening method that typically uses permanent cell lines for ranking acute toxicities of parent compounds based on the basal cytotoxicity theory chemicals exert their acute toxic effects by interfering with basic cellular functions that are common to all mammalian cells (Mingoia R.T. 2007) In vitro drug cytotoxicity may be variable among different cell lines and. one parameter for cell death is the integrity of the cell membrane, which can be measured by the cytoplasmic enzyme activity released by damaged cells (Weyermann J. 2005).

The large number of experimental results reported by different groups worldwide has lead to the accumulation of huge amounts of information in this sense. This in turn, has allowed the creation of large databases available online for public research. One of the more outstanding cases with respect to drug cytotoxicity/biological effects over macrophage cells is the enormous database ChEMBL. ChEMBL is an Open Data database containing Binding (B), Functional (F), and Absorption, Distribution, Metabolism, and Excretion - Toxicity in Pharmacokinetics ADMET (A) information for a large number of drug-like bioactive compounds. These data are manually abstracted from the primary published literature on a regular basis, then further curated and standardized to maximize their quality and utility across a wide range of chemical biology and drug-discovery research problems. Currently, the database contains 5.4 million bioactivity measurements for more than 1 million compounds and 5200 protein targets. Access is available through a web-based interface, data downloads and web services at: https://www.ebi.ac.uk/chembldb (Gaulton et al. 2012).

ChEMBL contains >10,000 outcomes for assays of drugs related somehow to macrophage with different degrees of curation (outputs obtained after using macrophage as keyword in a simple search). As a consequence, the search of computational models to predict the possible results for new drugs in all these assays have become a goal of the major importance to reduce experimentation costs. In addition, despite of the large number of assays described many drugs have been assayed only for some selected tests. Consequently, predictive models may become also an important tool to carry out an "*in silico*" mining of ChEMBL predicting new results for drugs already released. The mining of ChEMBL using different computational tools have been recognized by Mok *et al*. as a very interesting source of new knowledge (Mok and Brenk 2011). In special, Quantitative Structure-Activity Relationships (QSAR) have been widely used to predict toxicity from chemical structure and corresponding physicochemical properties (Kuzmin V.E. 2008). Unfortunately, almost current QSAR models are able to predict new outcomes only for one specific assay. In our opinion, we can circumvent this problem using High-throughput multi-target Quantitative Structure-Activity (High-throughput mt-QSAR) techniques to model complex datasets determined in multiplexing assay conditions (m_j) as is the case of ChEMBL (Riera-Fernandez et al. 2012, Prado-Prado et al. 2011). In particular, the method TOSS-MODE was introduced by Estrada *et al.* (Estrada et al. 2000b, Estrada and Peña 2000, Estrada, Gutierrez and González 2000a, Estrada 2000, Estrada and Uriarte 2001b) and implemented in the

software MODESLAB (renamed as TOPS-MODE). TOPS-MODE have been demonstrated to be successful in both QSAR (Estrada et al. 2002b, Estrada, Quincoces and Patlewicz 2004, Estrada et al. 2006, Estrada et al. 2010, Pisco et al. 2006) and QSTR (Quantitative Structure-Toxicity Relationships) models as well (Estrada and Uriarte 2001a, Estrada, Molina and Uriarte 2001, Estrada et al. 2003, Estrada, Patlewicz and Gutierrez 2004). More recently TOPS-MODE have applied to High-throughput mt-QSAR studies by our group (Marzaro et al. 2011) and also Molina & Speck-Planche *et al.* (Molina et al. 2012b). However, there are not High-throughput mt-QSAR models of multiplexing assay endpoints for drug effects over macrophages using TOPS-MODE or other technique.

The main objective of the present work is to develop a valid High-throughput mt-QSAR model for predicting the biological effect of drugs over macrophages in a large set of m_j assay conditions. Another important goal is to illustrate the use of the new method in a real-life example. Fort it, we are going to download and calculate TOPS-MODE selected descriptors for the large dataset reported in ChEMBL. Next, we shall fit and validate a new High-throughput mt-QSAR Linear Discriminant Analysis (LDA) model using the software STATISTICA. After that, we report, by the first time, the experimental study of the effect of the drug G1 over Balb/C mouse peritoneal macrophage population using flow cytometry. Last, we carry out the prediction of other multiplexing assay endpoints for G1, not experimentally determined in this work. The results obtained are very important because they complement the toxicological studies of this important anti-bacterial, anti-fungal, and anti-parasite drug. In addition, they open a new door for the multiplexing "*in silico*" screening of large libraries of compounds.

2. Materials and Methods

2.1. Computational methods

2.1.1. ChEMBL dataset

A general data set composed of >10,000 multiplexing assay endpoints was downloaded from the public database ChEMBL (Gaulton et al. 2012, Heikamp and Bajorath 2011). In any case, after a carefully curation of the dataset we retain 9000 multiplexing assay endpoints (statistical cases) after elimination of all cases with missing information or very low representation. This dataset includes Number drug (N_d) = 7,903 drugs and/or organic compounds previously assayed in different multiplexing assay conditions (m_j). Every drug evaluated in different mj conditions were assigned to 1 out of 2 possible activity classes: active (C = 1) or non-active compounds (C = 0). One compound may lead to 1 or more statistical cases because it may give different outcomes (statistical cases) for alternative biological assays carried out in diverse sets of multiplex conditions. In this work, we defined mj according to the ontology m_j => (a_u, c_l, o_t, t_e, s_x). The different conditions that may change in the dataset are: different: organisms (o_t), biological assays (a_u), molecular or cellular targets (t_e), or standard type of activity measure (s_x). In closing, we analyzed N = 9000 statistical cases conformed by the above mentioned N_d = 7,903 drugs; which have been assayed each one in at least one out of assays Number (N_a) = 1418 possible assays. For each one of these assays the dataset studied present for each drug at least one out of Number Standart Types (N_s) = 46 standard types of biological activity measures in turn carried out in at least one out of Number Target N_t = 36 molecular or cellular targets. These values have been reported in ChEMBL as results of experiments carried out on at least 1 out of 3 possible organisms. Number Organism (N_o) = 3 (*Homo sapiens*, and *Mus musculus*). The values are reported in ChEMBL with three different levels of Curation Number (N_c) = 3 (expert, intermediate, or auto-curation level). Please, see details on the assignation of cases to different classes in results and discussion section.

2.1.2. Theoretical model

In order to seek the High-throughput mt-QSAR model we used the LDA module of the software package STASTICA 6.0 (StatSoft.Inc. 2002). The model developed presented the general form.

$$S_i(m_j) = b_0 + b_1 \cdot p(a_u) \cdot p(c_l) \cdot {}^{std}\mu_5^i + \sum_{j=2}^{4} b_j \cdot \Delta\mu_5^i(m_j) \qquad (1)$$

$$= b_0 + b_1 \cdot p(a_u) \cdot p(c_l) \cdot {}^{std}\mu_5^i + \sum_{j=2}^{4} b_j \cdot \left({}^{std}\mu_5^i - \left\langle {}^{std}\mu_5^i(m_j) \right\rangle \right)$$

Where, $S(m_j) = S(d_i, a_u, c_l, o_t, t_e, s_x)$ is a real-valued variable that scores the propensity of the drug to be active in multiplex pharmacological assays of the drug depending on the conditions selected mj. The statistical parameters used to corroborate the model were: Number of cases (N), Canonical Regression coefficient (R_c), Chi-square statistic (χ^2), and error level (p-level); which have to be < 0.05 (Van Waterbeemd 1995). In this

model, stdμ5i is the spectral moment or order k = 5 calculated with Modeslab. We used standard bond distance (std) as entries of the main diagonal of the bond adjacency matrix. The parameter p(au) is a probability, calculated *a priori*, with which any drug is expected to give a positive results in the uth assay au. The parameter p(cl) is a probability, calculated *a priori*, of confidence for a given data value into the ChEMBL dataset studied. The structural deviation terms $\Delta\mu^i_5(m_j) = {}^{std}\mu^i_5 - \langle {}^{std}\mu_i{}^5(m_j)\rangle$ represent the hypothesis H_0. H_0: the different deviations of the i^{th} drug (d_i) with respect to the average of all positive drugs for different multiplexing assay conditions (m_j) predict the final behavior of the compound. See a detailed discussion of terms and m_j conditions in results and discussion section. This type of deviation-like High-throughput mt-QSAR models has been used successful by other groups to solve different problems (Molina et al. 2012a, Speck-Planche et al. 2011).

2.2. Biology assays

2.2.1. Reagents and antibody

1-5-Bromofur-2-il-2-bromo-2-nitroethene (G1); CAS number 35950-55-1, was kindly supplied from the Chemical Bioactives Centre, Sample purity was 99.93%. G1 was dissolved in dimethylsulfoxide (DMSO), which was purchased in turn from Sigma–Aldrich Co. (DF, México). Macrophages were stained with phycoerythrin (PE), labelled monoclonal antibodies according to the manufacturers' instructions. Flow cytometry was performed using a FACalibur cytometer (Becton Dickinson, México). Thereafter, FACS data were analyzed with FlowJo 7.6.5 software. Both, anti-CD14 antibody (used to label CD14 receptor) and 7 – aminoactinomycin (7-AAD) at 5 μg/mL viability solution were purchased from BD (BD Biosciences, México).

2.2.2. Animals.

Female Balb/C mice weighing 18–20 g were purchased from the UNAM-Harlan laboratories (DF, México). All animals (n=6) were allowed to acclimate to our laboratory facilities for at least 7 days before their inclusion in an experiment. They were housed in standard laboratory conditions (22 3 °C; relative humidity 50–55%; 12h light/dark cycle) and given *ad libitum* access to food and water. This work agreed with Ethical Principles in Animal Research adopted by México (NOM 1999).

*2.2.3. Peritoneal macrophages isolation and cell culture***.**

Peritoneal macrophages were obtained from mice euthanized by cervical dislocation. The peritoneal of the animals were surgically exposed using a midline incision. Peritoneal fluid was harvested by injecting 10 mL of ice-cold PBS into the peritoneal cavity followed by syringe aspiration. Cell suspensions were washed twice by centrifugation. Cell viability (over 95%) was determined using trypan blue exclusion. Macrophage numbers were adjusted to 1 × 106 cell/mL and plated 100 μL/ well in 96-well flat-bottomed tissue culture plates (UNIPARTS, Toluca, México). Cells were incubated in RPMI 1640 complete medium containing 10% FBS, and incubated for 24 h at 37 °C under 5% CO2 in a humidified chamber. Non-adherent cells were removed by gently washing with PBS and fresh RPMI 1640 complete medium was replaced. The efficiency of macrophage enrichment was monitored by 7AAD assay and routinely exceeded 90%. Cells were equilibrated for 24 h before commencing the experiment.

2.2.4. Determination of cytotoxicity percentage by flow cytometry analysis

In all cases, dimethyl sulfoxide (DMSO) was used as the diluting solvent, and dosage solutions were prepared immediately prior to testing. Incubations were carried out in triplicate; solvent controls were run with each experiment. The percentage of formation of cytotoxicity cells was determined by evaluating 7-Amino-actinomycin D (7AAD) stained preparations of macrophages treated with the dosed chemical (G1) at 10, 8, 6, 4 and 2 μg/mL in 24 h.

$$\text{Cytotoxicity}(\%) = 100 \cdot (\text{Ma}^* - 7\text{AAD}^*)/(\text{Total event Ma}) \qquad (2)$$

Ma*= Positive Macrophages labeled CD14PE
7AAD*= Positive 7AAD (Dead macrophages)
Total event Ma = Total macrophages labeled and unlabeled CD14 with CD14

Briefly, 1X 106 cells were washed twice with 1 mL ice-cold PBS. Cytotoxicity was determined using flow cytometry with a FACSCalibur cytometer (Becton Dickinson, USA) equipped with an argon-ion laser at 488

nm wavelength. Tubes 21 and 22, isotypic controls and tubes with antibodies alone were used to adjust PMT and fluorescence compensation. Fluorescence compensations were also occasionally adjusted with Compbeads (BD Biosciences) by determining the median of both positive and negative populations. Percent cytotoxicity was determined by the following formula (Tario J. D.. and K 2011), where Ma mean macrophages count, the symbol * indicates a positive answer to CD14Pe and Negative mean negative to 7ADD staining for living cells.

Last, was fitted a response curve *vs.* concentration (MFI_i *vs.* c_i) in order to calculate the EC50 values using the software MasterPlex 2010, 2.0.0.73 created for the MiariBio group (*www.miraibio.com*). The MasterPlex includes Readerfit to calculate the EC50 and adjust the curve. ReaderFit is a free online application for adjustment of the curve that allows two fitting curves and optionally interpolates unknown values of the curve. The ReaderFit contain several equations for the model: 4 parameters logistic (4PL), 5 parameters logistic (5PL), quadratic log-logit, log-log or linear and one out four optional weighting algorithms: 1/Y, $1/Y^2$, 1/X and $1/X^2$ to minimize the error. In our case, Y variable contains the different Mean Fluorescence Intensity (MFI_i) response values and X the different concentrations (c_i) for different samples. The parameters of 5PL model are: A, B, C, D, and E. A is the MFI value for the minimum asymptote. B is the Hill slope. C is the concentration at the inflection point. D is the MFI for the maximum asymptote. E is the asymmetry factor ($E \neq 1$ for a non-symmetric curve). MFI is the. MFI values are obtained after exposition of the biological sample to one volume of 100 µL of G1 at different c_i values. This equation is represented through a sigmoid curve:

$$MFI_i = A + \frac{D}{\left(1+\left(\left(X/C\right)^B\right)\right)^E} = \left(MFI_i\right)_{\min} + \frac{\left(MFI_i\right)_{\max}}{\left(1+\left(\left(\frac{c_i}{EC_{50}}\right)^B\right)\right)^E} \quad (3)$$

$$\left(\frac{1}{MFI_i^{\,2}}\right) = A + \frac{D}{\left(1+\left(\left(X/C\right)^B\right)\right)^E} = \left(\frac{1}{MFI_i^{\,2}}\right)_{\min} + \frac{\left(\frac{1}{MFI_i^{\,2}}\right)_{\max}}{\left(1+\left(\left(\frac{c_i}{EC_{50}}\right)^B\right)\right)^E} \quad (4)$$

MFI = Mean Fluorescence Intensity.
A= is the MFI/RLU value for the minimum asymptote
B=is the hill slope
C= EC50 is the concentration at the inflection point
D is the MFI/RLU value for the maximum asymptote

E is the asymmetry factor

2.2.5. Statistical Analysis of experimental assays

Data were analyzed using Statistica 6.0 software. Significant differences between treatments were determined by analysis of variance (ANOVA), followed by t test. Statistic significances were accepted when $P < 0.05$. The Tukey test with 95% confidence was applied to compare the means.

3. Results and Discussion

3.1. Multiplexing model of drug effect over macrophage

3.1.1. Model training & validation

It is well known that biological outcomes in multiplex cell viability assay for drugs effect over different cellular lineages depend not only on drug structure but also on the set of assay conditions selected (mj) (Gerets, Dhalluin and Atienzar 2011). In this work we developed a simple High-throughput mt-QSAR model

with only four variables able to assign each drug to 1 out of 2 possible activity classes: active (C = 1) or non-active compounds (C = 0); given the molecular structure and several multiplex assay conditions mj. This model is expected to give different classification probabilities of the compound for different: organisms (ot), biological assays (au), molecular or cellular targets (t_e), or standard type of activity measure (s_x). It is also desirable to use an algorithm that takes into consideration the different degrees of accuracy or level of curation (c_l) in the experimental data. We fit the classifier using LDA. The best equation found was:

$$S_i(m_j) = 5.8261 \cdot {}^{*}\mu_5^i - 0.2617\Delta\mu_5^i(o) - 2.2122 \cdot \Delta\mu_5^i(t) + 0.6819 \cdot \Delta\mu_5^i(s) - 4.0372 \quad (5)$$

$$N = 6747 \qquad R_c = 0.7 \qquad \chi^2 = 4571.16 \qquad p < 0.05$$

$S(m_j) = S(d_i, a_u, c_l, o_t, t_e, s_x)$ is a real-valued variable that scores the propensity of the drug to be active in multiplex pharmacological assays of the drug depending on the conditions selected mj. The statistical parameters for the above equation are: Number of cases (N), Canonical Regression coefficient (R_c), Chi-square statistic (χ^2), and error level (p-level); which have to be < 0.05 (Van Waterbeemd 1995). The different parameters in the equation were introduced to codify specific information that is known to be determinant in the final value of biological activity. This discriminant function presented good results both in training and external validation series with overall Accuracy higher than 90%. According to previous reports in the QSAR literature (Patankar and Jurs 2003, Garcia-Garcia et al. 2004, Marrero-Ponce et al. 2005a, Marrero-Ponce et al. 2005b, Casanola-Martin et al. 2007, Casanola-Martin et al. 2008, Casanola-Martin et al. 2010) values Accuracy higher than 75% are acceptable. All the statistical data of this model are resumed in **Table 1**.

Table 1 comes about here

The reader should be aware that N here is not number of compounds but number of statistical cases. One compound may lead to 1 or more statistical cases because it may give different outcomes for alternative biological assays carried out in diverse sets of multiplex conditions defined by the ontology m_j => (a_u, c_l, o_t, t_e, s_x). This type of ontology introduced here allows us to clearly define the multiplex conditions for one assay in our dataset following the same line of thinking used for other ontology-like datasets in the literature (Martinez-Romero et al. 2010). The above equation was written in a compact form. At follow we expand the equation n order to better explain the meaning of the different parameters:

$$S_i(m_j) = 5.8261 \cdot p(a_u) \cdot p(c_l) \cdot {}^{std}\mu_5^i - 0.2617 \cdot \left({}^{std}\mu_5^i - \left\langle {}^{std}\mu_5^i(o_t) \right\rangle\right) - 2.2122 \cdot \left({}^{std}\mu_5^i - \left\langle {}^{std}\mu_5^i(t_e) \right\rangle\right) \quad (6)$$

$$+ 0.6819 \cdot \left({}^{std}\mu_5^i - \left\langle {}^{std}\mu_5^i(s_x) \right\rangle\right) - 4.0372$$

$$N = 6747 \qquad R_c = 0.7 \qquad \chi^2 = 4571.16 \qquad p < 0.05$$

The first parameter ${}^{*}\mu^i_5 = p(a) \cdot p(c) \cdot {}^{std}\mu^i_5$ codify the influence of the chemical structure of the compound over the biological activity. It is known that the spectral moment of order 5 codify information about all types of structural fragments with five or less bonds in the molecule. In addition to the topological information wμi5 codify also information about the physicochemical properties of the atoms and bonds in the molecule. It depends on the type of atomic or bond weights w_{ij} used. In our equation we set w_{ij} equal to the values of standard bond distance (std) in order to incorporate geometrical information (Estrada et al. 2001, Estrada and Uriarte 2001a, Estrada et al. 2002a, Estrada and González-Díaz 2003, Estrada et al. 2003). Consequently, ${}^{*}\mu^i_5$ codify the effect of the structure of the drug over the biological activity but depending on the type of assay carry out. In this sense, we pre-multiplied μi5 by the parameters $p(a_u)$ and p(cl). The parameter p(a) is a probability (*a priori*) that codify the propensity of one assay to yield positive results. We defined $p(a_u) = n_l(a_u)/n_{tot}(a_u)$; where $n_1(a_u)$ and $n_{tot}(a_u)$ are the number of positive or total results for the i^{th} pharmacological assay ai in the ChEMBL dataset studied, respectively. The parameter $p(c_l)$ is a probability (*a priori*) of confidence for a given data value into the ChEMBL dataset studied. We defined p(c) as follow p(c) = 1, 0.75, or 0.5 for data values reported as being curated at expert, intermediate, or auto-curation level respectively. In **Table 2** we give some example of assays and their $p(a_u)$ values. In the Table SM1 of the online supplementary material file we list exhaustive values of these parameters.

Table 2 comes about here

The other three terms in the equation express the structural dissimilarity between one specific compound and a group of active compounds that have been assayed in specific multiplex conditions defined by the sub-ontology m_j => (o_t, t_e, s_x). We quantify this effect in terms of the deviation $\Delta\mu^i_5(m_j) = {}^{std}\mu^i_5 - \langle {}^{std}\mu^i_5(m_j) \rangle$. This deviation terms represent the hypothesis: H0 the structural dissimilarity between one compound with respect to the average of all compounds in a group predict the final behavior of the compound. For instance, $\Delta\mu^i_5(o_t)$

$= {}^{std}\mu^{i}_{5} - <{}^{std}\mu^{i}_{5}(o_t)>$ measure the deviation from the average value $<\mu^{i}_{5}(t_e)>$ of μ^{i}_{5} for all active compounds (C = 1) assayed in the organism o_t => t = 1, 2 for Human or Mouse, respectively. The three possible values for this parameter are $<\mu^{i}_{5}(o_1)>$ = 18139.7, and $<\mu^{i}_{5}(o_2)>$ = 18149.6. This type of model able to model/interpret cross-species activity is of the major importance in order to reduce assays in humans (Meinel et al. 2011). By analogy, $\Delta\mu^{i}_{5}(t_e) = {}^{std}\mu^{i}_{5} - <{}^{std}\mu i5(t_e)>$ is the dissimilarity between the structure of compound i^{th} (expressed by stdμi5) with respect to all compounds active against the molecular or cellular target t_e. In **Table 3** and **Table 4** we give the values of $<\mu^{i}_{5}(t_e)>$ and $<\mu^{i}_{5}(s_x)>$ for the different targets or standard measure types respectively.

Please insert both Table 3 and Table 4 near here

3.1.2. Domain of application of the model

A QSAR model is only valid within its calibration domain or domain of applicability (DA), and new objects must therefore be assessed as belonging to this domain before the model is applied (Oberg 2004). The valid DA can easily be defined with the LDA model, as outlined in previous works (Gonzalez-Diaz et al. 2007). In this data set, a total of only 355 out 9000 total objects (statistical cases) fall outside of the DA. This DA may be geometrically defined as the rectangular area inside the 5% confidence bound for the ± 2 residuals interval and the leverage limit of $h = 3\cdot p'/N = 3\cdot(Nv + 1)/N = 3\cdot(4 + 1)/6746 = 0.00223$. Where, Nv is the number of variables in the model and N the number of cases used to train it. The DA can be visually illustrated in the so called Williams' graph (see **Figure 1**) (Papa and Gramatica 2008). All of the remaining 8645 objects (96.1% of the data set) fall within the valid DA. We found similar error for both train and prediction sub-sets with 6747 and 2253 objects (6747 + 2253 = 9000) respectively. Interestingly, 93.8% of drugs tested in some macrophage cytotoxicity assay lie within the DA as well. Similar behavior was found for other sub-sets of objects (see **Table 5**).

Figure 1 comes about here

In order to predict the classification of one compound one have to substitute in the High-throughput mt-QSAR model in first instance the structural parameter of the compound μ^{i}_{5}. However, this not sufficient to obtain different outputs for the same compound assayed in diverse conditions. In addition, we have to substitute the parameters characteristics of the given assay conditions $p(a_u)$, $p(c_l)$, $<\mu^{i}_{5}(o_t)>$, $<\mu^{i}_{5}(t_e)>$, and $<\mu^{i}_{5}(s_x)>$.

The models is expected to be more accurate for those m_j based on the more representative as possible number of cases (N_j); taking into consideration the influence of N_j in multiplex assays (Atienzar et al. 2011). In **Tables 2, 3,** and **4** we report values of these parameters. In total we analyzed N_a = 1418 assays, Nt = 36 molecular or cellular targets, N_s = 46 standard types of biological activity measures. Considering that we have determined this values independently our High-throughput mt-QSAR model is able to predict a huge number of combinations of biological assay conditions m_j. However, we strongly recommend using the model only for those m_j with at least 10 known cases. The number N_j of m_j that fulfill this stronger requisite are: Na = 437 assays, N_t = 22 targets, Ns = 20. The max number of outputs with this constrain $S_{max} = N_a \times N_t \times N_s \times N_o$ = 437 x 22 x 20 x 2 = 384,560 multiplex conditions m_j. Notably, N_o = 2 is the number of organisms susceptible to be studied with this model - Human (*Homo sapiens*) and Murine (*Mus musmuculus*). Consequently, our model is expected to be successful in the predictive extrapolation of experimental data from Murine species to Human.

Table 5 comes about here

3.2. Experimental-Theoretic Study of G1 anti-microbial drug

3.2.1. Experimental results

The compound G-1 is one of the members of a new family of furylethylene derivatives with both anti-bacterial and anti-fungal properties (Blondeau et al. 1999). More recently anti-parasite activity has been also reported (Marrero-Ponce Y 2005). The compound was synthesized in the laboratories of the Chemical Bioactives Center (CBQ) at the Universidad Central de Las Villas (UCLV), Cuba. Nitrovinilfurans compounds are widely used in medicine, industry and agriculture Interest in the study of these compounds has increased in recent years due to the potent microcidal activity shown by compounds with this type of chemical structure Nitrofurans constitute an important group of chemicals with antimicrobial properties that are currently used in human and veterinary medicine (Perez Machado Giselle. 2004).

3.2.2. Cytotoxicity assays

The cytotoxicity is defined as the response of toxicity of a compound on the cell. The kinetic cell viability measurement provides the temporal information as to when a drug of interest induces its cytotoxic effect

(Colombo P 2001). Quantifying cell viability or cytotoxicity is crucial for understanding cancer biology, compound toxicity and cellular response to cytokines and other biological questions. The endpoint measurements and assays used in cytotoxicity tests very considerably. An important consideration when undertaking *in vitro* cytotoxicity tests concerns the length of exposure of the cells to the test material A distinction has been made between 'short-term' and 'long-term' tests: the short-term tests involve exposure to chemicals for periods from I min to about 4 hr (endpoint measurements are typically focused on cell viability and cell membrane damage), whereas in the long-term tests cells are exposed for 24 hr or more before measuring, for example, cell survival or cell proliferation (Skowron and Zapor 2004).The specific method used will greatly influence the interpretation of the data. While many viability methods have been used for decades, there have been recent developments which offer increased sensitivity, throughput, and specificity. The particular type of cell death, apoptotic or necrotic, is becoming increasingly important. This requires multiplexing of methods, or methods that are able to distinguish between the different cell states and different endpoint evaluated (Cao LF 2010, Riss TL 2004).

In our study we used only the detection of membrane integrity by staining with 7AAD and flow cytometry. Several parameters were analyzed for dramatic views on the cytotoxicity of the drug. Viability dye 7AAD is routinely used in four-color flow cytometry assays, and therefore its use in conjunction with fixation should be carefully evaluated (Jacques Nathalie 2008). The analyses with flow cytometry were performed; in order to follow the percentage of live macrophages present in the macrophages populations treated with G1 at different concentrations we observed changes in the viability of the macrophages after 24 hours. The assay shows a significant increase of dead cells, Cytotoxicity (%) = 23.6%, compared to the group untreated (2.85 %) and the DMSO group (3.23%) at c_{max} = 10 µg/mL. The treatment of 6 and 8 µg/mL results in a dose-dependent significant increase in cytotoxicity (16.5%) and (19.4%) respectively (**Figure 2**). The percent of cytotoxicity is similar in concentrations 2 and 4 µg/mL (approximately 10%). It is noted further that there is an increased cytotoxicity in a dose-dependent this phenomenon has been reported in several studies using other drugs (Savaşan S 2005). These resulted indicate slight toxicity of G1 (10 pg / mL) because the percentage of cytotoxicity calculated was 23.6% <50%. Furthermore the estimated EC50 for this product was 21.82. (OECD 2010). In other studies with the product in lymphocyte populations the concentration 15µg/mL was observed cytotoxicity (González Borroto JI 2005).

Figure 2 comes here

Identification of ‘viable’ or ‘healthy’ cells by light-scatter (a common practice as perceived in a core laboratory) is purely empirical, and relies on the shape of the Forward Scatter *vs.* Side Scatter (FSC / SSC) cluster. Essentially, gating is set on the cloud-like distribution of cells with low to medium side-scatter, excluding cells with low forward scatter and high side-scatter. Sometimes this procedure provides a remarkable correlation between the percentage of excluded cells and the percentage of dead cells as identified by a viability stain such as 7-aminoactinomycin D (7-AAD) or propidium iodide (PI) (Petrunkina A.M. 2011). Secondly, we investigated the MFI on highly homogenous macrophages populations defined by the expression of CD14, obtained from the peritoneal macrophage of healthy mouse. These macrophages were exposed to different concentrations of G1 with DMSO.

In **Figure 3,** we depict a pseudo-color smooth projection of mean Intensity Fluorescence (MFI). This Figure represents plot FSC vs. SSC after exposure of G1 at 10µg/mL. In Figure 3A shows a 11.4% of the cell population of the total acquisition. The figure 3B has shown the cell population alone. The 3C and 3D shows the regions (R2 and R3) of macrophages labeled with CD14Pe (98.6%) of the total population. This figure shows the similarity of the dispersion values using foward (FSC) and side scatter (SSC). Cytotoxicity studies were used both forward scatter and side scatter because has shown high correlation (Veselá R 2011). This same methodology was used to represent the regions (R4, R5) stained with 7AAD this cell population represents 53.3% of total (3E; 3F).The SSC-H +, FSC-H + shows no significant differences at $p < 0.05$ compared to control. On the other hand no significant differences are observed in CD14Pe+7AAD+ at 10µg/mL compared to control (CN) which shows that there is some cytotoxicity in macrophages thus corresponds to the results of cytotoxicity percentage calculated for this population. It is known from literature that the forward light scatter versus side scatter 90th is a measure of cell size and cell granularity respectively, the Latter Being dependent upon the presence of intracellular structures that change the refractive index of light (McGowan P 2012). The number of labeled cells with 7AAD indicates a slight cytotoxicity G1 but the

actual calculation of cytotoxicity was 23.6%.Statistical analysis also confirms that there are slight cytotoxicity since no significant differences between the treated and control.

Figure 3 comes here

In **Table 6**, we show the average values of Mean fluorescence intensity (MFI) in SSC and/or FSC scattering mode, for all samples (Negative Control, DMSO, and CD14Pe phenotypic marker macrophages exposed to G1).for all concentration of product G1. MFI and cell count (Event count) in FSC scattering mode give an idea of cellular size, while the same parameters but in SSC scattering mode measure internal cellular damage (Gorczyca W 2011). The events average in the dose 10 µg/mL was 1034. Moreover, the respective averages of MIF in quadrant 2 (Q2) for FSC and SSC are 550.83 ± 103 and 313.83 ± 94.4 (see **Figure 3**). In conclusion it was observed events classified by size and granularity for this concentration.

At a given a concentration, each experiment was carried out two times (repeated two times) using different animals (three animals) and the measure obtained for each animal was replicated three times (see materials and methods). **Table 6** we show the averages of repetitions. We used the software STATISTICA for both means and ANOVA analysis (Hill and Lewicki 2006). In general, the results show not significant differences ($p \leq 0.05$) between the mean values of MFI for G1-treated samples at different concentrations (2 - 10 µg/mL) with respects to the negative control (NC) and DMSO groups. In particular, there are not significant differences between the mean values of MFI for G1-treated samples labeled with anti-CD14Pe and stained with 7AAD (living macrophages) with respects to both control groups (see **Table 6**). The ANOVA analysis was carry out applying Tukey's method. We confirm that there not significant differences for treated samples of living macrophages with respect to control groups. The numbers of cells are in a range between 500 and 1500 events in general. The Figure 4 shows significant differences between groups.

Table 6 and Figure 4 comes about here

In addition, CD14 PE was used as a macrophages marker in the presence of 7AAD; as described in the Materials and methods section. In total, 52.7% of macrophages were marked with CD14Pe and 7AAD. The MFI average was 32.55 ± 9.3 and 130.35 ± 29.4 respectively. It means that more than 45% of macrophage were still alive after treatment with G1 at the higher concentration c_{max} = 10 µg/mL. In **Figure 5**, we show two parameters CD14Pe (FL2) and 7AAD (FL3) of the population of macrophages at this concentration. These additional results are consistent with the previous paragraph.

Figure 5 comes here

Finally, EC50 calculations using different methodologies have been shown below. The results show that the best dose-response curve was the Five parameter logistic ($1/Y^2$) with an $R^2 = 0.956$. The Root Mean Square Error (RMSE = 0) and EC_{50} = 21.82. This study calculated the EC_{50} being observed that these values differ with relation to the methodology applied (**Tabla7**). Fitting nonlinear models to observed data is often complicated by non-constant or heterogeneous variability. Heterogeneous variability or *heteroscedasticity* occurs in most types of observed data. This is especially true for biochemical assays where concentration or dose is the predictor. The best curve fit is reached when the curve is pulled as close as possible to each data point without breaking the actual curve model. The nonlinear least square algorithm accomplishes this task. The nonlinear (or linear) least square algorithm assumes that all points have the same variability, so all points influence the curve fit equally (Manivannan and Prasanna 2005). The nature of the data entails a variation of the dependent variable that changes over the data is known as heteroscedasticity. Many methods of regression analysis is based on the assumption of equal variances, but MasterPlex ReaderFit software used to calculate the EC_{50} offers 4 different weighting algorithms to account for heteroscedasticity. The five parameter logist is the optimal model equation and weighting algorithm with different parameters (Root Mean Square Error (RMSE), R-Square, and Standard Deviation of % Recovery). One way to counterbalance no constant variability is to make them constant again. To accomplish this, weights are assigned to each standard sample data point.These weights are designed to approximate the way measurement errors are distributed. By applying weighting, points on the lower part of the curve are given more influence on the curve again. One of algorithms of assigning weights: is $1/Y^2$ – Minimizes residuals (errors) based on relative Mean Fluorescence Intensity and Relative light Unit, (MFI/RLU) values. Many functions have been tried as curve models for immunoassays, but few of them possess all of these properties. The need for a curve model that accommodates asymmetry has been necessitated by improvements in instrument and laboratory technology. The development

of sandwich assays led to dose–response curves that tend to be more asymmetric than earlier types of assays. Additionally, because of improvements in signal-to-noise ratios, asymmetry is an issue even for assays whose dose–response relationships are not as highly asymmetric. The reason for this is that even modest levels of lack-of-fit error caused by fitting mildly asymmetric data to a symmetric model can dominate the pure error due to random variation in low-noise modern assays. For symmetric immunoassay and bioassay data, it can be argued that no curve model has been as successful as the four-parameter logistic function. Despite its utility, the 4PL function is generally not an adequate curve model for much of the asymmetric response data commonly observed in immunoassay and bioassay applications. The five-parameter logistic function, which includes a fifth parameter, permits asymmetry to be effectively modeled (Shin KJ 2006). The formula for analysis is:

$$\left(\frac{1}{MFI_i^{\,2}}\right) = 47.85819 - \frac{130.509}{\left(1+\left(\left(\frac{c_i}{21.82}\right)^{-7.17925}\right)\right)^{0.51856}} \qquad (5)$$

In conclusion two of the highest concentrations showed some cytotoxicity but I note that the EC_{50} is above the concentrations used in our study. In general, the cytotoxicity EC_{50} values for each compound were lower after 24h exposure. The best method used for the analysis was the 5PL using ($1/Y^2$). The **Figure 6** displays MasterPlex program used to calculate the EC50. It observes where the parameters that makes up the formula of equation 5PL.

Figure 6 come here

This study evaluated the cytotoxicity by calculating the percentage of cytotoxicity and EC50. by Equation 5 PL. The best equation showed a R = 0.95. A comparison between the MFI of the groups treated with the negative control for parameters that reads the flow cytometer. The evaluated product showed slight cytotoxicity

3.2.3. Prediction of G1 cytotoxicity for other assays

In total we predicted 1,265 multiplexing assay endpoints for G1 biological activities. Notably the model predict very low probability (0.28) for G1 cytotoxicity (cutoff of TC_{50} < 100 μM) against human macrophages. The model also predicts only 7 positive endpoints for G1 out of 1,251 cytotoxicity assays (0.56% of probability of cytotoxicity in multiple assays), see **Table 8**. Interestingly, the predictive probability obtained for this compound in the cytotoxicity assay against WEHI cell line was 0.84. WEHI cell line is a biological model for leukemia and has been used to test anti-carcinogenic activity. (Lin CC 2011)

Several predictions were conducted in J774 macrophages cell line (170 assays). In all cases the model predicts low probability of G1 to present cytotoxicity effect against J774 macrophages. Macrophages are highly motile cells capable of chemotaxis and pathogen engulfment (Costa Lima S 2012). J774 and Raw 264.7 macrophage cell lines; which are well-established model systems in cell biology and immunology. The resistance of passive J774 cells to expansion of their surface areas is about one order of magnitude higher than that of human neutrophils (Lam J. 2009). The J774 has been used (Ganfon H 2012) to assess drugs anti-parasitic activity against diverse parasite species such as Plasmodium parasites, *Trypanosoma brucei brucei*, and *Leishmania mexicana mexicana*. Other research reported this cell line to assess anti-leishmanial activity of compounds against both the promastigote and intracellular amastigote stages of *Leishmania infantum* and *L. donovani* (Wert L 2011). This is of a great importance if we know that the G1 have been demonstrated experimentally to be active against bacteria and parasites (Marrero-Ponce Y 2004).

Table 8 comes about here

Some of these positive results in predictive tests included the evaluation of the cytotoxicity in RAW264.7 (Monocytic-macrophage leukemia cells) cell lines. The same **Table 8** shows that the G1 could inhibit with 89% of probability such cells in some specific assay conditions. However, the model predicts low probabilities of cytotoxicity in other assays using RAW264.7 cell. The RAW264.7 cell line was derived about 30 years ago from a tumor developing cells in a BAB/14 mouse, a BALB/c IgH congenic strain, inoculated with Abelson murine leukemia virus (MuLV), a defective transforming virus containing the v-abl tyrosine kinase oncogene, and replication-competent Moloney (Mo-MuLV) that served as helper virus (Raschke WC 1978) In addition, because of ease of cell propagation, high efficiency for DNA transfection, sensitivity to RNA interference,

possession of receptors for many relevant ligands, and other properties, RAW264.7 has been chosen by the Alliance for Cellular Signaling as the primary experimental system for their large-scale study of signaling pathways (Shin KJ 2006, Park HY 2012).

Appendix A. Supplementary data

Supplementary data to this article can be found online

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TABLES TO BE INSERTED IN THE TEXT

Table 1. Overall results of the classification model

Statistics	%	Sub-set	negative	positive
		Train		
Specificity	97.4	negative	3438	90
Sensitivity	85.6	positive	464	2755
Accuracy	91.8	total		
		CV		
Specificity	97.9	negative	1138	25
Sensitivity	85.0	positive	163	927
Accuracy	91.7	total		

Table 2. Some examples p(a) values for different assays

ID of a_u [i]	$p(a_u)$	n_1	n_{tot}	Cutoff	Relation	Type	Units	Assay Description
1002955	0.263	54	208	81814.3	>	K_i	nM	Inhibition of MMP12
964734	0.263	54	208	81814.3	<	IC_{50}	nM	Inhibition of MMP12
924957	0.263	54	208	81814.3	<	$\log(1/K_i)$		Inhibition of MMP12
970762	0.743	74	100	21281.7	<	IC50	nM	Inhibition of TGH
660813	0.711	68	96	2992.4	<	IC50	nM	Inhibitory activity against recombinant human Chemokine receptor type 3 (CCR3) expressed in chinese hamster ovary cells
1776768	0.821	77	94	55936.7	<	ID50	nM	Cytotoxicity against mouse J774 cells
1261026	0.821	77	94	55936.7	<	EC50	nM	Cytotoxicity against mouse J774 cells
940865	0.021	1	93	30.1	>	Inhibition	%	Inhibition of CCR1 at 10 uM
1674458	0.430	39	92	73346.2	<	TC50	uM	Cytotoxicity against mouse RAW264.7 cells after 24 hrs by MTT assay
1175699	0.430	39	92	73346.2	<	IC50	nM	Cytotoxicity against mouse RAW264.7 cells after 24 hrs by MTT assay
1657211	0.430	39	92	73346.2	<	IC50	ug mL-1	Cytotoxicity against mouse RAW264.7 cells after 24 hrs by MTT assay
860201	0.224	16	75	1961.4	>	Ki	nM	Inhibition of CSF1R
1025517	0.224	16	75	1961.4	<	IC50	nM	Inhibition of CSF1R
1664436	0.413	30	74	12.7	>	Inhibition	%	Inhibition of mouse recombinant iNOS at 1 mM after 40 mins by colorimetric assay
867926	0.840	62	74	229.4	<	IC50	nM	Inhibition of LPS-induced TNFalpha production in human monocytes
1285558	0.222	15	71	178344.1	>	Ki	nM	Inhibition of mouse recombinant iNOS
957262	0.222	15	71	178344.1	<	IC50	nM	Inhibition of mouse recombinant iNOS
921708	0.130	8	68	2046.7	>	Selectivity ratio		Inhibition of cFms
998565	0.130	8	68	2046.7	<	IC50	nM	Inhibition of cFMS

[i] ChEMBL ID for the assay a_u

Table 3. Values of $<\mu^{i}_{5}(t_e)>$ for all molecular or cellular targets studied

t_e	Target name	$<\mu^{i}_{5}(t_e)>$	n_1	n_{total}
1	RAW264.7 (Monocytic-macrophage leukemia cells)	20060.3	1630	3376
2	C-C chemokine receptor type 3	23855.95	700	1185
3	J774 (Macrophage cells)	16309.1	601	1001
4	Cyclooxygenase-2	14102.64	558	1061
5	C-C chemokine receptor type 1	20864.24	440	825
6	Nitric oxide synthase, inducible	13168.54	420	1082
7	J774.A1 (Macrophage cells)	22090.65	375	694
8	MCSFreceptor	16919.75	343	752
9	Matrix metalloproteinase 12	13775.03	280	566
10	Acyl coenzyme A:cholesterol acyltransferase	12571.06	271	486
11	Macrophage migration inhibitory factor	11088.6	257	461
12	Macrophage-stimulating protein receptor	16863.76	74	159
13	Monocytes	12711.64	68	84
14	Dipeptidyl peptidase IV	16785.55	50	116
15	EL4 (Thymoma cells)	24952.45	48	128
16	Interleukin-8	15053.32	40	107
17	Interleukin-5	21910.88	28	74
18	C-C motif chemokine 5	32323.55	27	34
19	Macrophage colony-stimulating factor 1 receptor	25464.67	21	29
20	RAC-alpha serine/threonine-protein kinase	13962.93	12	38
21	Serine/threonine-protein kinase TAO3	21791.05	12	26
22	PMNL (Polymorphonuclear leukocytes)	18763.72	12	15
23	Macrophages	42504.04	9	24
24	Monocytes (Monocytic cells)	18649.45	7	15
25	Scavenger receptor type A	48625.99	6	21
26	eosinophils (Eosinophils)	15608.45	6	11
27	WEHI (Macrophages)	13590.42	5	8
28	Human macrophage cell line	16607.23	4	6
29	EOL1 (Eosinophilic cells)	8907.19	2	6
30	Granulocyte colony stimulating factor receptor	23935.49	1	2
31	Macrophage scavenger receptor types I and II	11803.07	1	2
32	Macrophage metalloelastase	16403.66	1	2

Table 4. Values of $<\mu^{i}_{5}(s)>$ for different standard type measures of biological activity

s_x	Standard Type	$<\mu^{i}_{5}(s)>$	n_1	n_{tot}	s_x	Standard Type	$<\mu^{i}_{5}(s)>$	n_1	n_{tot}
1	IC_{50}	960.45	3641	6070	23	Ratio EC_{50}	494.89	8	23
2	Inhibition	810.03	809	1997	24	TC_{50}	1203.74	7	15
3	Activity	892.48	721	1615	25	Ratio CC_{50}/IC_{50}	734.58	7	12
4	K_i	748.39	355	1045	26	NO formation	318.8	6	19
5	EC_{50}	1123.73	176	218	27	TD_{50}	619.19	6	11
6	CC_{50}	964.87	143	205	28	Ratio IC_{50}	955.38	5	34
7	Selectivity	761.4	83	240	29	Emax	1299.51	4	10
8	ED_{50}	1208.73	42	75	30	LD_{50}	809.01	4	5
9	ID_{50}	727.11	39	67	31	Count	390.21	4	6
10	K_d	1016.32	37	92	32	Initial rates	354.7	4	12
11	Ratio	743.76	21	92	33	SI	811.54	4	12
12	GI_{50}	607.89	19	60	34	$MNTD_{70}$	557.23	3	12
13	Efficacy	994.28	16	33	35	Specific activity	1051.26	3	6
14	K_m	759.45	15	57	36	Selectivity index	772.11	3	6
15	Selectivity ratio	869.72	12	41	37	k_{cat}	501.13	2	11
16	FC	3483.18	12	20	38	IC_{90}	1421.46	2	3
17	NOHA	271.55	12	37	39	RBA	1078.46	2	7
18	$MNTD_{90}$	524.19	10	12	40	Ratio K_i	970.76	2	3
19	Fold change	558.31	10	35	41	K_b	1214.19	1	3
20	Residual activity	1167.52	9	18	42	pIC_{50}	809.49	1	1
21	LC_{50}	841.72	8	22	43	K_{inact}	423.27	1	2
22	Survival	642.2	8	18	44	Cytotoxicity	366.49	1	3

n_1=number of active(C=1) cases for standart type ,n ($_{Total)}$= Total cases for standart type

Table 5. Results of the study of Domain of Applicability (DA) for the model

Endpoints Sub-set[a]	DA count	Total Sub-set count	DA %
Train	6481	6747	96.1
CV	2164	2253	96.0
Positive effect	4134	4309	95.9
Negative effect	4511	4691	96.2
Cytotoxicity	1174	1251	93.8
Human	3405	3506	97.1
Mouse	5234	5485	95.4
IC_{50}	4313	4494	96.0
EC_{50}	166	180	92.2
All	8645	9000	96.1

[a] Positive effect indicates that C = 1, this sub-set includes all cytotoxicity endpoints together with other biological effects.

Table 6. Effect on cytotoxicity for G1-treated samples at different concentrations *vs.* control groups

Group 1 Conc of G1 µg/mL	Macrophage Cytometry Parameter	Group 2							
		NC				DMSO			
		$Mean_1$	$Mean_2$	t	p	$Mean_1$	$Mean_2$	t	p
10	MFI	502.9	548.8	-0.57	0.59	502.9	480.1	0.35	0.73
	MFI SSC	416.0	432.0	-0.11	0.92	416.0	426.0	-0.10	0.92
	MFI SFC	669.2	665.5	0.09	0.93	669.2	604.8	1.44	0.18
	Anti-CD14PE	38.6	21.2	2.06	0.08	38.6	29.9	1.94	0.08
	7AAD	157.7	166.0	-0.18	0.86	157.7	174.8	-0.55	0.59
	Anti-CD14PE + 7AAD	91.5	93.6	-0.07	**0.95**	91.5	108.1	-0.99	0.35
8	MFI	516.9	548.8	-0.44	0.68	516.9	480.1	0.60	0.56
	MFI SSC	409.0	432.0	-0.17	0.87	409.0	426.0	-0.18	0.86
	MFI SFC	688.5	665.5	0.76	0.48	688.5	604.8	1.99	0.07
	Anti-CD14PE	41.2	21.2	2.65	0.04	41.2	29.9	2.82	0.02
	7AAD	153.2	166.0	-0.33	0.75	153.2	174.8	-0.78	0.45
	Anti-CD14PE + 7AAD	89.5	93.6	-0.17	0.87	89.5	108.1	-1.31	0.22
6	MFI	537.7	548.8	-0.14	0.89	537.7	480.1	0.89	0.39
	MFI SSC	444.0	432.0	0.08	0.94	444.0	426.0	0.17	0.86
	MFI SFC	705.3	665.5	0.84	0.43	705.3	604.8	2.16	0.06
	Anti-CD14PE	38.9	21.2	2.27	0.06	38.9	29.9	2.16	0.06
	7AAD	175.2	166.0	0.19	0.85	175.2	174.8	0.01	0.99
	Anti-CD14PE + 7AAD	116.2	93.6	0.85	0.43	116.2	108.1	0.53	0.61
4	MFI	498.8	548.8	-0.69	0.52	498.8	480.1	0.30	0.77
	MFI SSC	474.2	432.0	0.30	0.78	474.2	426.0	0.49	0.63
	MFI SFC	594.5	665.5	-0.56	0.60	594.5	604.8	-0.13	0.90
	Anti-CD14PE	42.8	21.2	2.40	0.05	42.8	29.9	2.67	0.02
	7AAD	214.5	166.0	0.89	0.41	214.5	174.8	1.14	0.28
	Anti-CD14PE + 7AAD	138.7	93.6	1.25	0.26	138.7	108.1	1.50	0.17
2	MFI	497.2	548.8	-0.69	0.52	497.2	480.1	0.27	0.79
	MFI SSC	398.5	432.0	-0.25	0.81	398.5	426.0	-0.29	0.78
	MFI SFC	668.2	665.5	0.08	0.94	668.2	604.8	1.48	0.17
	Anti-CD14PE	40.7	21.2	2.41	0.05	40.7	29.9	2.50	0.03
	7AAD	199.7	166.0	0.80	0.45	199.7	174.8	0.85	0.41
	Anti-CD14PE + 7AAD	124.8	93.6	1.18	0.28	124.8	108.1	1.09	0.30

$Mean_1$=mean group 1; mean $_2$= mean group2

Table 7. Results of Dosis *vs.* Effect EC_{50} curve fitting by different algorithms

Curve Fitting	R2	RMSE	a	b	c	d	e
5PL ($1/Y^2$) *	0.9588	0.0000	47.85819	-7.1792.	21.8259	130.5090	0.5185
5PL (1/Y)	0.1833	5.8115	47.7578	-12.029	18.2210	-76.5320	0.3032
4PL (1/Y)	0.9583	1.2316	-214.1114	-3.7650	25.8656	47.8645	
Log-Log	0.6222	2.3871	0.0948	1.1722			
Quadratic (1/Y)	0.9526	0.9381	-0.1505	0.9424	46.5202		
Linear (1/Y)	0.8154	1.4961	-0.8616	50.7310			

* Best fit model, 5PL is Five Parameters Logistic, 4PL is Five Parameters Logistic

Table 8. Theoretic-experimental determination of some endpoints for G1 cytotoxicity in multiplexing assays

C	p(1)	Type	Rel	Cutoff	Units	Assay Description b
Endpoints for G1 cytotoxicity experimentally determined in this work						
0	0	EC_{50}	<	21.58/10	μg	7ADD mouse peritoneal macrophages after 24h
0	0	Cytotoxicity	>	23.6 / 50	%	7ADD mouse peritoneal macrophages after 24 hrs at 10 μg/mL
0	0	Cytotoxicity	>	19.4 / 50	%	7ADD mouse peritoneal macrophages after 24 hrs at 8 μg/mL
0	0	Cytotoxicity	>	16.5 / 50	%	7ADD mouse peritoneal macrophages after 24 hrs at 6 μg/mL
0	0	Cytotoxicity	>	9.6 / 50	%	7ADD mouse peritoneal macrophages after 24 hrs at 4 μg/mL
0	0	Cytotoxicity	>	9.9 / 50	%	7ADD mouse peritoneal macrophages after 24 hrs at 2 μg/mL
0	0	MFI	<	p < 0.05	%	7ADD mouse peritoneal macrophages after 24 hrs at 10 μg/mL
0	0	MFI	<	p < 0.05	%	7ADD mouse peritoneal macrophages after 24 hrs at 8 μg/mL
0	0	MFI	<	p < 0.05	%	7ADD mouse peritoneal macrophages after 24 hrs at 6 μg/mL
0	0	MFI	<	p < 0.05	%	7ADD mouse peritoneal macrophages after 24 hrs at 4 μg/mL
0	0	MFI	<	p < 0.05	%	7ADD mouse peritoneal macrophages after 24 hrs at 2 μg/mL
Predicted multiplexing endpoints for G1 cytotoxicity						
0	**0.28**	TC_{50}	<	100	μM	Cytotoxicity against human macrophages
1	**0.84**	ED_{50}	<	11.4	μM	CAM WEHI cell line by MTT assay
1	**0.63**	IC_{50}	<	33.4	μM	Cytotoxicity against WEHI cell lines.
1	**0.72**	EC_{50}	<	164.7	uM	CAM RAW264.7 cells (MMLC) assessed as cell survival after
1	**0.69**	EC_{50}	<	23.83	ug ml-1	CAM RAW264.7 cells (MMLC) by MTT colorimetric assay in
1	**0.89**	IC_{90}	<	13.1	μM	Cytotoxicity against rat RAW264.7 cells by MTT assay
1	**0.78**	EC_{50}	<	10.2	μM	In vitro cytotoxicity against J774.2 cells after 72 h incubation.
1	**0.60**	IC_{50}	<	26.7	μM	CAM J774 cells expressing RANKL signaling by Alamar blue
1	**0.63**	IC_{50}	<	143.8	μM	Cytotoxicity against J774.1 cell line after 48 hrs
1	**0.74**	EC_{50}	<	9.19	uM	Cytotoxicity against macrophage cell line (J774)
0	0.33	CC_{50}	<	133.23	μM	CAM RAW264.7 cells after 48 hrs by MTT assay
0	0.37	CC_{50}	<	28.87	uM	CAM J774A1 cells assessed as cell viability after 72 hrs by
0	0.37	CC_{50}	<	50	ug.ml-1	Cytotoxicity against human J774A1
0	0.40	CC_{50}	<	79.03	ug.ml-1	CAM J774A1 cells after 48 hrs by Geimsa staining method
0	0.44	CC_{50}	<	76.79	ug ml-1	CAM J774A1 cells after 24 to 72 hrs by MTT assay
0	0.44	CC_{50}	<	57.64	ug ml-1	CAM J774A1 cells after 72 hrs by MTT assay
0	0.45	CC_{50}	<	42.49	ug ml-1	CAM J774A1 cells
0	0.46	CC_{50}	<	43.1	ug ml-1	CAM J774A1 cells by MTT assay
0	0.39	IC_{50}	<	90.93	uM	Cytotoxicity against LPS-stimulated mouse RAW264.7 cells
0	0.40	IC_{50}	<	68.76	ug ml-1	CAM J774A1 by MTT assay
0	0.42	IC_{50}	<	13.34	ug ml-1	CAM J774A1 cells by rapid colorimetric assay
0	0.42	IC_{50}	<	236.7	uM	Cytotoxicity against LPS-stimulated mouse RAW264.7 cells
0	0.42	IC_{50}	<	30.94	uM	Cytotoxicity in mouse RAW264.7 cells
0	0.43	IC_{50}	<	6.76	ug ml-1	*In vitro* cytotoxicity of compound against EL4. mouse
0	0.43	IC_{50}	<	73.35	uM	CAM RAW264.7 cells after 24 hrs by MTT assay
0	0.43	IC_{50}	<	231.62	uM	CAM J774 cells after 24 hrs by by resazurin reduction test
0	0.44	IC_{50}	<	23.8	ug ml-1	CAM J774A1 cells after 72 hrs by cell-titer assay
0	0.44	IC_{50}	<	48.22	uM	CAM RAW264.7 cells assessed as cell viability after 24 hrs by
0	0.45	IC_{50}	<	103.9	uM	Cytotoxicity against LPS-stimulated mouse RAW264.7 cells
0	0.45	IC_{50}	<	10	uM	CAM RAW264.7 cells assessed as cell viability after 24 hrs by
0	0.45	IC_{50}	<	6.3	ug ml-1	Cytotoxicity against human EL4 cells
0	0.46	IC_{50}	<	57	uM	CAM RAW264.7 cells assessed as reduction in cell viability
0	0.46	IC_{50}	<	175	ug ml-1	CAM J774A1 cells after 24 hrs by trypan blue exclusion assay
0	0.46	IC_{50}	<	42.72	uM	CAM RAW264.7 cells by MTT assay
0	0.47	IC_{50}	<	207.5	uM	CAM macrophage RAW264.7 cells after 48 hrs by MTT assay
0	0.49	IC_{50}	<	12.17	ug ml-1	CAM RAW264.7 cells after 2 days by MTT assay
0	0.50	IC_{50}	<	27.5	uM	CAM RAW264.7 cells after 72 hrs by resazurin assay

0	0.25	Inhibition	>	17.3	%	CAM J774A1 cells assessed as reduction in metabolic activity
0	0.37	LC_{50}	<	12.28	ug ml-1	CAM RAW264.7 cells
0	0.09	$MNTD_{70}$	<	88.51	uM	CAM RAW264.7 cells assessed as maximum non-toxic dose
0	0.12	Survival	>	68.14	%	CAM RAW264.7 cells assessed as cell survival rate at 10 uM
0	0.12	Survival	>	94	%	CAM RAW264.7 cells at 21 uM
0	0.33	Activity	>	100	%	CAM J774 macrophage assessed as cell viability at 1 ug/mL
0	0.35	Activity	>	65.3	%	CAM J774A1 cells assessed as macrophage number at 40 to 60
0	0.35	Activity	>	64.3	%	CAM J774A1 cells assessed as macrophage number at 2.3
0	0.35	Activity	>	23.27	%	CAM J744A.1 cells at 0.5 ug/mL by MTT assay
0	0.35	Activity	>	99.5	%	CAM J774A1 cells assessed as macrophage number at 4 ug/ml
0	0.35	Activity	>	5	%	CAM J774A1 cells assessed as dead cells at 10 uM by Sytox
0	0.35	Activity	>	94	%	CAM J774A1 cells assessed as live viable cells at 10 uM by
0	0.35	Activity	>	93	%	CAM J774A1 cells assessed as live cells at 50 uM by calcein
0	0.35	Activity	>	58.9	%	CAM J774A1 cells assessed as live cells at 100 uM by calcein
0	0.35	Activity	>	94.4	%	CAM J774A1 cells assessed as macrophage number at 10.5
0	0.35	Activity	>	73.4	%	CAM J774A1 cells assessed as macrophage number at 3 ug/ml
0	0.35	Activity	>	16	%	CAM J744A.1 cell assessed as survival rate at 10 uM
0	0.35	Activity	>	12.3	%	CAM J774A1 cells assessed as macrophage number at 3.2
0	0.35	Activity	>	55.9	%	CAM J774A1 cells assessed as macrophage number at 1.7
0	0.35	Activity	>	50	%	CAM J774 macrophage at 40 uM
0	0.36	Activity	>	100	%	Cytotoxicity against murine J774 cells (MC) assessed as cell
0	0.36	Activity	>	100	%	Cytotoxicity against murine J774 cells assessed as cell viability
0	0.36	Activity	>	100	%	Cytotoxicity against murine J774 cells assessed as cell viability
0	0.39	Activity	>	11.35	%	CAM J744A.1 cells at 0.005 ug/mL by MTT assay
0	0.39	Activity	>	12.35	%	CAM J744A.1 cells at 0.05 ug/mL by MTT assay
0	0.41	Activity	>	70.83	%	CAM J744A.1 cell assessed as survival rate
0	0.43	Activity	>	0.95	%	CAM J774 cells at 100 uM relative to 5-
0	0.43	Activity	>	42.21	%	Unspecific cytotoxicity against murine J774 macrophages at
0	0.43	Activity	>	41.5	%	CAM J774 cells assessed as cell viability at 100 ug/ml by MTT
0	0.44	Activity	>	50	%	CAM J774 macrophage at 400 uM
0	0.44	Activity	>	88.2	%	CAM J774 cells infected with Mycobacterium bovis BCG
0	0.44	Activity	>	0.76	%	CAM J774 macrophages at 2.1 uM after 24 hrs by resazurin
0	0.44	Activity	>	4.89	%	CAM J774 macrophages at 8.6 uM after 24 hrs by resazurin
0	0.44	Activity	>	18.88	%	CAM J774 macrophages at 21.7 uM after 24 hrs by resazurin
0	0.44	Activity	>	51.8	%	CAM J774 cells at 400 uM after 48 hrs by MTT assay
0	0.44	Activity	>	2.11	%	CAM J774 macrophages at 4.3 uM after 24 hrs by resazurin
0	0.45	Activity	>	51.17	%	CAM J774 cells assessed as cell viability at 100 ug/mL after 24
0	0.45	Activity	>	95.34	%	CAM RAW264.7 cells assessed as cell viability at 1 uM after
0	0.45	Activity	>	48.86	%	CAM J774 cells assessed as cell viability at 10 ug/ml by MTT
0	0.46	Activity	>	95.42	%	CAM RAW264.7 cells assessed as cell viability at 100 uM after
0	0.46	Activity	>	75.95	%	Cytotoxicity against Mycobacterium bovis Bacillus Calmette-
0	0.47	Activity	>	81.68	%	Cytotoxicity against Mycobacterium bovis Bacillus Calmette-
0	0.47	Activity	>	89	%	Cytotoxicity against Mycobacterium bovis Bacillus Calmette-
0	0.47	Activity	>	93.57	%	CAM J774 cells assessed as cell viability at 1 ug/ml by MTT
0	0.33	IC_{50}	<	6	uM	Cytotoxicity against EL-4 cell line (mouse thymoma cells)
0	0.39	IC_{50}	<	21.41	uM	Inhibition of Bacillus anthracis lethal toxin-induced
1	0.54	IC_{50}	<	221.07	uM	CAM J774 cells after 48 hrs by MTT assay
1	0.54	IC_{50}	<	2256.3	uM	CAM J774 cells after 24 hrs by resazurin assay
1	0.54	IC_{50}	<	565.75	ug ml-1	CAM J774 cells after 24 hrs by MTT assay
1	0.55	IC_{50}	<	508.6	uM	CAM RAW264.7 cells assessed as cell viability after 4 hrs by
1	0.56	IC_{50}	<	25.51	uM	In vitro CAM J774 macrophages.
1	0.56	IC_{50}	<	736.2	uM	CAM J774 cells assessed as cell viability after 48 hrs by alamar

1	0.57	IC_{50}	<	72.79	uM	CAM J774 macrophages after 48 hrs by MTT assay
1	0.58	IC_{50}	<	0.24	uM	Inhibitory concentration required for cytotoxicity in J774.2
1	0.58	IC_{50}	<	416.67	ug ml-1	CAM J774 cells after 24 hrs

[a] Cutoff used was the threshold value recommended by REACH for this assay (in experimental outcomes) or the average value for all compounds in ChEMBL for this assay (in predicted outcomes). The J774 cell lines are Macrophage Cells (MC) and RAW264.7 is a murine macrophage-like cells (MMLC). CAM is Cytotoxicity Against Macrophage.

Table 9. Theoretic prediction of some endpoints for G1 interaction with human protein targets

Target name	ID	p(1)	Res.	Lev.	Type	Rel	Cutoff	Units
C-C chemokine receptor type 3	3473	0.78	-0.63	0.002	E_{max}	>	56.25	%
C-C chemokine receptor type 3	3473	0.63	-0.53	0.002	K_b	>	0.01	uM
C-C chemokine receptor type 1	2413	0.71	-0.58	0.001	ED_{50}	<	0.00.1	uM
Matrix metalloproteinase 12	4393	0.66	-0.55	0.001	IC_{50}	<	5.8	uM
Interleukin-8	2157	0.64	-0.54	0.000	IC_{50}	<	5.3	uM
MSR I and II	5811	0.63	-0.53	0.001	IC_{50}	<	38.3	μM
Acyl-CoA: cholesterol Acyltransferase	2265	0.64	-0.54	0.001	IC_{50}	<	21.3	μM
MSPR	2689	0.62	-0.52	0.001	Activity	>	78.67	%
MSPR	2689	0.60	-0.52	0.000	K_d	>	9.74	uM
MCSFreceptor	1844	0.62	-0.52	0.001	Activity	>	100.33	%
MCSFreceptor	1844	0.61	-0.52	0.000	IC_{50}	<	1.38	μM
MMIF	2085	0.70	-0.57	0.001	IC_{50}	<	65.1	μM
MMIF	2085	0.60	-0.51	0.001	Activity	>	36	%

[a] MMIF is Macrophage Migration Inhibitory Factor, MCSF is Macrophage Colony Stimulating Factor, MSPR is Macrophage-Stimulating Protein Receptor, MSR is Macrophage Scavenger Receptor I and II

FIGURES TO BE INSERTED IN THE TEXT

Figure 1. Analysis of the Domain of Application of the model

Figure 2. Dose-Response of cytotoxicity in Balb/C mouse peritoneal macrophages marked with CD14Pe/7AAD exposed to different concentrations of G1

Figure 3. Pseudo-color smooth projection of MFI values over FSC *vs.* SSC plot after administration of G1 at cmax. Total cell populations (A) or Macrophages population (C.B.D.E.F)

Figure 4. Effect of G1 on Balb/C mouse macrophages culture primary and were exposed to different concentrations ((10, 8, 6, 4, and 2 µg/mL) for a period of 24 hours The results are expressed as Mean Intensity Fluorescence (MFI) of control values N = 6 animals per group and 1.106 Cell; NS= Not Statistically significant differences $\boldsymbol{p} \leq 0.05$ for the same groups. Dark Green=macrophages labeled with CD14PE and stained with 7AAD (Dead), Dark Purple = macrophages labeled CD14 PE (live)

Figure 5. Results of flow cytometry for Balb/C mouse peritoneal macrophages exposed to G1 at 10 µg/mL

Figure 6. Masterplex interface illustrating MFI *vs.* conc. effect of G1 in Balb/c mouse peritoneal macrophages **(A)** and Sigmoidal curve representative of the 5PL model **(B)**.