Arh. farm. 2021; 71: 257 – 278 *Review article/Pregledni rad*

In silico toxicology methods in drug safety assessment

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Abstract

While experimental animal investigation has historically been the most conventional approach conducted to assess drug safety and is currently considered the main method for determining drug toxicity, these studies are constricted by cost, time, and ethical approvals. Over the last 20 years, there have been significant advances in computational sciences and computer data processing, while knowledge of alternative techniques and their application has developed into a valuable skill in toxicology. Thus, the application of *in silico* methods in drug safety assessment is constantly increasing. They are very complex and are grounded on accumulated knowledge from toxicology, bioinformatics, biochemistry, statistics, mathematics, as well as molecular biology. This review will summarize current state-of-the-art scientific data on the use of *in silico* methods in toxicity testing, taking into account their shortcomings, and highlighting the strategies that should deliver consistent results, while covering the applications of *in silico* methods in preclinical trials and drug impurities toxicity testing.

Keywords*:* software, databases, drug preclinical trials, drug impurities, safety assessment

doi.org/10.5937/arhfarm71-32966

Introduction

Animal studies have historically been the most conventional approach conducted to assess drug toxicity and are currently considered the main method for assessment of possible toxic effects of drug candidates (1, 2). Yet these studies are constricted by cost, time, and ethical considerations (1). Nevertheless, as stated by the U.S. Environmental Protection Agency (US EPA), around 20,000 to 100,000 animal study requests are submitted annually, including species like mice, rats, rabbits, guinea pigs, dogs etc. (3). In order to predict nine different hazard classifications, traditional testing uses up to 57% of the total animals in safety testing in Europe, or almost 600,000 animals annually (4). In accordance with Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) directive and the European Chemicals Agency (ECHA) objective to promote non-animal testing methods and other alternatives, experiments on vertebrates are only allowed as a last resort. Usually, *in vitro* tests are also conducted in order to assess drug safety, together with a range of toxicities and adverse drug effects. Recently, the development of *in vitro* models such as "organ on a chip" has become one of the major aims to reduce the overall cost of analysis (5). Nevertheless, these approaches are time-consuming and still not developed enough. With the aim of further reducing animal testing by 2035, the US EPA recommended *in silico* modeling for assessing various toxicity end-points of the tested compounds, particularly drug candidates (3), suggesting the implementation of various computational approaches that are able to simulate, visualize, analyze, or even predict toxicity (6). It is important to keep in mind that *in silico* models are usually extremely complicated and are grounded on accumulated knowledge from mathematics, bioinformatics, statistics, biochemistry, as well as molecular biology (7, 8).

In this review, we will present the current state-of-the-art *in silico* methods used in toxicity testing for drug safety assessment, taking into account their shortcomings, and presenting strategies that should be able to offer consistent results.

Historical overview: the need for *in silico* methods in toxicology

Over the last 20 years, advances in bioinformatics, virtual reality, and internet have occurred, while expertise in alternative techniques and their application in toxicology has become a useful skill for every modern toxicologist (9). Moreover, the advances in alternative methods to experiments on animals have led to excessive replacement and modifications of traditional toxicology tests (6). Alternative methods are methods established in accordance with the '3R' principle first defined by W.M.S. Russell and R.L. Burch in "The Principles of Humane Experimental Techniques" in 1959. This principle aims to: 1) "reduce" (refers to the number of animals necessary in a test), 2) "refine" (refers to making sure that toxicology procedures are less painful or stressful to experimental animals), or, 3) "replace" (meaning that, whenever possible, animals should be substituted with non-animal techniques such as *in vitro*, *ex-vivo*, and/or *in silico* systems) (10). Furthermore, regulatory frameworks, such as the seventh amendment to the EU Cosmetics Directive (11), foresees the elimination of *in vivo* testing for cosmetic

products, whereas the marketing ban has been in effect since March 2009. This directive encourages the use of alternative methods for the prediction of human health effects caused by cosmetic ingredients, including, but not limited to, skin sensitization, carcinogenicity, and developmental toxicity (11).

In 2006, with the revision of EU regulatory framework regarding registration, evaluation, authorization, and restriction of chemicals (REACH) (12), the necessity of alternative testing methods emerged in order to reduce the number of animals used in *in vivo* experiments. The REACH regulation covers non-testing methods for "predictive toxicology", risk assessment, and evaluation of safety endpoints of commercially available chemicals in the EU. Even though this revision contains only a partial set of *in silico* methods, the Guidance on information requirements and chemical safety assessment issued by the ECHA provides comprehensive background information and recommendations for the use of computational, non-animal methods and grouping of chemicals (13). Chemicals that could be examined by new, alternative approaches include not only human pharmaceuticals, but also food ingredients, environmental agents and other substances humans are constantly exposed to (14). Hence, many researchers predict a future in which almost all routine toxicity testing would be conducted not only *in vitro*, on human cells or cell lines, but also by *in silico* methods, which would save time and resources, while appreciating the '3R' principle. Thus, the development of new and improvement of existing computational tests, various *in silico* methods and models, should be highly beneficial not only for toxicologists, but also for the global scientific community.

The development and discovery of a new drug is a long, expensive and interdisciplinary process, while advances in technology and hardware solutions have enabled *in silico* methods to lead to the optimization of this process. Computer toxicology is widely used today in the development of chemicals, and provides valuable information in the drug discovery process. All of this shortens the time required for a drug to be released to the market, reduces the number of animal experiments, enables strategic planning of the development of new pharmaceutical and chemical products, and is supported by regulatory frameworks, including REACH (15).

The use of *in silico* methods in preclinical drug toxicity assessment

Undesirable bioavailability of drugs due to improper pharmacokinetic and pharmacodynamic properties, followed by the undesirable safety, mainly caused by the absorption, distribution, metabolism, excretion, and toxicity characteristics, are the main causes of excessive failure rate in the process of discovering new drugs (16). For a drug to proceed from lab to clinics, a minimum of 12–15 years of development cycle is necessary. Prior to that, the product's performance, stability, effectiveness, and safety are assessed by conducting preclinical testing (7). Preclinical studies are conducted by *in vitro*, *in vivo*, *ex vivo*, and *in silico* methods, while following GLP/GSP guidelines (good laboratory practice/good scientific practices), to obtain essential data regarding the safety and biological efficacy of a drug candidate before it is tested in the final target population

- humans (7). Approximately, drug candidates which are being subjected to clinical trials have merely an 8% likelihood of being put on the market, while about 20% of failures in the later stages of drug development happen because of the occurrences of toxicities (2). *In silico* predictive methods are aimed at rationalizing the preclinical drug development, while enabling the reduction of not only animal experiments, but also associated time and costs (2). Toxicity predictions may range from predictions of various toxic endpoints (e.g. acute toxicity or carcinogenicity) to predictions of the basic mechanisms of toxicity (defining the targets implicated in adverse drug reactions, as well as their toxic effects) (2). Some of the most often applied databases and software in pre-clinical studies are described below.

ADME software in preclinical studies

In early preclinical drug development, the prediction of ADME properties (i.e. absorption, distribution, metabolism, and excretion) is of value not only for drug-like effects, but also for toxicity assessment (17). In literature, while ADMET is used as a comprehensive term which integrates ADME and toxicity predictions (T), quantitative structure-activity relationship (QSAR) methods and related approaches have been used to investigate the molecular features that influence these processes (18). A significant advance has been made in recent years in the area of structure-based *in silico* modeling of ADMET properties, accompanied by the release of a huge variety of commercial and freely available *in silico* prediction tools. While dynamic modeling of toxicokinetics is frequently conducted to find appropriate explanations behind the adverse events in preclinical regulatory toxicology, it is, likewise, progressively being applied together with predictive toxicology (19). An expert workgroup considered the contribution of kinetics as a part of animal-free systemic toxicity testing (20). It has been concluded that *in silico* kinetics testing cannot be considered alone, but should be viewed as a useful tool for screening and prioritization and integrated with *in vitro* and *in vivo* tests. Additionally, *in silico* approaches that are currently in use need further optimization in the light of more quality control in data collection (19, 20). Furthermore, accurate prediction of ADMET parameters depends on choosing an appropriate modeling method, molecular descriptors of ADMET endpoints, and extensive experimental data sets associated with these endpoints (16).

Some of the software which could be used for this purpose include ADME-Tox, ADMETlab, admetSAR, vNN-ADMET, etc. The use of the ADME-Tox software, licensed by ACD/Lab, covers not only pharmacokinetic and metabolic, but also toxicological issues connected with the disposition and fate of drugs, with a well-recognized role in assessing preclinical toxicity of new drugs (17). Another web-platform which covers not only physicochemical properties and ADME processed, but also toxicity, is ADMETlab, licensed under a Creative Commons Attribution-Noncommercial License. It is grounded on the wide-ranging database of around 288,967 chemicals and 31 optimized QSAR models, which contains 27 toxicity endpoints and 8 toxicophore rules (751 substructures) (3, 21). This platform was recently expanded with the addition

of the Toxicology in the 21st Century (Tox21) dataset to include biological target-based pathways. The software now contains data about twelve different biological targets which belong to the nuclear receptor pathway and the stress response pathway as the two main groups. In addition, eight other toxicophore rules were added to this segment, such as environmental, human and comprehensive toxicity (21). Another comprehensive, prediction web-based tool for ADMET predictions is admetSAR, built by a team from the Shanghai Key Laboratory of New Drug Design, accessible free of charge at http://lmmd.ecust.edu.cn/admetsar2. It has the ability to predict not only 50 significant ADMET endpoints, but also multiple ecotoxicity endpoints by using QSAR models (3). AdmetSAR includes data on properties such as CYP450 substrates and inhibition, skin sensitivity, drug-induced liver injury, acute toxicity on rats, mutagenicity, carcinogens, reproductive toxicity, biodegradability, bioconcentration factors, etc., while detailed biological endpoints stored in this database include IC50 (median inhibitory concentration), LC50 (median lethal concentration), LD50 (median lethal dose), TD50 (median toxic dose), etc. (22). The vNN-ADMET is a publicly accessible online platform created in conjunction with the Telemedicine and Advanced Technology Research Center (TATRC) and US Army Medical Research and Development Command (USAMRDC). This platform is capable of predicting 15 ADMET properties, such as chemical mutagenicity (AMES Test), cytotoxicity, cardiotoxicity, drug-induced liver injury, MMP Disruption (mitochondrial toxicity), and drug-drug interactions (3, 23). The models in this software are constructed according to the variable nearest neighbor (vNN) methodology. The vNN method analyses the similarity structural distance between compounds and forms a distance threshold on which predictions are based (23).

In silico methods for predicting acute and repeated-dose toxicity

Acute toxicity is most often represented by the 'median lethal dose' (LD50), a statistically derived dose at which 50% of the animals will be expected to die in the period of 24 hours (24). However, LD50 is considered difficult to predict because of its complexity, having in mind that it depends on the variability of the biological mechanisms (24). Nevertheless, a number of commercial and freely available software provide options for LD50 predictions, such as ACD/LABS ToxSuite, US EPA Toxicity Estimation Software Tool (T.E.S.T.), Accelrys TOPKAT, ProTox, etc.

ToxSuite (from ACD/Labs) generates potential LD50 in mice and rats for different exposure routes, such as oral, intraperitoneal, intravenous, and subcutaneous. This software was built using experimental data for more than 100,000 compounds present in the US Registry of Toxic Effects of Chemical Substances (RTECS) and the European Chemical Substances Information System (ESIS) databases (24). This software identifies and visualizes specific structural toxicophores, while its predictions can be linked to the confidence intervals and probabilities, delivering reliable predictions (25). The acute toxicity option provided by the ACD/Labs ToxSuite was made by integrating expert knowledge of various effects, like the inhibition of cholinesterase and adenosine triphosphate synthesis, disruption of central nervous system, and QSAR analysis (24).

Toxicity Estimation Software Tool (T.E.S.T.), from the US US EPA, is a predictive system developed by the Environmental Protection Agency, which is based on the QSAR mathematical models (26). US EPA T.E.S.T. contains a model that can be used for predicting rat oral acute toxicity, which is comprised of both predicted and experimental LD50 values. A variety of toxicity endpoints are available in the T.E.S.T. software and can be applied for predictions of acute toxicity values. This is accomplished by applying a simple linear function of molecular descriptors (e.g. octanol–water partition coefficient, steric and/or electronic parameters, parameters related to the presence/absence of a given chemical group) (26). TOPKAT (Toxicity Prediction by Komputer Assisted Technology), licenced by Accelrys, contains a module which can be used for rat oral LD50 assessment. This module, along with the oral rat chronic lowest observed adverse effect level (LOAEL), rodent carcinogenicity, Ames test on mutagenicity, and developmental toxicity, can be considered one of the most frequently used models at The National Center for Environmental Assessment (NCEA) (27). The software uses experimental LD50 of approximately 4000 chemicals extracted from the literature. The LD50 module of the TOPKAT package is made of 19 statistically significant, cross-validated models. Every QSAR model evaluates rat oral LD50 for a specific chemical class, allowing TOPKAT to produce a plausible toxicity value for a chemical structure delivered from a QSAR linear equation (24). ProTox, licensed under a Creative Commons Attribution-Noncommercial License, freely available at http://tox.charite.de/tox, is a web server that allows the prediction of oral toxicity in rodents by analysis of the similarity of compounds with known LD50 (2). This web server is capable of identifying toxic fragments and includes an indication of possible toxicity targets, based on a set of protein–ligand pharmacophore models, the so-called ‘toxicophores’ (2).

Repeated-dose toxicity studies aim to investigate the effects of repeated oral, dermal, or inhalation exposure to a substance over a certain time period, delivering comprehensive information about the adverse effects, possible target organs or systems (liver, kidney, endocrine, central nervous, reproductive system, etc.), as well as the perseverance or reversibility of the effects (28). As the main outcome of the repeated-dose toxicity studies, no observed adverse effect level (NOAEL) continues to be used in various regulatory guidance documents for choosing the suitable dose levels, i.e. maximum recommended starting dose (MRSD), for conducting the co-called ‘first in human’ studies (7, 28). Thus, the existence of various databases and computational models for NOAEL and LOAEL prediction should be mentioned. The measurement unit of these parameters is expressed as mg/kg body weight/day, while the quality of the chemical structures and data is crucial, as well as the route and duration of exposure, the species and strain used, space between doses, and organ level effects (29). Hence, the prediction of human safe doses is heavily dependent on the accessibility of validated animal models for each of the effects of interest (30).

For instance, a freely available database for repeated dose toxicity, the RepDose database (http://fraunhofer-repdose.de/), developed by Fraunhofer ITEM, consists of NOAEL and LOAEL values for 655 chemicals from to oral or inhalation studies in

rodents repeatedly exposed to the investigated substances for a period of at least 14 days (29). Thus, the database contains three essential data sets for every chemical: physico–chemical data and structural features, study design data, as well as the results of each study (31). Hence, as a part of the study design data, the database provides specification of the animals (number per dose group, strain, sex), as well as the exposure (dose groups, duration and route, postexposure observation period) (29, 31). Previously mentioned software, TOPKAT, developed by Accelrys, also contains the ability of LOAEL predictions. This software uses continuous and dichotomous (binary) measures to predict various endpoints, including skin and eye irritation, skin sensitization, LD50, carcinogenicity, mutagenicity, developmental toxicity, but also maximum tolerated dose and LOAEL (32, 33). The TOPKAT LOAEL model is aimed at predicting rat chronic oral LOAEL (only for studies lasting 12 months or longer) (29). TOPKAT LOAEL model contains US EPA data, which consist of peer reviewed LOAEL values, as well as NCI/NTP data, which contains values obtained from the text tables using the lowest dose at which an adverse effect was first noted (27).

However, having in mind that the NOAEL/LOAEL approach has certain well-known limitations, effort has been put into developing new alternatives to accompany the use of this method in regulatory science. Some of these include *in silico*-based models completely implemented by prominent regulatory agencies to support human safety, the Benchmark Dose (BMD) (34, 35), as well as adverse outcome pathways (AOPs) (36). There is still some hesitation about nearly all of these concepts, but the near future might witness their full application and acceptance (7). The BMD approach is considered a scientifically more forward-thinking method in comparison with the NOAEL approach for dose-response modelling of the toxicological data in order to obtain a Reference Point (Point of Departure) in the risk assessment process (35). The two most commonly used software for BMDL determination include BMDS (Benchmark Dose Software, BMDS), created by the US Environmental Protection Agency (US EPA), and PROAST, created by the National Institute of Public Health and the Netherlands National Institute for Public Health and the Environment (RIVM) (34). On the other hand, the adverse outcome pathway (AOP) seeks to draw a line between molecular initiating events and adverse events at the level of organ/organism. The AOP Knowledge Base (AOP-KB) is a database, available at www.aopkb.org, which contains experimentally derived AOPs. This database is an OECD initiative, which is executed as a close collaboration among the European Commission's Joint Research Centre (JRC), the United States Environmental Protection Agency (EPA), and the US Army Engineer Research & Development Center (ERDC) (37). It delivers a combination of organized and free-text inputs whose aim is to accumulate data about each AOP component, including graphical representations of AOPs (38). It also allows the scientific community to distribute, build, and discuss AOPs and provides knowledge management for information supporting all phases of development, including putative, formal, and quantitative AOP (qAOP) (39). Integrating the AOP framework into preclinical drug development has many benefits, including the estimation of drug metabolism and its importance for toxicity assessment

(37). AOP framework can be viewed as a tool for drawing biological connections and summarizing key data across different levels of biological organization to bridge biological disturbances at the molecular level with adverse outcomes either for individuals or an entire population (40). Thus, the Comparative Toxicogenomics Database (CTD) should be mentioned as another important source which could be helpful in generating AOPs. The CTD database curates and integrates data describing links between chemicals, genes, and human diseases. According to the latest update, CTD includes 45 million toxicogenomic relationships for over 16300 chemicals, 51300 genes, 5500 phenotypes, 7200 diseases and 163 000 exposure events, from 600 different species (41). While inferences in CTD are mostly based on information obtained from animal studies, CTD directs at environmental chemicals and outcomes important for human health. Thus, genes/proteins of interest are included in the database only if they are also present in humans (42).

The use of *in silico* investigation for testing different toxicity end-points in pre-clinical studies

Having in mind that hepatotoxicity, mutagenicity, genotoxicity and carcinogenicity are among the most crucial toxicity end-points tested in the drug development cycle, the implementation of *in silico* software in pre-clinical trials for the assessment of these effects is explained in more detail.

Hepatotoxicity

Liver toxicity plays a significant role in the process of drug development, while hepatotoxicity can be regarded as one of the main reasons for drug attrition and may lead to the discontinuation of both preclinical and clinical studies. It can even result in organ failure and can lead to fatal outcome (43). Many drugs have been withdrawn from the market because of their ability to cause liver injuries (44), which may occur through several different mechanisms, including oxidative stress, mitochondrial dysfunction, transporter inhibition, adduct formation (covalent binding), as well as transcription disorder. These mechanisms are important for providing pharmacologically relevant endpoints and may be the core of assessing the effect of predictive models for liver toxicity. One of the main indicators of the response of liver cells to drugs is the regulation of enzymes that metabolize drugs and drug transporters. The activity and expression of these proteins may be altered by systemic exposure to certain drugs, which may also cause recurrent liver dysfunction, manifested by various liver tissue injuries (45). Drug-induced liver injury (DILI) studies have led to the collection of significant data, which enabled the development of a number of databases which may be useful in hepatotoxicity testing. Databases specializing in processing and collecting data on liver injury offer access to a large amount of useful information. Hence, several databases on the topic have been built, including LiverTox, Liver Toxicity Knowledge Base (LTKB), Open TG-GATEs, Hepatox, DILIsym (44), as well as eTOX (46). eTOXsys (by Lhasa Limited and Molecular Networks) is a good example of software which might be used for predicting

drug hepatotoxicity, along with various other toxicological end-points, by bringing together different tools, databases and results (47). It was compiled by Molecular Networks GmbH and contains a retrospective analysis of the preclinical toxicology data obtained as a part of the eTOX project, whose main goal was to deliver background rates and treatment-related analysis on both clinical pathology and histopathology datasets (48). eTOXsys enables access to predictive models and databases by a single user interface which supports hazard identification and risk assessment of drug candidates from the input. Compounds can be entered into the system by name or SMILES files, or even by being sketched in the molecule editor. Database prediction results can be further refined and analyzed by users and exported in compatible standard file formats (Excel document). eTOXsys offers data regarding the toxicity of molecules deposited in the eTOX database, but even toxicity prediction for molecules with no suitable data in this database (47). eTOXsys interface allows toxicity searches, while single/multi-parameter scientific hypothesis can be set through an interactive builder. Information related to toxicity can be searched by species, study design, duration and route of exposure, as well as target organ. The query builder for formulating searches about toxicology allows users to conduct their searches by the inserting terms. Searches by toxicology and chemistry can be performed combining focus on a particular chemical with specific toxicological effects (47). Structures of the target chemicals are shown on the left side of the software interface, along with histopathology findings in the liver as a target site that might be related to the investigated compounds. Chemical names are presented in the software, along with their IDs and registry numbers, mode of action and directory of existing studies on different species (47).

Genotoxicity and carcinogenicity

Carcinogens and mutagens cannot be regarded in a traditional sense of chemistry, considering that, even though these chemicals have structural commonalities, they are complex and subtle. There are hypotheses which postulated that mutagenicity can be regarded as the ability of the molecule to reacts with the DNA bases, which can be connected with the existence of an electrophilic center present in the molecule (33). These structures may be apparent if the electrophilic group is a part of the molecule structure, or may be latent and formed by metabolism. Thus, there have been a lot of attempts to identify possible electrophilic attack sites which might be connected to mutagenicity and carcinogenicity (33). The most often used assay for mutagenicity testing is the Ames experiment. This test is a short-term bacterial assay for detecting compounds that induce genetic damage and point mutations. Low reproducibility rate of *Salmonella typhimurium*, which is around 85%, can be viewed as its major limitation (49). Nevertheless, the Ames test is one of the most applied methods for mutagenic activity assessment. Most of the *in silico* methods available for such genotoxicity/mutagenicity predictions are based on existing *in vitro* tests. There are two main groups of these tests. The first are developed from human derived structure activity relationship (SAR), while the second are developed by the computer algorithm QSAR (50). By applying QSAR, it

is possible to study biological activities associated with the structure of the molecule. QSAR plays a role in explaining how the structures of molecules are related to biological activities (51). Statistical learning algorithms which have been used for this purpose include support vector machine (SVM), random forest (RF), k-nearest neighbours (kNN), as well as artificial neural network (ANN). These statistical learning algorithms have good predictive capability, but they are complicated for use (49). Carcinogenicity predictions can be accomplished by including a large number of factors, which usually contain chemical and information about metabolism of the tested compound, toxicity and genetic toxicity interaction, the possibility of non-targeted mutagenesis, etc. Extrapolation is possible through the activities within a series of congeners. However, predictions within dissimilar classes of chemicals are demanding. Artificial intelligence systems are able to predict parameters listed above. However, predicting every aspect of carcinogenicity is not possible (33). Various knowledge-based software can be used for this purpose, while some of them include DEREK, HazardExpert, Oncologic and COMPACT. On the other hand, statistically-based systems may also be used, which include ADAPT, TOPKAT, CASE, MultiCase, QSAR-ES and common reactivity pattern (COREPA). DEREK software (by Lhasa Limited) was created based on toxicity and structure relationship. It includes a database which covers various endpoints, such as mutagenicity, carcinogenicity, teratogenicity, as well as skin and respiratory sensitization. DEREK incorporates a series of 'structural alerts' interrelated with types of toxicity, which are included in the system operation rules (52). When the software analyses an unknown structure, the system performs pattern recognition to identify structurally similar characteristics. ADAPT (Jurs Research Group, Pennsylvania State University, Philadelphia, PA, USA) was used to investigate a nitrogenous cyclic chemicals and establish a relationship between mutagenicity and their chemical structure. It characterizes chemicals by an extensive range of molecular descriptors. There are four major classes of descriptors which are used (topological, geometric, electronic, and physical), while substances are classified into mutagens and non-mutagens (33, 53). Computer automated structure evaluation (CASE), developed by Klopman and Rosenkranz (1984), makes investigating the distribution of each fragment among active/inactive molecules possible. This software also identifies fragments whose distribution is different from an ideal symmetrical distribution. CASE can also provide tests for significance of certain continuous molecular descriptors. It is based on structural and physical-chemical factors that might be related to the detected activity. The program also includes an expert system known as META, which helps to detect sites in the molecule that are responsible for metabolic transformation. Computer optimized molecular parametric analysis of chemical toxicity (COMPACT), developed at the University of Surrey (UK), was created with aim of indirectly identifying carcinogens by the shape and molecular orbital energy levels of a chemical structure (e.g. planarity level) and evaluate if the chemical will interact with cytochrome P450I (33), or with the binding site of the Ah receptor, and hence manifest carcinogenicity/toxicity (54, 33). The Common reactivity pattern (COREPA) method, by US EPA, can be used for identifying

reactivity patterns of chemical structure that exert similar biological effects, as well as for the identification of structural requirements to cause mutagenic effects. The factors controlling mutagenicity are in combination with the ability of chemical to take part in local electrophilic reactions, such as electronic charges and reactive fragments (33). COREPA may also evaluate 3D similarity between chemicals from the input, while also defining and investigating various distributions of chemical conformers in molecular descriptor space (55).

Examples of the use of *in silico* methods in drug safety assessment

As mentioned earlier, ADME profiling is often combined with toxicity predictions, in order to gain comprehensive insight into the potential toxic effects of drug candidates. For example, Bhati et al. (2019) applied *in silico* techniques to investigate the ADMET profile of newly designed thiohydantoin derivatives linked with piperazine, which can potentially be used as an androgen antagonist for treating prostate cancer. OSIRIS DataWarrior, developed at Actelion/Idorsia Pharmaceuticals Ltd, an online cheminformatics tool, was used to reveal pharmacokinetic parameters like solubility and toxic properties (i.e. tumorigenicity, mutagenicity, irritant and reproductive effect) (56). DataWarrior allows predictions of physicochemical and other properties straight from chemical structures, while toxicity risks are predicted from already formed fragment lists (57). The prediction was grounded on the functional group similarity between the inserted molecules and *in vitro*/*in vivo* validated compounds accessible in the database. AdmetSAR was used for identifying ADMET profile of the ligands, including blood–brain barrier penetration, CYP inhibitory promiscuity, human intestinal absorption (HIA), Caco-2 cell permeability, mutagenicity, carcinogenicity, as well as acute toxicity in rats. The results have demonstrated possible mutagenic properties of some of the ligands, along with the non-carcinogenic nature of the designed thiohydantoin derivatives. The predicted rat LD50 was between 2.72 and 2.91 mol/kg. Health effect probability and maximum recommended daily dose prediction were also performed in this study by using ACD/I-Lab. Some of the ligands have shown a health effect probability score at a moderate level, while the maximum recommended daily dose (MRDD) was found in the range between 1.4 and 4.6 mg/kg/day (56). Furthermore, Attwa et al. (2020) performed toxicity screening of the new poly (ADP-ribose) polymerase (PARP) inhibitor talazoparib (TZB) by using DEREK software, while toxicity predictions were also accompanied by ADME predictions, particularly metabolism. Structural alerts shown by the DEREK software indicated that the side effects of the investigated drug might comprise of nephrotoxicity and hERG channel inhibition because of the presence of halogenated benzene and hERG pharmacophore II in its structure. *In silico* metabolic vulnerability prediction was performed by the WhichP450TM software, which indicated that C1 and C5 on the 1H-1,2,4-triazole ring in the TZB structure could be viewed as moderate labile sites of metabolism, while CYP3A4 was found to have a vital function in TZB metabolism. From these results, the authors predicted that accumulation of the investigated drug after multiple doses might be possible, while liver would have a minor

role in its excretion (58). Lawal et al. (2018) conducted a study to characterize naringenin (NAR) as a novel inhibitor of collapsin response mediator protein-2 in the treatment of Alzheimer's disease. Toxicity risks of the investigated substances were assessed by the ProTox webserver. The predicted LD50 value was 2000 mg/kg, while naringenin was estimated to belong to toxicity class 4 according to the globally harmonized system (GHS) of labeling chemical compounds. The Molsoft program (http://molsoft.com/mprop/) and OSIRIS DataWarrior property explorer were used to predict the physicochemical properties of the investigated substance. As estimated, NAR has a molecular weight of 272 g/mol (Da), which is within the tolerable threshold of a CNS drug. The logP of NAR was estimated with the aim of predicting its lipophilic property related to the blood–brain barrier (BBB) permeability and bioactivity as a neurogenic drug. The results have demonstrated that NAR had a log P value of 2.3, which is within the acceptable threshold of 1.5–2.7 for a BBB permeable substance (59).

The use of *in silico* methods in impurity assessment in drugs

Bearing in mind that drug impurities have no beneficial effects and therefore only pose a risk for human health, the regulatory review process of pharmaceutical contaminants and degradants that may be found in drug products should always strive to lessen those impurities to the lowest concentration levels (those that are technically feasible or that should bear no significant health risk) (60).

Guidelines for assessing the quality of active pharmaceutical ingredient (API) and medicinal products which have been developed by The International Council for Harmonisation (ICH), Food and Drug Administration (FDA), World Health Organization (WHO) or European Medicines Agency (EMA) are focused on evaluating the stability of the API by establishing different stress tests to confirm the presence of the impurities (61). The identification of impurities is mainly focused on determining their structure, with simultaneous determination of physicochemical properties, followed by the toxicity estimation (62). Former ICH regulations concerning the quantitation limit of impurities in a drug depended on the API daily dose, routes of administration, as well as duration of therapy. The determination of impurity in concentrations lower that 0.1% was unrequired and there has also been a lack of mindfulness about impurities existing in the drug substance, especially in the final product (ICH Q3B (R), 2000) (63) The main concept of impurity qualification is given in the guidelines for active substances (Q3A, Impurities in New Active Substances) (64) or medicinal products (Q3B, Impurities in New Medicinal Products) (63), while qualification can be defined as the process of acquiring and evaluating data that establishes the biological safety of an individual impurity or a given impurity profile at the level(s) specified. In the case of potentially genotoxic impurities, the determination of acceptable dose levels is thought to be a particularly critical issue. Having in mind the necessity for the identification of the genotoxic impurities and determination of their limits in the API, EMEA proposal resulted in a significant change in standards and thresholds of impurities (ICH M7 guidelines (2014)) (65).

In the absence of data which are typically required for risk assessment methods application (i.e. data from carcinogenicity long-term studies or data providing evidence for a threshold mechanism of genotoxicity), the implementation of a generally applicable approach as defined by the Threshold of Toxicological Concern (TTC) is proposed. A TTC value of 1.5 μg/day intake of a genotoxic impurity is thought to be an acceptable risk (excess cancer risk of <1 in 100,000 over a lifetime) for most pharmaceuticals. From this threshold value, a permitted level of impurity in the active substance can be determined based on the daily dose (ICH M7 guideline) (65).

To meet such requirements, recent advances in computational methods have provided extra resources for safety assessment of drug impurities, various screening processes and software specific algorithms connected to the chemical structure (66).

In the following text, an insight into the *in silico* methods in the assessment of impurities in APIs / drugs is given, along with examples of how computational toxicology methods is currently being applied.

The DEREK software, as already mentioned, delivers a prediction by associating structural features of the target compound with the so-called alerts. Each alert contains a toxicophore (a substructure known or thought to be responsible for the toxic effect) and is linked to not only literature sources, but also notes and examples.

Zhu et al. (2013) applied DEREK for toxicity assessment in their evaluation research of an impurity in levofloxacin, descarboxyl levofloxacin. The prediction results indicated quinoline as a target compound - alert in this impurity (67). Genotoxicity of descarboxyl levofloxacin was evaluated as positive due to *in vitro* (bacteria, *E. coli* and *S. typhimurium*) and *in vivo* (dogs, guinea pigs, hamsters, humans, mammals, monkeys, mice, primates, rabbits, rats, and rodents) results in which the mutagenicity endpoints were persuasive.

Nagulakonda et al. (2019) utilized two complementary QSAR programs, DEREK Nexus and TOPKAT, in accordance with ICH M7 standards. Their DEREK analysis showed similar alerts elicited for alpha-2-mu-globulin nephropathy, carcinogenicity, photo allergenicity, skin sensitization, hepatotoxicity, mitochondrial dysfunction, nephrotoxicity and mutagenicity in the analysis of drug compound and targeted six impurities. However, the authors concluded that these findings can be considered “no alert” because they are not relevant to the human organism and the alpha-2-mu-globulin protein can be found only in rats. Moreover, in mammals, rats and rodents, alpha-2-mu-globulin nephropathy was alerted at the “doupted” level by drug compound and targeted impurities and the compounds which have the molecular volume considerably more than 0.2 nm^3 were rated as incapable to bind to the protein and cause the disorder. In favor of that conclusion is the fact that ICH M7 categorize all impurities in Class 4 (alerting structure) including non-mutagenic and examined drug compounds (68).

TOPKAT models are created by combining a variety of molecular, chemical, physical, and spatial descriptors assessing the certainty of prediction with the proprietary

Optimal Predictive Space validation technique (OPS), a specific multivariate descriptor space in which a particular model is thought to be relevant (69).

Nagulakonda et al. (2019) used TOPKAT as the statistical-based methodology to assess tazarotene and its impurities due to toxicophores (consecutive triple bonds, sulfone functional group) (68).

Toxtree, a versatile and easily accessible program created by Ideaconsult Ltd. and the European Commission's Joint Research Centre, sorts chemicals into categories and forecasts hazardous effects by using decision tree techniques. In the study conducted by Pikul et al. (2016) the calculation of the probable degradation metabolites of ivabradine was determined by applying the Toxtree program. Their results have demonstrated that there were no mutagenic effects exerted by degradation products with slight effects on cytochromes (62).

The PreADMET package is a software for toxicity prediction which includes information based on studies in which mice were treated with a particular chemical for two years, to assess cancer development and information obtained by applying the Ames test, to assess genotoxicity (70).

Abdelwahab et al. (2020) performed toxicity examination for cinnarizine and its impurities using the preADMET software. The results demonstrated that Ames mutagenicity test was positive for all impurities in one of the examined salmonella strains. In the case of [1-(diphenylmethyl)piperazine] the mutagenicity test was positive for both strains. Additionally, all of the contaminants were assessed to be carcinogenic in rats, mice, or both, with a moderate risk of hERG inhibition. The OSIRIS® Property Explorer program is an online software program that allows the prediction of toxicity risk and factors such as mutagenicity, tumorigenicity, irritation, effects on different systems in organism, as well as physicochemical characteristics using Chou and Jurs algorithm (71 72).

The OECD QSAR Application Toolbox is a freely available software tool developed by the Organization for Economic Co-operation and Development (OECD, Paris), the European Chemicals Agency (ECHA, Helsinki) and the Laboratory of Mathematical Chemistry (LMC, Bourgas University, Bulgaria). It is intended for substances categorized in groups based on their chemical and mechanistic properties, retrieving the information from experimental studies for categorized substances and categorized substances properties prediction (73). To predict the structural alerts leading to genotoxicity of ceftazidime and its impurities, Han et al. (2019) applied the OECD QSAR Application Toolbox and assessed toxicity mechanisms and toxicity endpoints. DNA and protein binding were noted as significant toxicity mechanisms while genotoxicity, *in vitro* mutagenicity tests (*in vitro* - Ames test and *in vivo*- micronucleus test) were noted as significant toxicity endpoints. The impurities that might be genotoxic in ceftazidime were targeted based on the presence of toxic functional groups (the beta-lactam ring, the quaternary amine group, and the acetates) (74).

MultiCASE/MC4PC (Multiple computer aided structure evaluation) is a system for activity prediction analyzing the presence or absence of specific fragments in the chemical structure. It uses statistical data and training sets including a number of compounds and the biological activity data linked with them. In MC4PC, a newly submitted molecule is separated into fragments, which are analyzed and compared to already discovered and stored biophores and biophobes. The software analyzes segments of active and inactive molecules and finds those that are predominantly linked with biophores - active molecules. The active segments are considered mutagenic and the inactive non-mutagenic (75 ,76, 61).

Advantages and disadvantages of *in silico* approach

In silico toxicology differs from traditional toxicology in many ways, while probably the most significant is the one considering the scale, especially in terms of the numbers of the studied substances, range of the endpoints and pathways that could be covered by *in silico* analysis, as well as the levels of biological organization examined, but also the variety of exposure conditions which are simultaneously considered (77). In many different situations *in silico* methods have an important role in hazard assessment, for both existing chemicals and new substances which are under development. These include: 1) urgent situations in which prompt explanations of potential toxicological outcomes of exposure is needed in the lack of existing toxicological testing data; 2) cases where the supply of available test material is incomplete; 3) scenarios which include challenges to conduct laboratory studies; 4) cases in which the synthesis of a complex test material is not possible; or 5) situations where a less time-consuming and less expensive high-throughput method is required (6). For example, our recent work has demonstrated the uses of *in silico* toxicogenomic approach with the aim of assessing the safety of drug combinations in COVID-19 treatment (78). The urgent need for COVID-19 treatment required a fast response from scientific community about the proposed combinational therapy. Therefore, *in silico* methods can scale up traditional toxicology testing and largely save time and other laboratory resources.

However, while some limitations of alternative tests are obvious, it is very challenging to overcome them. Among the recognized restrictions, the most important ones are related to the quality of the gathered data, which depends strictly on online sources and previously published results (79). Similar to other experiments, *in silico* methods first require the collection of available data and deep literature mining. Publicly available databases used for alternative toxicity testing do not have standardized protocols for generation and interpretation of experimental results. For example, chemicals from different manufactures are represented based on their generic names and CAS Registry Numbers; the statistical analysis might be carried out or interpreted differently, or the dose used in reported experiment may be missing. Moreover, individual sensitivity of exposed subjects is not taken into account, as well as the complexity of the living organisms (80). Therefore, the need to assess the data quality is an important requirement for making a decision when and how to use alternative methods in toxicology testing.

Until then, the combination of alternative and traditional tests may be suggested as optimal for 'toxicity prediction' and risk assessment.

Conclusion and future perspectives

The application of *in silico* methods in drug safety assessment is constantly increasing, while many *in silico* toxicity prediction software and databases are updated on the regular bases in accordance with consumer needs. However, there is no universal *in silico* software/database for toxicity assessment, and each of them is developed for a specific purpose. That is why, when choosing an appropriate software/database, it is of great importance to know its pros and cons, the domain of applicability and correct interpretation of the obtained results. Data obtained by *in silico* methods are currently mostly used merely as a support to other relevant scientific data. For example, it is suggested that predictions of some toxicity endpoints (e.g. genotoxicity and carcinogenicity) should be based on a battery of models, merging high-sensitivity models (low rate of false negatives) with high-specificity ones (low rate of false positives) and *in vitro/in vivo* assays in an integrated manner (81). Additionally, if the *in silico* system itself is not able to provide a valid decision about the importance of a certain prediction, expert knowledge is still required to assess whether this data is relevant or not (61). Nevertheless, it is evident that the role of *in silico* methods will become much more prominent in the near future, with a strong tendency to become widely applied as a stand-alone evidence, completely replacing *in vitro* and *in vivo* toxicity testing.

Acknowledgement

This work was partially supported by The Ministry of Education, Science and Technological Development of the Republic of Serbia (451-03-9/2021-14/200161).

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In silico metode u toksikologiji za procenu bezbednosti lekova

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Kratak sadržaj

Ispitivanja na eksperimentalnim životinjama ne samo da su u prošlosti bila smatrana najkonvencionalnijim pristupom za procenu bezbednosti lekova, već su i trenutno osnovna metoda za utvrđivanje njihove toksičnosti. Međutim, ova ispitivanja su skupa, vremenski zahtevna i za njihovo sprovođenje neophodne su etičke dozvole. Tokom poslednjih 20 godina došlo je do napretka u računarskoj nauci i kompjuterskoj obradi podataka, dok se znanje o alternativnim tehnikama i njihovoj primeni razvilo u dragocenu veštinu u toksikologiji. Stoga, primena *in silico* metoda u proceni bezbednosti lekova neprestano raste. Ove metode su veoma složene i zasnivaju se na saznanjima iz toksikologije, bioinformatike, biohemije, statistike, matematike i molekularne biologije. Ovaj pregledni rad će rezimirati trenutna naučna saznanja koja se tiču upotrebe *in silico* metoda u ispitivanju toksičnosti lekova, uzimajući u obzir njihova ograničenja i ističući strategije pomoću kojih se mogu dobiti konzistentni rezultati, sa posebnim osvrtom na primenu *in silico* metoda u pretkliničkim ispitivanjima i ispitivanjima toksičnosti nečistoća u lekovima.

Ključne reči: softveri, baze podataka, pretklinička ispitivanja lekova, nečistoće u lekovima procena bezbednosti