Preclinical toxicological profiling of novel psychoactive cyclopropylindole-based synthetic cannabinoids (SCs): UR-144, XLR-11 and XLR-12: molecular aspects and key toxicity endpoints

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Abstract

Cyclopropylindole-based synthetic cannabinoids, including UR-144, 5F-UR-144 (XLR-11), and XLR-12, represent a structurally atypical subgroup of novel psychoactive substances that remain insufficiently characterized in terms of their toxicological properties, despite increasing relevance in clinical and forensic toxicology. The lack of systematic data on their toxicity constitutes a critical gap, particularly given their unique molecular architecture, which may influence biological interactions and toxicological outcomes. In this study, we performed a comprehensive preclinical toxicological characterization with a focus on molecular aspects of toxicity, using an integrative set of qualitative and quantitative in silico approaches. Qualitative analyses (STopTox, ADMETlab 3.0, admetSAR 3.0, ProTox 3.0) were applied to evaluate acute toxicity (oral, dermal, inhalation), genotoxicity (Ames test), and organ-specific toxicity, including effects on the cardiovascular, renal, pulmonary, and hematological systems, alongside identification of structural toxicophores. Quantitative methods (VEGA QSAR, Percepta) provided estimates of acute toxicity across species and exposure routes, as well as cardiotoxic potential related to hERG channel inhibition. The results indicate that specific molecular features play a key role in shaping the toxicological profiles of the investigated compounds. In particular, the cyclopropyl moiety was consistently associated with increased cardiotoxic potential, while fluorinated substituents (5-fluoropentyl in XLR-11 and trifluorobutyl in XLR-12) modulated predicted toxicity, including acute toxicity and genotoxicity-related endpoints. All compounds demonstrated a high probability of pulmonary toxicity (> 85%) and moderate to high nephrotoxic potential (~ 70%), suggesting potential relevance for inhalation exposure and renal risk observed in clinical settings. Importantly, the integration of toxicophore analysis with quantitative toxicity parameters enabled the identification of structure–toxicity relationships at the molecular level, providing a basis for mechanistic interpretation and supporting the development of testable hypotheses for future preclinical studies. This work contributes to the toxicological characterization of emerging psychoactive substances and highlights the importance of molecular features in determining their potential health risks.

1. Introduction

New psychoactive substances (NPS) are a highly complex, diversified and problematic group of substances in toxicological considerations - both from the point of view of clinical and forensic toxicology (Shafi et al., 2020a). Here is no doubt that the rise of NPS represents a critical and ongoing public health challenge. First identified in the early 2000s, these substances quickly gained attention as "legal highs" or research chemicals, designed to mimic the effects of controlled drugs while evading existing regulations (Deluca et al., 2012). From chemical points of view, NPS are mostly analogs of already existing and controlled drugs and pharmaceutical products, or are newly synthesized chemicals designed to circumvent current drug control laws (Soussan and Kjellgren, 2016). This kind of substances are easily available globally, especially through online marketing. Two types of NPS have received considerable attention, i.e. synthetic cathinones known as ’bath salts‘ and synthetic cannabinoids known as ‘spice’. The first of these produce effects similar to amphetamine or cocaine by activating monoamine systems in the brain and periphery (Baumann et al., 2014; De Felice et al., 2014). The second group, i.e. synthetic cannabinoids, on the other hand, produce effects that resemble those after marijuana use by activating the endocannabinoid system (Fattore and Fratta, 2011; Wiley et al., 2014). The profile of NPS on the market is rapidly changing and therefore raises concerns about uncertainty and ambiguity regarding their chemical and metabolic profile, but also about their toxicity and associated side effects on the body (Shafi et al., 2020b). Over the past two decades, the rapid emergence of NPS has outpaced scientific research and regulatory frameworks, with hundreds

of new compounds appearing each year (Zaami, 2019). Despite significant advancements in monitoring systems, the lack of robust toxicological data continues to hinder effective public health responses.

One of the most intriguing groups are cannabinoids, which are structurally varied substances which based on ‘Drug Wheel’ by Alcohol and Drug Foundation (ADF) consist of – Fig. 1A): butane hash oil, cannabidiol, cannabis, medicinal cannabis and synthetic cannabinoids (SCs) (Hanuš et al., 2016; Marzo and Petrocellis, 2006).

It should be underlined that SCs are a group of new psychoactive substances (Roque-Bravo et al., 2023) which structure and nomenclature are summarized very different. Initially, synthetic cannabinoids (SCs) were given non-scientific names by users and vendors as part of a marketing strategy. For instance, the designation "XLR-11" likely originates from an internal naming system used by Abbott Laboratories, which was the first to synthesize this compound. Subsequently, the nomenclature established by the International Union of Pure and Applied Chemistry (IUPAC) is used to describe the structures of SCs and their metabolites. While cyclopropylindole-based SCs have long been recognized (probably invented by Abbott Laboratories in 2006 (Barth et al., 2007) as a significant issue and remain at the forefront of forensic and diagnostic identification, their vast structural diversity poses challenges in terms of nomenclature and classification. Several classification approaches are used for SCs, including the European Monitoring Centre for Drugs and. Drug Addiction (EMCDDA) pharmacophore model(Shevyrin et al., 2016a), which is based on the four-group (Shevyrin et al., 2016b; Wang et al., 2023) pharmacophore concept and categorizes structures according to the "core," "side moiety" (or tail), "linker," and "secondary moiety" (or head). A less complex classification system, the Cayman "flipbook" pharmacophore model (three elements: head, core and tail), is also widely applied. However, while these classification models are generally suitable for most cases, they prove problematic for atypical substances like cyclopropylindoles – Fig. 1B): UR-144, 5F-UR-144 (XLR-11) and XLR-12. It is surprising that these substances were likely synthesized as early as 2006 by Abbott Laboratories, as referenced in patent 2006/069196, although this matter remains somewhat unclear. Since then, several publications have emerged that include these substances, but primarily from an analytical perspective - focusing on identification and quantification methods for toxicological diagnostics and forensic toxicology - while entirely overlooking aspects of their physico-chemical properties and chemical structure. The summary of known physico-chemical properties of investigated cyclopropylindole-based synthetic cannabinoids are summarised in Table 1.

Since chemical structure determines toxicological properties, this aspect remains completely overlooked in the case of these substances, despite the uniqueness of their molecular architecture. Regardless of classification, the core of these molecules is particularly important - it consists of an indole ring rather than an indazole ring, which is the predominant scaffold in most SCs. It should be underlined that cannabinoids derived from the indole ring, featuring substitutions in place of the prototypical naphthoyl group, have been less extensively studied (Wiley et al., 2013, 2012). However, increasing legal restrictions have driven the exploration of novel structural frameworks, either inspired by existing scientific literature or developed through entirely new synthetic approaches. Additionally, the linker is a carbonyl group rather than an amide structure, which is emphasized in the latest Cayman classification. Notably, this recent classification does not account for the presence of an indole ring in SC categorization at all (but it was mentioned earlier) (Shevyrin et al., 2016c). Furthermore, these substances are the only SCs that feature an atypical cyclopropane moiety as their "head," which is of significant importance due to its influence on narcotic effects (Hirose et al., 1979). In contrast, the "tail" consists of a simple, saturated five-carbon chain (*n*-propyl). Studies indicate that substitution of the indole at the *N*-1 position with various alkyl groups can influence the pharmacokinetics and bioavailability of compounds, which is crucial in the design of new drugs (Hutchinson et al., 1993). What is more, a study on synthetic cannabinoid designer drugs found that fluorinated

analogs exhibited increased potency at CB1 receptors and altered toxicity profiles *in vivo*, suggesting that fluorine substitution in alkyl chains could modulate the intensity and duration of pharmacological effects (Banister et al., 2015a). It should be underlined that SCs have been linked to severe adverse effects, including acute toxicity, cardiovascular complications, and psychomotor impairment, however toxicological profiles remain poorly understood.

Interesting and important example is UR-144 ([(1-pentyl-1H-indol-3-yl)(2,2,3,3 tetramethylcyclopropyl)methanone]; CAS: 1199943-44-6) which is a synthetic cannabinoid (Adamowicz et al., 2017) that has gained attention as a significant synthetic cannabinoid due to its widespread recreational use and potential public health implications studies have reported that its effects on the central nervous system are similar to those of THC in terms of addiction (Pennings et al., 2017). UR-144 is most commonly smoked and mixed with herbs, tobacco or can be taken orally, vaporized or inhaled (Adamowicz et al., 2017). The lack of scientific data related to the induction of both non-fatal and fatal poisonings by UR-144 has put the compound under surveillance. The first reports of UR-144 emerged around 2012 when it was detected in herbal products sold online as "legal highs" or research chemicals. The compound quickly gained attention due to its widespread availability and use as an alternative to THC-containing cannabis (Choi et al., 2013). Despite its popularity, its toxicological profile has remained poorly understood, prompting several studies to explore its effects on human health and forensic implications.(Choi et al., 2013). A 2017 study investigated fatalities linked to UR-144 use, highlighting its potential for severe toxic effects and behavioral consequences, including accidental poisoning and suicide (Rojek et al., 2017). Moreover, team from Institute of Forensic Research in Kraków published in 2017 first communication about the effects on the human body based on a review of 39 cases in Poland (Adamowicz et al., 2017). Further research in 2022 described the cardiotoxic mechanisms of UR-144, including calcium dysregulation and autophagy-related necrosis in cardiomyoblast cells, which underline its hazardous impact on cardiac health (Akar et al., 2023). On the other hand, forensic and clinical analyses conducted in 2013 identified UR-144 metabolites in blood and urine samples from intoxicated individuals (Adamowicz et al., 2013). These findings linked its use to psychomotor impairment and other toxic outcomes, raising concerns about its presence in drug-related emergencies. Additionally, UR-144 has been implicated in cases of driving under the influence, demonstrating effects on impairment that are comparable to or worse than those of THC(Adamowicz and Lechowicz, 2015).

An interesting derivative of UR-144 is 5F-UR-144 or XLR-11 (tentatively named by Abbott Laboratories as XLR-11; [1-(5-fluoropentyl)indol-3-yl]-(2,2,3,3-tetramethylcyclopropyl)-methanone; CAS: 1364933-54-9), which differs only by the presence of a highly electronegative fluorine atom located at the 5-position of the *n*-pentane (see Fig. 1B). This is an important derivative from forensic toxicology point of view because it was first identified by laboratories in 2012 as an ingredient in synthetic cannabis smoking blends and appears to be a novel compound specifically invented for grey-market recreational use (Wilkinson et al., 2015). It should be noted that XLR-11 was first detected in Korea in 2012 and, by 2013, had become the most commonly identified synthetic cannabinoid(Chung et al., 2014). Its use has been linked to serious health effects, such as acute kidney injury (AKI) and cerebral ischemia (Buser et al., 2014; Chung et al., 2014).

Finally, XLR-12 ((2,2,3,3-tetramethylcyclopropyl)-[1-(4,4,4-trifluorobutyl)indol-3-yl]-methanone; CAS: 895155-78-9) was s invented by Abbott Laboratories in 2006 as structural analogue of XLR-11, where the 5-fluoropentyl chain has been substituted with a 4,4,4-trifluorobutyl chain (see Fig. 1B). XLR-12 exhibits notable selectivity for the CB2 receptor, with a Ki value of 0.09 nM and 167-fold selectivity over the CB1 receptor. However, it still demonstrates significant affinity for CB1, with a Ki of 15 nM. Additionally, XLR-11-2, a fluoropentyl isomer, features a 2-fluoropentyl side chain and is the only compound in this series with fluorine introduced at the second position of

the pentyl chain. Notably, when compared to the non-fluorinated analogue UR-144, displayed nearly identical CB1 receptor affinity but was less potent than XLR-11, which possesses fluorination at the fifth position (Hess et al., 2016).

Considering all aspects of the chemical structure of these three substances, their unique molecular framework, which undoubtedly plays a crucial role in determining their specific toxic properties, it is justified, in the absence of any experimental data, to determine the key toxicity parameters. Hence, the aim of this study is to provide the first toxicity prediction for problematic and atypical cyclopropylindole-based SCs, specifically UR-144, 5F-UR-144 (XLR-11), and XLR-12. For this purpose we applied comprehensive approach using different *in silico* methods (qualitative: STopTox, Admetlab 3.0, AdmetSAR, ProTox 3.0, and ToxTree 3.1.0 and quantitative: TEST 5.1.2, Percepta, VEGA QSAR 1.2.3, and SL-tox) for prediction of key toxicological parameters, including: acute toxicity (qualitative and quantitative), skin and eye irritation, chosen health effects (toxicity effects on internal organs: gastrointestinal system, kidney, cardiovascular system, liver, lungs and blood), genotoxicity based on the Ames test, and cardiotoxicity by testing hERG inhibitors. It should be underlined that in our study, not only were functional toxicological data obtained in numerical form (eg. median lethal dose), but for the first time, special attention was given to specific structural motifs responsible for the toxicity of these substances (toxicophores) for investigated cyclopropylindole-based SCs. These toxicophores were visualized for each type of toxicity, providing a novel structural perspective on the toxicological properties of UR-144, 5F-UR-144 (XLR-11), and XLR-12. The workflow of conducted *in silico* studies is schematically presented in Fig. 2.

2. Methods

2.1 Qualitative *in silico* methods

2.1.1 STopTox

STopTox (Systemic and Topical chemical Toxicity) is a open-source software that has been designed to predict both systemic and topical toxicity of chemicals. It can be found at: https://stoptox.mml.unc.edu/. Its main goal is to provide a reliable alternative to traditional toxicity tests performed on animals, particularly those known as 6-pack tests. These tests, also known as 6-pack toxicity tests, involve the evaluation of six key toxicity endpoints of chemicals. These include acute oral toxicity, acute dermal toxicity, acute inhalation toxicity, skin irritation and corrosion, eye irritation and corrosion, and skin sensitization. These classic tests are commonly used to assess the risks of new chemicals and their potential health effects. STopTox uses a set of Quantitative Structure-Activity Relationship (QSAR) models that have been developed based solely on data from previous animal tests. These models are based on data collected from tests that follow OECD (Organization for Economic Co-operation and Development) protocols, which are standards for toxicity testing of chemicals. By using data from such tests, STopTox makes it possible to predict toxicity risk without the need for new animal tests, thus contributing to the reduction of in vivo testing (Borba et al., 2020).

Additionally, STopTox offers advanced qualitative analysis features, including the visualization of toxicophores - structural motifs associated with specific toxicological responses (red regions). By highlighting these substructures within the molecular framework, the software enables users to gain insight into the mechanistic basis of its QSAR-based predictions.

2.1.2. AdmetSAR 3.0

admetSAR 3.0 is an open-source software (http://lmmd.ecust.edu.cNAdmetsar3/) designed to facilitate the evaluation of ADMET properties (absorption, distribution, metabolism, elimination and toxicity), which play a key role in assessing the safety of chemicals. admetSAR 3.0 supports various input data formats, such as SMILES strings, chemical structures and molecular files in batch format. The program also generates various types of results, including numerical data, graphical visualizations and downloadable files. The platform contains more than 370,000 ADMET experimental data for more than 100,000 chemical compounds. In addition, it enables similarity searches of chemical structures to facilitate comparisons. admetSAR 3.0 includes a broad set of 119 endpoints, which are divided into five main categories: basic properties, ADME properties, human health toxicity, environmental risk assessment and cosmetic risk assessment (Gu et al., 2024). In the context of these studies, the admetSAR 3.0 software was employed to generate a qualitative assessment of acute toxicity, thus complementing and enhancing the interpretation of the quantitative findings. Additionally, admetSAR 3.0 can provide probability estimates (in %) for acute oral toxicity, acute dermal toxicity, and acute inhalation toxicity, offering users a more detailed understanding of a chemical's potential health risks.

2.1.3. Admetlab 3.0 3.0

Admetlab 3.0 3.0 is one of the most widely used and extensive ADMET property prediction platforms, offering comprehensive tools for analyzing chemicals for absorption, distribution, metabolism, elimination and toxicity. The platform includes a total of 119 endpoints, which are divided into various categories, such as: 21 physicochemical points, 20 related to medicinal chemistry, 9 related to absorption, 9 related to distribution, 14 related to metabolism, 2 related to elimination, 36 related to toxicity, and 8 toxicophore rules. One of the key features of Admetlab 3.0 3.0 is the uncertainty assessment function for predicted results, which is an important aspect in the process of assessing the quality and reliability of predictions. Uncertainty assessment allows users to gain a more accurate understanding of the precision and reliability of the results obtained, which in practice allows better interpretation of prediction results and more informed decision-making. Such functionality is extremely useful, especially in the context of developing new chemicals and drugs, where the precision of the results is critical to safety. An advanced methodology called multi-task deep message passing neural networks (DMPNN), which is combined with a variety of molecular descriptors, was used to develop predictive models in Admetlab 3.0 3.0. This approach allows for more accurate modeling of complex chemical interactions and prediction of chemical properties under different conditions. The software is freely available to all users, making it a widely accessible tool for performing ADMET analyses without charge. It can be found at: https://admetlab3.03.scbdd.com. (Fu et al., 2024). Thanks to the use of Admetlab 3.0 3.0, several endpoints in the toxicological analysis were evaluated, such as the probability of a positive result in the Ames test (genotoxicity) of the tested compounds, and it also allowed us to perform a prediction of the toxicity of causing eye and skin irritation.

2.1.4. ProTox 3.0

ProTox 3.0 is an advanced in silico software for predicting the potential health risks caused by various chemicals. The system enables the evaluation of the toxicity of chemicals based on

a wide range of data on both the properties of the compound itself and its interaction with molecular targets, metabolism and mechanisms of side effects. To obtain a toxicity prediction, the user must provide the name of the compound, which can be done by entering its canonical SMILES notation or by using the chemical structure drawing tool provided by the ChemDoodle service (https://www.chemdoodle.com/). This graphical tool allows the user to enter chemical structures quickly and easily, a convenient addition to the analysis process. ProTox 3.0 offers various predictive models that classify chemicals according to their level of toxicity. Among the parameters

evaluated are oral toxicity (LD50), organ toxicity, including hepatotoxicity, as well as assessments of mutagenicity, carcinogenicity and other toxicological endpoints. Users have the option of selecting a single model or using a set of all available models, which increases the accuracy of predictions and allows for more comprehensive results. Version 3.0 of ProTox is based on state-of-the-art methods such as molecular similarity analysis that use pharmacophores, molecular fragments and machine learning models. The platform includes 61 different models that have been developed to evaluate various aspects of chemical toxicity. Importantly, ProTox 3.0 is a publicly available and open-source platform, making it an accessible tool for a wide range of users, including scientists, researchers and industrialists who need fast and accurate toxic risk assessments of chemicals. With ProTox 3.0, it is possible to obtain information on a wide spectrum of toxic effects, as well as on the potential mechanisms of action of substances on organisms. Thanks to its extensive database and the use of modern computer techniques, the platform is a valuable tool in the processes of assessing the safety of chemicals in the context of their use in industry, medicine or environmental protection (Banerjee et al., 2024).

2.2 Quantitative in silico methods

2.2.1. VEGA QSAR 1.2.3

The ability to predict the toxicity of chemicals using computational models is becoming increasingly popular, as it allows the identification of potential biological hazards without the need for experiments. In this context, the concept of SAR (structure-activity relationship), which assumes that the biological properties of a chemical are closely related to its molecular structure, plays a key role. When this relationship is quantified, one speaks of the Quantitative Structure-Activity Relationship (QSAR) method. QSAR models use experimental data on the toxicity of various chemicals to make predictions based on the chemical structure of a compound. The goal is to combine measured toxic effects with molecular descriptors, which are mathematical representations of a molecule's structural properties. The VEGA QSAR program is one of the tools supporting this methodology, offering a broad collection of more than 70 models to help predict the toxic effects of various substances on organisms. Among the predicted toxic endpoints are such effects as carcinogenicity, mutagenicity, hepatotoxicity, endocrine disruption or skin sensitization. Input data in the VEGA QSAR program can be provided in various formats, such as SMILES notation or SDF (Structure Data File) files, which provides flexibility in entering information about the substances being analyzed. Predictions obtained using this tool can provide important support in the process of toxic risk assessment and safety evaluation of new chemical compounds, including in the context of health and environmental regulations. It is worthy of note, that the QSAR approach is widely used in the field of in silico toxicology, as it enables rapid, inexpensive and accurate prediction of potential chemical risks, providing an important tool in chemical safety research (Benfenati et al., n.d.).

2.2.2. Percepta

Percepta (ACD/labs Percepta 2023.1.2) is an advanced prediction platform that serves to predict in silico ADME/TOX and furthermore physicochemical properties. It allows for insight into the relationship between chemical structure and ADME, toxicological and physicochemical properties. Percepta allows the use of more than 25 parameters, which may include acute toxicity, pKa, genotoxicity, hERG inhibitors, skin and eye irritation, etc. (“ACD/Labs Releases Percepta,”). The work in Percepta starts by entering SMILES notation. After a short while, a user receives the results along with a reliability index, which helps to estimate how reliable the obtained results are. In addition, the user also receives visualizations of chemical structures with color mapping on the analyzed structure. Percepta is professional, licensed software. Research was carried out in Percepta version 2023.1.2 with a license purchased by the Institute of Medical Expertises in Lodz.

2.3. Validation

The validation of the applied *in silico* methods was a crucial step to ensure the reliability and accuracy of toxicity predictions for investigated SC. This process involved comparing computational predictions of acute toxicity with experimental data obtained from structurally similar compounds. The primary objective was to confirm that the *in silico* methods accurately reflect the actual toxicological properties of the tested substances. For validation, all investigated *in silico* methods were assessed. The similarity index for each method was calculated using the MCS Tanimoto coefficient (Zhang et al., 2015) and was compared to experimental LD_{50} values reported in the literature. For instance, for the compound UR-144, the similarity with Psilocin was 0.41, and the prediction similarity percentages varied between methods, with values ranging from 30.31% (VEGA) to 94.38% (TEST Consensus). The complexity of validation was influenced by the unique structural characteristics of the compounds, which sometimes posed challenges when selecting appropriate structural analogs. However, by integrating multiple *in silico* methods and considering various similarity indices, a comprehensive validation approach was achieved. Brief summary of conducted validation studies is described in Supplementary Materials 1 (SM1) as Table S1 - the validation process of acute toxicity estimation for applied *in silico* methods.

3. Results and Discussion

3.1. Acute Toxicity

3.1.1. Qualitative prediction of acute toxicity

Qualitative predictions of acute toxicity, provides initial of how harmful these compounds could be if ingested or exposed to organisms, which is a critical aspect of their overall safety profile including not only probabilistic predictions (in %) but also qualitatively visualizing (through the intensity of red shading) the fragments and structural motifs responsible for toxicity – toxicophores, within the molecule of a given substance. Toxicophores should be understand as ‘hot zones’, i.e. specific functional groups, molecular motif or molecular fragments in a molecule that are responsible for its toxicity properties (Hakimelahi and Khodarahmi, 2005; Kalgutkar et al., 2008; Singh et al., 2016; Williams and Park, 2003). At the beginning of the prediction of the acute toxicity including different route of exposure (oral, dermal and inhalation) of the investigated SCs was the application of three independent *in silico* scientific softwares, ie.: StopTox, Admetlab 3.0 3.0 and admetSAR 3.0. The results - Table 2., indicate variations in predicted acute oral toxicity (AOT) levels among the compounds, which can be attributed to structural differences. Due to the complexity of the obtained results, the findings for each compound were analyzed and presented according to the specific route of administration to ensure the most relevant toxicological interpretation, as shown below.

STopTox indicates all three compounds are characterized by AOT at a similar level, with

a high level of confidence, however, the probability of toxicity varies across the models (in the range: 69% – 75%). Each SCs structural toxicophores (red regions) were identified, highlighting the molecular fragments contributing to toxicity. A consistent pattern of toxicophores was observed across all three compounds. The indole core was identified as a primary toxicophore, as it is known to disrupt membrane potential, leading to cytotoxic effects (Nielsen et al., 2016). It should be noted that indole, which is

a heterocyclic aromatic compound made up of conjugated benzene ring and pyrrole ring, could have been visualized as a potential toxicophore, as it is known that its high concentrations are toxic to cells due to disruption of the membrane potential (Kim et al., 2013). Additionally, the pentyl/fluoropentyl side chain was highlighted as another key structural feature affecting toxicity, primarily due to its lipophilic nature, which enhances interaction with cannabinoid receptors, increasing psychoactive effects and toxicity (Banister et al., 2015b). In the case of UR-144, the indole scaffold and alkyl chain were identified as the primary toxicophores, contributing to its predicted toxicity. Scientific software analyses yielded varying results, with admetSAR predicting the lowest probability of toxicity at 17.6%, suggesting that UR-144 may exhibit a lower toxicity profile compared to its fluorinated analogues. In contrast, 5F-UR-144 (XLR-11) exhibited structural modifications that significantly altered its toxicological properties. The addition of a fluorine atom at the pentyl chain (5F) increased the molecule's lipophilicity, a factor known to enhance interactions with biological membranes and receptors (Banister et al., 2015). This structural modification was reflected in higher toxicity predictions, with admetSAR estimating a 78.3% probability and Admetlab 3.0 predicting an even higher 79.5% probability. The toxicophores were more extensively distributed across the indole scaffold and fluorinated alkyl chain, suggesting that fluorination enhanced the overall toxic potential of the molecule compared to UR-144. Experimental studies have shown that fluorination increases CB1 receptor affinity and functional potency of synthetic cannabinoids, which could account for the observed increase in toxicity (Banister et al.,. The more pronounced shift in toxicity observed with XLR-12 can be attributed to its three fluorine atoms and a structural modification in its alkyl chain, which now lacks a methylene (-CH_2-) group compared to its structural analogue

(XLR-11). This alteration results in a shortened, highly fluorinated side saturated hydrocarbon chain, which is expected to enhance interactions with CB receptors, potentially increasing the compound’s bioactivity and toxicity (Wohlfarth et al., 2013).

For acute dermal toxicity (ADT), obtained results indicate that UR-144, 5F-UR-144 (XLR-11), and XLR-12 are classified as non-toxic, although with varying confidence levels and probability estimates. Despite their structural differences, all three compounds share similar ADT, as evidenced by their relatively low toxicity probabilities and the absence of strongly highlighted toxicophores. For

UR-144, the model predicted non-toxicity with 72% confidence and an admetSAR probability of 48.8%. The absence of intense red regions in the toxicophore visualization suggests that no significant structural motifs contribute strongly to dermal toxicity. Instead, the green-highlighted regions, particularly along the alkyl chain and carbonyl moiety, indicate areas that may influence molecular interactions but do not exhibit significant toxic potential upon dermal exposure. On the other hand,

5F-UR-144 (XLR-11) showed similar results, with a 66% confidence level for non-toxicity and a notably higher admetSAR probability of 80%, suggesting a lower likelihood of dermal toxicity compared to its oral profile. The fluorinated pentyl chain is again highlighted in green, but its impact on dermal penetration and absorption remains uncertain. Given that fluorination increases lipophilicity, it may enhance percutaneous absorption, but the current predictions do not indicate a significant increase in dermal toxicity risk (Barth et al., 2007). Surprisingly, XLR-12 presents a more complex case, as its toxicophore visualization exhibits more pronounced red-shaded regions, particularly within the indole scaffold and fluorinated alkyl chain. Despite this, the model still predicts non-toxicity, although with

a lower confidence level of 56% and an admetSAR probability of 74.3%. The presence of three fluorine atoms and a shortened alkyl chain may influence dermal absorption rates and metabolic stability, but there is no clear indication of enhanced toxicity. It should be noted that only the benzene ring shows toxicophoric character in this case.

For acute inhalation toxicity (AIT), applied *in silico* methods predictions classify UR-144,

5F-UR-144 (XLR-11), and XLR-12 as toxic across all cases, with confidence levels ranging from 65% to 71%. The visualized toxicophores (red-shaded regions) indicate that specific structural motifs contribute significantly to toxicity when the compounds are introduced via inhalation. In the case of

UR-144, the AIT probability was predicted with 71% confidence, making it the compound with the highest confidence level for toxicity in this category. Interestingly, the nitrogen atom in indole scaffold (and neighboring carbon atom in hydrocarbon chain) was identified as the primary toxicophoric motif (the highest red shading indicating high contribution to AIT). Identified nitrogen atom can affect the molecule's interaction with receptors including cannabinoid receptors (CB1 and CB2). In one study (Qiao et al., 2016) it was confirmed that a compound containing nitrogen in its structure, showed the highest binding affinity to the CB1 receptor. The way a molecule binds to receptors is important for its pharmacological effects and for its potential toxicity. It should be underlined that the tetramethylcyclopropyl moiety and the alkyl chain, shown in green, appear to have a lesser role in toxicity but may influence physicochemical properties affecting absorption and distribution. For 5F-UR-144 (XLR-11), AIT toxicity was predicted with a confidence of 66%, slightly lower than UR-144. The fluorinated alkyl chain appears prominently in the molecular visualization, but the indole scaffold remains the dominant toxicophore. The addition of fluorine in the pentyl chain enhances lipophilicity, potentially increasing lung absorption and prolonging receptor interactions, leading to an overall elevated toxicity profile. Despite this, the probability of AIT remains comparable to UR-144, suggesting that fluorination alone does not drastically alter inhalation toxicity but may impact its duration of action. XLR-12, featuring three fluorine atoms and a shortened alkyl chain, displayed an AIT toxicity prediction confidence of 65%, the lowest among the three compounds. However, the toxicophore visualization reveals a more extensive red-shaded region in the indole scaffold, further supporting its critical role in toxicity. The three fluorine atoms and modified alkyl structure, highlighted in green, may influence metabolic stability and lung retention. The presence of fluorinated groups and enhanced lipophilicity suggests a greater potential for metabolic activation, possibly leading to reactive metabolites contributing to lung toxicity.

3.1.2. Quantitative prediction of acute toxicity

Despite ongoing controversies, acute toxicity remains a fundamental toxicological endpoint, commonly quantified by the median lethal dose (LD_{50}). This parameter is crucial for the assessment of

a substance's toxicity, including pharmaceuticals, as it provides essential information regarding the dose at which 50% of test subjects are expected to succumb. The determination of LD_{50} is still a required step in the evaluation of drug safety, offering critical insight into the potential health risks associated with exposure to chemical compounds (Morris-Schaffer and McCoy, 2021). The methods that are increasingly being used to determine the mentioned LD_{50} as theoretical value (t-LD_{50}) focus on the use of prediction by *in silico* methods.

The acute toxicity predictions (t-LD_{50}) for the cyclopropylindole-based synthetic cannabinoids, UR-144, 5F-UR-144 (XLR-11), and XLR-12, were evaluated across different species and routes of administration using several in silico

methods. The obtained quantitative acute toxicity predictions for UR-144, depending on the species and route of administration, are summarized in Table 3.

Table 3
Results of acute toxicity prediction (t-LD_{50}) for investigated cyclopropylindole-based synthetic cannabinoids: UR-144, 5F-UR-144 (XLR-11) and XLR-12 depending on the species and administration route.

Results for UR-144				
Software/Method	Species	Administration route	t-LD_{50}, mg/kg bw	Reliability/Prediction accuracy
Percepta 2023.1.2	Mouse	Intraperitoneal	110	Not Reliable (RI = 0.14)
		Oral	970	Borderline (RI = 0.33)
		Intravenous	23	Moderate (RI = 0.62)
		Subcutaneous	830	Moderate (RI = 0.57)
	Rat	Intraperitoneal	250	Moderate (RI = 0.65)
		Oral	470	Borderline (RI = 0.45)
ProTox 3.0	Rat	Oral	200	PA – 69.26%
VEGA QSAR 1.2.3	Rat	Oral	185.42	The predicted compound is outside the Applicability Domain of the model
Results for 5F-UR-144 (XLR-11)				
Software/Method	Species	Administration route	t-LD_{50}, mg/kg bw	Reliability/Prediction accuracy
Percepta 2023.1.2	Mouse	Intraperitoneal	14	Not Reliable (RI = 0.13)
		Oral	66	Not Reliable (RI = 0.18)
		Intravenous	16	Borderline (RI = 0.5)
		Subcutaneous	75	Borderline (RI = 0.38)
	Rat	Intraperitoneal	19	Borderline (RI = 0.39)
		Oral	21	Borderline (RI = 0.31)
ProTox 3.0	Rat	Oral	200	PA -69.26%
VEGA QSAR 1.2.3	Rat	Oral	NA	Unable to perform Applicability Domain check
Results for XLR-12				
Software/Method	Species	Administration route	t-LD_{50}, mg/kg bw	Reliability/Prediction accuracy
Percepta 2023.1.2	Mouse	Intraperitoneal	16	Borderline (RI = 0.42)
		Oral	750	Borderline (RI = 0.36)
		Intravenous	24	Moderate (RI = 0.68)

RI = Reliability Index, PA – Prediction accuracy, t-LD_{50} – theoretical (estimated) median lethal dose rate 50;

Results for UR-144				
		Subcutaneous	630	Borderline (RI = 0.4)
	Rat	Intraperitoneal	230	Moderate (RI = 0.64)
		Oral	220	Moderate (RI = 0.58)
ProTox 3.0	Rat	Oral	200	PA = 69.26
VEGA QSAR 1.2.3	Rat	Oral	NA	Unable to perform Applicability Domain check
RI = Reliability Index, PA – Prediction accuracy, t-LD_{50} – theoretical (estimated) median lethal dose rate 50;				

For UR-144, the predicted t-LD50 values in mice ranged from 23 mg/kg for intravenous administration (RI = 0.62) to 970 mg/kg for oral administration, with intermediate values of 110 mg/kg and 830 mg/kg for intraperitoneal and subcutaneous routes, respectively. Notably, a result that was determined to be unreliable (RI = 0.14) was obtained for the intraperitoneal route in mice, whereas the rest of the values, oscillating between an RI of 0.33 and 0.65, are thus defined as borderline to moderate. In rats, the t-LD_{50} values were 250 mg/kg for intraperitoneal administration and 470 mg/kg for oral administration. The lowest predicted t-LD_{50} dose was 23 mg/kg (intravenous route, RI = 0.62). In addition, the ProTox 3.0 software predicted a higher oral toxicity of 200 mg/kg in rats with a prediction accuracy of 69.26% than did Percepta (which predicted 970 mg/kg for mouse/oral and 470 mg/kg for rat/oral), thus representing the more accurate result in terms of prediction accuracy. Moreover, VEGA QSAR predicted for rats via the oral administration route a dose of 185.42 mg/kg, but this result may not be reliable, as the predicted compound is outside the applicability domain of the model. In contrast, 5F-UR-144 exhibited markedly lower t-LD_{50} values, indicating significantly higher acute toxicity; in mice, the values were as low as 14 mg/kg for intraperitoneal administration and 75 mg/kg for subcutaneous administration, while in rats the values were 19 mg/kg for intraperitoneal and 21 mg/kg for oral administration, with reliability indices generally ranging from 0.13 to 0.5, reflecting considerable uncertainty in these estimates. Previous studies indicate that XLR-11, a fluorinated analogue of

UR-144, may exhibit enhanced receptor binding potency and acute toxicity compared to its non-fluorinated counterpart (Banister et al., 2015). For the last compound, XLR-12, Percepta predicts

a divergent t-LD_{50} depending mainly on the route of administration, ranging from a low of 16 mg/kg (mouse, intraperitoneal) to a high of 750 mg/kg (mouse, oral), while in rats the t-LD_{50} values were 230 mg/kg for intraperitoneal and 220 mg/kg for oral administration. Notably, the ProTox program indicated the same t-LD_{50} dose and the same prediction accuracy (200 mg/kg with 69.26% accuracy) for UR-144 and XLR-12, suggesting that it does not recognize the differences in chemical structure among the three tested compounds, even though such differences are present; this, in effect, shows that the software has its limitations in terms of recognizing the structure of the compounds and distinguishing them from one another. It is necessary to be cautious in interpreting these results, as the outcomes vary depending on the in *silico* methods in question, and the indicators of reliability and prediction accuracy are not at a very high level; additionally, any result that falls outside the domain of applicability cannot provide an accurate or reliable outcome. Differences in the results obtained may be due to distinct mathematical models, varying databases, different uses of molecular descriptors, or diverse validation methods. Research has shown that synthetic cannabinoids like UR-144 and XLR-11 can have significant

toxic effects, including hypothermia, reduced heart rate, and organ toxicity, which may contribute to their varying toxicity profiles (Kronstrand et al., 2013).

3.2. Health Effects

The popularity of SCs, which exhibit effects similar to natural cannabis, poses a serious health risk to users. In addition, their potential effects can be stronger and more unpredictable than those of natural substances, putting them at greater risk (Cohen and Weinstein, 2018). SCs are often synthesized in clandestine laboratories, then sold online or in smoke stores. Also, work on new SC groups with more and more structurally diverse compounds may not be covered by current regulations and thus raise concerns (Castaneto et al., 2014a). Many different and adverse health effects of SCs have been reported. SCs poisoning can cause, among other things, perceptual changes or mild cognitive impairment (Wilson et al., 2013). Side effects of SCs also include nausea, vomiting, tachycardia, elevated blood pressure, seizures, and hallucinations ("Synthetic Drugs (a.k.a. K2, Spice, Bath Salts, etc.)"). One of the reported cases of hallucinations was that of two patients with untreated schizophrenia who, after being drugged with K2, committed a self-inflicted eye injury (Malik et al., 2021). Fatal effects of SC use by men aged 17–59 have also been reported (Patton et al., 2013; Saito et al., 2013; Wikström et al., 2013).

In view of the above, it is very important to conduct detailed studies on their impact on public health in order to properly assess the consequences of their use. Currently, there is significant development in the field of toxicological diagnostics and new methods for forensic toxicology, but there is virtually no basic research on the organ toxicity of this type of substance. Hence, the knowledge about key health effects important from toxicological point of view, such as organ/system toxicity, eg. lungs, blood, kidney, cardiovascular system, gastrointestinal system and liver are desired and important. For better readability, the obtained predictions for the health effects by the Percepta software have been presented below, organized in order from the highest to the lowest prediction probability – Table 4. Due to the large number of results, each effect has been categorized into separate subsections.

3.2.1. Health effects related to lungs

Among all the analyzed health effects, the highest probability of adverse effects was observed in the lungs at 89%, with Percepta predicting similar levels. Percepta predicts similar levels of lungs toxicity for all investigated SC (see Table 4). The results indicate a high predicted probability of lung-related health effects for UR-144, 5F-UR-144 (XLR-11), and XLR-12, with values of 89%, 89%, and 80%, respectively. Identified toxicophores suggest potential structural contributors to pulmonary toxicity. In UR-144, the cyclopropyl group with methyl substitutions and the carbonyl group are key toxicophores, with their lipophilic nature potentially facilitating deep penetration into lung tissues and disrupting cellular functions. Similarly, 5F-UR-144 (XLR-11) shares these features but also includes a fluorine atom in the aromatic ring, which may enhance metabolic stability and bioavailability, leading to prolonged tissue exposure and increased toxicity. XLR-12, although showing slightly lower toxicity probability, contains fluorinated side chains that could interact with pulmonary tissues, potentially causing irritation or inflammatory responses. These structural features suggest significant toxic potential for these compounds in the respiratory system.

The high predicted probabilities for lung toxicity, especially for UR-144 and 5F-UR-144 (both at 89%), suggest a significant potential for these compounds to induce pulmonary damage. This may manifest as irritation, inflammation, or oxidative stress in lung tissues. The presence of fluorinated groups, particularly in 5F-UR-144 and XLR-12, is noteworthy as fluorinated compounds have been associated with increased toxicity due to their stability

and lipophilicity, which can lead to bioaccumulation and persistent exposure in lung tissues. Existing literature has reported that inhalation of synthetic cannabinoids can lead to severe respiratory issues, including acute respiratory distress syndrome (ARDS), pulmonary inflammation, and airway obstruction (Castaneto et al., 2014b). Furthermore, the reactive nature of certain molecular fragments, such as cyclopropyl groups, may exacerbate inflammatory responses or cause cellular membrane disruption in the lungs.

3.2.2. Health effects related to blood

Blood is one of the most important body fluids, and its functions include maintaining intracellular homeostasis, providing intercellular communication, defense and repair mechanisms (Weiss and Jelkmann, 1989). Toxins that affect the blood can contribute to damaging its components, e.g. red blood cells, white blood cells, platelets, etc. Detecting toxicity to the blood enables action to be taken to prevent complications. In addition, understanding the effects of a substance on the blood is essential when assessing its safety in the context of long-term use.

Using *in silico* methods, a prediction of UR-144 blood toxicity with a probability of 82% was obtained using the Percepta program (Table 4.) For XLR11 and XLR12, very similar results of 84% and 86% respectively were obtained. This *in silico* tool further visualized the toxicophores in the molecule, which focus on the cyclopropane fragment and the methyl groups. The marking of methyl groups as potential toxicophores may be related to the fact that the methyl group presumably plays an important role in improving biological activity and drug susceptibility due to its ability to modulate the molecular conformation, in addition to physical and chemical properties, e.g., lyophilicity and metabolism (Mao et al., 2021). The identified toxicophore in UR-144 as the cyclopropyl group with three methyl substitutions, which may contribute to toxicity through its lipophilic nature and interaction with biological membranes, potentially disrupting cellular processes. In 5F-UR-144 (XLR-11), the addition of a fluorine atom in the side chain likely enhances metabolic stability and membrane permeability, increasing systemic bioavailability and potential toxicity. XLR-12, with the highest predicted blood-related toxicity (86%), combines the effects of fluorinated alkyl chains and the cyclopropyl group, suggesting that fluorination enhances biological interactions, while the cyclopropyl structure may further destabilize cellular components, contributing to its overall toxic potential.

3.2.3. Health effects related to kidney

For the kidney, with an average probability of 70% probability of health effects associated with UR-144, XLR-11 and XLR-12 (Table 4), potential toxicophores have been visualized.

The presented results indicate that fluorinated derivatives of synthetic cannabinoids, such as 5F-UR-144 (XLR-11) and XLR-12, exhibit a relatively higher probability of inducing nephrotoxic effects compared to UR-144 (73% and 76% respectively in comparison to UR-144–70%). The identification of toxicophores, particularly the cyclopropyl fragment in 5F-UR-144 (XLR-11), suggests that this structural group, and additionally the shared carbon atoms of the pyrrole and benzene rings, may significantly contribute to the toxic effects on the kidneys.

The kidney is an organ that plays an essential role in many important functions in the body such as detoxification, regulation of extracellular fluids, internal homeostasis and excretion of toxic metabolites(Stevens et al., 2006). Nephrotoxicity is a major problem associated with the deterioration of renal function due to the toxic effects of substances(Al-Naimi et al., 2019). There are many different mechanisms that lead to this type of toxicity including renal tubular toxicity, inflammation or glomerular damage(Al-Kuraishy et al., 2019). Literature studies confirm that XLR-11 can lead to acute kidney injury (AKI) by disrupting mitochondrial function, causing mitochondrial membrane depolarization, and activating apoptotic pathways in renal tubular cells (Silva et al., 2018). Additionally,

clinical cases have demonstrated that the use of products containing XLR-11 and UR-144 has been associated with AKI, manifested by nausea, vomiting, and elevated serum creatinine levels (Thornton et al., 2013). Hence, in cases of poisoning and toxidromes involving investigated SC early detection of nephrotoxicity is essential. It should be underlined that traditional markers like serum creatinine and blood urea nitrogen (BUN) may fail to detect initial kidney damage, delaying diagnosis and treatment. Therefore, the use of more sensitive biomarkers is recommended. Kidney Injury Molecule-1 (KIM-1) is effective for identifying early proximal tubular injury, while Neutrophil Gelatinase-Associated Lipocalin (NGAL) provides rapid detection of acute kidney injury (AKI) in both urine and blood samples. Additionally, Cystatin C (Cys C) offers a reliable assessment of glomerular filtration rate, being less influenced by external factors such as muscle mass. Incorporating these biomarkers into diagnostic protocols can significantly improve the early diagnosis of AKI resulting from synthetic cannabinoid exposure, enable faster clinical intervention, and reduce the risk of irreversible kidney damage. (Bonventre et al., 2010). Moreover, in the context of forensic toxicology, histopathological examination of renal tissues, focusing on mitochondrial damage, apoptosis, or tubular injury, could provide essential evidence supporting nephrotoxicity as a contributing factor to death. Furthermore, post-mortem analysis of sensitive biomarkers such as Kidney Injury Molecule-1 (KIM-1) and Neutrophil Gelatinase-Associated Lipocalin (NGAL) may offer valuable insights into pre-mortem kidney injury. These biomarkers have demonstrated reliability in early detection of nephrotoxic changes and could enhance the accuracy of forensic investigations (Ichimura et al., 2004). Integrating such analyses into forensic protocols may provide a stronger evidentiary framework in legal cases involving synthetic cannabinoid intoxication.

3.2.4. Other health effects

The results indicate low predicted probabilities of adverse effects for the gastrointestinal system and liver, while moderate risks were observed for the cardiovascular system concerning UR-144, 5F-UR-144 (XLR-11), and XLR-12. Cardiovascular toxicity was predicted at 59% for UR-144, 70% for 5F-UR-144, and 72% for XLR-12, with toxicophores like cyclopropyl groups and fluorinated side chains potentially contributing to these effects. Gastrointestinal toxicity showed lower probabilities (27%, 19%, and 14%, respectively), suggesting limited direct risk but not excluding potential indirect effects under higher or chronic exposure. Similarly, hepatotoxicity predictions were low (24–32%), though the presence of fluorinated groups in XLR-12 could imply a risk of bioaccumulation and delayed toxicity.

3.3. Genotoxicity

Genotoxicity relates to the toxic effects of compounds on the DNA structure of somatic and/or reproductive cells (Menz et al., 2023). If somatic cells are affected, the effect can be the development of various diseases including cancer or neurodegenerative diseases, while damage in reproductive cells can manifest as either hand defects or hereditary changes, ultimately leading to genetic diseases (Hasselgren et al., 2019; Nikinmaa, 2014). It should not be confused with mutagenicity, because all mutagenic substances are genotoxic, but in turn not all genotoxic compounds are mutagenic (Seukep et al., 2014). The Ames test, i.e. the Salmonella reverse mutation test, developed by Bruce Ames and colleagues has become one of the most popular tests in the field of genotoxicity testing (Ames et al., 1973). It should be underlined that the Ames test primarily detects point mutations, including base substitutions, where one DNA base is replaced by another, and frameshift mutations, involving the insertion or deletion of nucleotides that disrupt the gene's reading frame. However, it does not identify larger genetic damage (Kirkland et al., 2011), such as chromosomal aberrations, DNA strand breaks, or aneuploidy (abnormal chromosome numbers). Consequently, substances causing genotoxic effects beyond point mutations - like structural chromosomal damage—may yield false-negative results in this test.

Genetic toxicology aims to conduct genotoxicity tests to identify and assess the potential risk of

a chemical agent and its ability or lack thereof to cause genetic changes (Sera et al., 2003). For the tested compounds, i.e. UR-144, XLR-11 and XLR-12, predictions of their potential genotoxicity as Ames test results were predicted using several *in silico* methods – Table 5.

Table 5
Genotoxicity results (Ames test) for investigated cyclopropylindole-based synthetic cannabinoids: UR-144, 5F-UR-144 (XLR-11) and XLR-12 received with various in silico methods.

***in silico* method**	**UR-144**		**5F-UR-144 (XLR-11)**		**XLR-12**	
	Results of Ames test %	**Reliability %**	**Results of Ames test**	**Reliability %**	**Results of Ames test**	**Reliability %**
Admetlab 3.0 3.0	58.4	NA	58.8	NA	60.7	NA
admetSAR 3.0	18.6	NA	9.2	NA	15.8	NA
VEGA QSAR 1.2.3 (CONSENSUS)	Mutagenic	Consensus score of 45%	Mutagenic	Consensus score of 20%	Non-Mutagenic	Consensus score of 35%
Percepta 2023.1.2	23%	Bordeline (RI = 33%)	21%	Borderline (RI = 0,32)	22%	Not Reliable (RI = 0,28)
RI – Reliability Index; NA – not applicable						

The genotoxicity as Ames test of investigated SCs was predicted using various *in silico* methods, including Admetlab 3.0 3.0, admetSAR 3.0, VEGA QSAR 1.2.3 (CONSENSUS), and Percepta 2023.1.2. According to Admetlab 3.0 3.0, the probability of genotoxicity was 58.4% for UR-144, 58.8% for 5F-UR-144, and 60.7% for XLR-12. In admetSAR 3.0, the predicted genotoxicity was lower, with probabilities of 18.6% for UR-144, 9.2% for 5F-UR-144, and 15.8% for XLR-12, also without reliability data. The VEGA QSAR 1.2.3 (CONSENSUS) model classified UR-144 and 5F-UR-144 as mutagenic, with consensus scores of 45% and 20%, respectively. In contrast, XLR-12 was deemed non-mutagenic with a consensus score of 35%. The Percepta 2023.1.2 model provided borderline genotoxicity predictions for UR-144 (23%, RI = 0.33) and 5F-UR-144 (21%, RI = 0.32). The prediction for XLR-12 (22%) was considered not reliable, with a low reliability index (RI = 0.28).

Among investigated SCs, only XLR-11 has been the subject of experimental genotoxicity studies available in the literature (Ferk et al., 2016) and even these data are relatively limited in scope. Specifically, experimental research on XLR-11 has focused on assessing its potential to cause DNA damage in human cells. Although XLR-11 did not show mutagenic effects in the Ames test, which is designed to detect point mutations in bacterial DNA, other assays indicated genotoxic potential. For instance, studies utilizing single-cell gel electrophoresis (SCGE) and micronucleus tests revealed that XLR-11 induces dose-dependent DNA damage in human lymphocytes and epithelial cells of the respiratory tract. These assays demonstrated DNA strand breaks and chromosomal aberrations, suggesting that exposure to XLR-11, particularly through inhalation, could pose genotoxic risks to tissues directly exposed to smoke (Ferk et al., 2016).

It is important to emphasize that the *in silico* predictions in the present study were based solely on the Ames test, which, while widely used for initial genotoxicity screening, only detects specific types of genetic mutations -

namely, point mutations in bacterial DNA. However, the Ames test has limitations, especially in assessing the broader spectrum of genotoxic effects, such as chromosomal damage or DNA strand breaks, which are better captured by assays like the SCGE or micronucleus tests. Thus, while the *in silico* models predicted mutagenic potential in the context of the Ames test, experimental data for XLR-11 suggest that its genotoxicity may manifest through mechanisms not detectable by this bacterial assay. In other words, this suggests that the genotoxic mechanisms of XLR-11 may involve direct DNA damage or chromosomal instability, which are not captured by the mutagenicity-focused scope of the Ames test.

On the other hand, for UR-144 and XLR-12, no experimental genotoxicity data were found in the literature. This highlights a significant knowledge gap and underscores the need for further *in vitro* studies employing a wider range of genotoxicity assays. Given the limitations of the Ames test, future research should include additional methods, such as comet assays and chromosomal aberration tests, to comprehensively evaluate the genotoxic potential of these compounds.

3.4. Eye and skin irritation

Testing the toxicity of a substance, in this case eye and skin irritation, is crucial in the context of SCs, because they are frequently consumed through smoking, which poses significant risks of exposure not only via inhalation but also through direct contact with the skin and eyes. Psychoactive substances can cause various types of irritation, which in effect is a threat to the skin and eyes, and in addition, they can also increase allergic reactions. For example exposure to second-hand marijuana smoke, which shares similarities with SCs, has been associated with eye irritation and discomfort. Environmental factors such as smoke concentration, ventilation, and the number of active smokers influence exposure levels and irritation risks (Holitzki et al., 2017). What is more, the acute adverse effects of synthetic cannabinoid smoking include physiological symptoms such as eye redness and dry mucous membranes, highlighting the risk of ocular and skin irritation due to direct contact with smoke or combustion residues in human (Sutlović et al., 2018) and animals (Wiebelhaus et al., 2012).With the development of *in silico* tools that have models for predicting this type of irritation, it is possible to simplify the process of assessing the toxicity of substances. This area of risk assessment is attractive because of its speed, low cost, and is a great alternative to animal testing, especially because eye and skin irritation testing are often two critical and primary endpoints of evaluation (Selvestrel et al., 2022). Hence, several *in silico* methods, including Admetlab 3.0 3.0, admetSAR 3.0, StopTox, and Vega QSAR, were used to predict potential eye and skin irritation by UR-144, XLR-11 and XLR-12. Due to the discussion of two organs, the discussion is divided into two separate sections: 3.4.1. eye irritation and 3.4.2. skin irritation, and for clarity, the results begin with qualitative methods and toxicophore predictions, followed by quantitative probability assessments. All obtained results are summarized in Table 6 and Table 7.

3.4.1. Eye irritation

Potential eye irritation is one of the most important parameters in assessing the safety of

a compound. Currently, according to the Organisation for Economic Co-operation and Development (OECD), one of the validated and widely recognized tests for assessing eye irritation potential is the OECD Test Guideline (TG) 492. This test utilizes a Reconstructed Human Cornea-like Epithelium (RhCE) tissue model to differentiate between substances that are irritants or corrosives and those that are non-irritants. It is an advanced *in vitro* method aimed at reducing animal testing, and it significantly contributes to the classification of ocular irritation potential of chemicals (Kaluzhny et al., 2015; Robinson et al., 2002; Wilhelmus, 2001). Given the possibility of

applying more environmentally friendly approaches for preliminary assessments, integrated *in silico* methodology were employed (*n* = 5; StopTox, Admetlab 3.0 3.0, admetSAR 3.0, Percepta 2023.1.2 and Vega QSAR 1.2.3 - Eye irritation CONCERT/Kode 1.0.0) to predict the eye irritation potential of SC, offering an efficient and ethical alternative that reduces the need for animal testing and aids in identifying potential toxicophores, particularly for compounds with limited experimental data.

Firstly, StopTox provided a qualitative evaluation by classifying all three compounds: UR-144, XLR-11, and XLR-12, as non-toxic concerning eye irritation. The average probabilities assigned were 58% for UR-144, 59% for XLR-11, and 50% for XLR-12. However, it's critical to highlight that only UR-144's result fell within the model's Domain of Applicability (AD), suggesting greater confidence in this prediction. The toxicophores identified suggest that bulky, hydrophobic groups (tert-butyl group) and reactive functional sites (carbonyl-adjacent region) might contribute to irritation potential. However, the overall non-toxic classification with a 58% probability implies that while these regions could pose minor irritation risks, they are not dominant enough to categorize the compound as a significant irritant. Nonetheless, it is noteworthy that (Adamowicz et al.), reviewed 39 cases involving UR-144 exposure and observed symptoms such as redness of the conjunctiva, suggesting possible ocular irritation. However, these observations are clinical and not derived from controlled experimental irritation tests.

On the other hand, for XLR-11 and XLR-12, the predictions were made outside the model's AD, indicating a lower reliability and a need for cautious interpretation. It should be noted that there are currently no experimental studies directly assessing eye irritation for XLR-11 and XLR-12, that’s why it is not possible to compare obtained results with experimental data.

Results obtained from other various *in silico* methods, reveal notable discrepancies, highlighting the complexity and variability of predictive algorithms. Admetlab 3.0 3.0 identified the highest irritation potential for UR-144 (78.2%), followed by XLR-11 (58%) and the lowest for XLR-12 (44.1%). The elevated probability for UR-144 could be attributed to its molecular structure, which may enhance interactions with biological membranes or ocular tissues. Similarly, admetSAR 3.0 predicted the highest irritation potential for UR-144 (88.3%), with significantly lower probabilities for XLR-11 (42.1%) and XLR-12 (49.9%). The consistency in UR-144's higher predicted risk across these two models suggests the presence of structural features that may be commonly associated with irritation potential. In contrast, Percepta 2023.1.2 predicted similar and notably lower irritation probabilities for all compounds—19% for UR-144 and XLR-11, and 20% for XLR-12. This uniformity suggests that Percepta's algorithm may generalize irritation risk across structurally similar compounds or that it may have lower sensitivity to subtle structural variations. These discrepancies underscore the importance of interpreting *in silico* predictions with caution and highlight the need for experimental validation to confirm the actual irritation potential of these compounds.

Given these discrepancies, particularly for XLR-11 and XLR-12, where the predictions are less consistent, further experimental validation, such as *in vitro* eye irritation tests following OECD TG 492 will be essential to confirm these computational assessments and ensure accurate risk evaluations.

3.4.2. Skin irritation

Potential skin irritation is also critical parameter in assessing the safety of chemical compounds, particularly for substances that may come into direct contact with the skin during handling or use. Currently, the Organisation for Economic Co-operation and Development (OECD) recommends the OECD Test Guideline (TG) 439 as a validated

and widely recognized method for evaluating skin irritation potential (Groeber et al., 2016; Han et al., 2020; Sugiyama et al., 2018). This test employs

a Reconstructed Human Epidermis (RhE) model to distinguish between irritant and non-irritant substances. As an advanced *in vitro* approach, it aligns with ethical research practices aimed at reducing animal testing while ensuring accurate and reliable toxicity classification. Given the possibility of applying more environmentally sustainable methods for preliminary skin toxicity assessment, an integrated *in silico* approach was employed similarity to eye irritation assessment (*n* = 5; StopTox, Admetlab 3.0 3.0, admetSAR 3.0, Percepta 2023.1.2, and VEGA QSAR 1.2.3 – Skin Irritation CONCERT/Kode 1.0.0) to predict the skin irritation potential of investigated compounds.

In the StopTox analysis for skin irritation, all three compounds were classified as non-irritants with probabilities of 60% for UR-144 and XLR-11, and 70% for XLR-12. Importantly, unlike in previous predictions for eye irritation, all three compounds were classified inside the model's domain of applicability, suggesting a higher reliability of these predictions. In all investigated SCs the identified toxicophores are primarily associated with hydrophobic side chains, where in UR-144 it is the flexible pentyl chain, in XLR-11 the fluorinated side chain, and in XLR-12 the fluorinated cyclopropyl ring, suggesting that hydrophobicity and the presence of fluorine may enhance interactions with skin tissues, potentially contributing to irritation risks through increased membrane penetration or structural rigidity.

Predictions for skin irritation using other *in silico* methods presented divergent outcomes, reflecting differences in algorithm sensitivity and model limitations. Admetlab 3.0 3.0 did not provide data for any of the compounds (UR-144, XLR-11, XLR-12), which could be due to limitations in its database or structural criteria for predictions. In contrast, admetSAR 3.0 predicted the highest probability of skin irritation for UR-144 (34.1%), followed by XLR-11 (15.7%) and XLR-12 (12.9%). This suggests that UR-144's molecular structure may present a slightly greater irritation risk, potentially due to differences in hydrophobicity or molecular interactions with skin tissues. Similarly, Percepta 2023.1.2 predicted low irritation probabilities, with 28% for UR-144 and XLR-11, and 29% for XLR-12, showing consistent and moderate risk profiles. This uniformity may indicate that Percepta's model emphasizes broader structural features, resulting in generalized predictions across structurally similar compounds. The VEGA QSAR 1.2.3 (Skin Irritation CONCERT/Kode 1.0.0) model classified UR-144 as a non-sensitizer, while XLR-11 and XLR-12 were predicted as active sensitizers. However, it is important to note that for all three compounds, the predictions were classified as outside the Domain of Applicability (AD). This limits the reliability of the results, as the model's training data did not sufficiently cover compounds with similar structures, thereby increasing the uncertainty of these assessments.

Studies indicate that synthetic cannabinoids can exhibit various forms of toxicity, including skin irritation, due to their chemical structure and interactions with biological tissues (Courts et al., 2016). Hence, while admetSAR suggests a higher irritation potential for UR-144, the low probabilities in Percepta and the AD limitations in VEGA QSAR emphasize the need for cautious interpretation. Given these discrepancies and uncertainties, especially for XLR-11 and XLR-12, experimental validation using OECD Test Guideline 439 (RhE model) is essential to accurately assess skin irritation risks and confirm the computational predictions.

3.5. Cardiotoxicity

Cardiotoxicity is a crucial safety aspect in the context of NPS (Radaelli et al., 2021). The hERG (human ether-a-go-go related gene) potassium channel plays an essential role in cardiac repolarization, and its blockage can lead to

QT interval prolongation and life-threatening arrhythmias. Studies confirm that many new psychoactive substances, such as 25D-NBOMe and 25C-NBOMe, exhibit the ability to block the hERG channel (Yoon et al., 2019), resulting in QT interval prolongation and potentially life-threatening cardiac rhythm disturbances. Furthermore, other research emphasizes that the structural characteristics of compounds influence their ability to inhibit the hERG channel, making this parameter critical for evaluating the safety of new substances (Koulgi et al., 2022). Therefore, the application of an hERG inhibition module in the NPS research process enables the rapid and effective identification of potentially hazardous compounds. Predictive models based on large experimental data sets can significantly improve the reliability of safety assessments (Radchenko et al., 2017). Additionally, early assessment of hERG toxicity is recommended by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA), among others (Garrido et al., 2020). In this part, key parameter associated with the potential risk of hERG (human Ether-à-go-go-Related Gene) channel inhibition was half-maximal inhibitory concentration ($IC50_{50}$). IC_{50} represents the concentration of a substance required to inhibit 50% of the hERG channel's activity. Lower IC_{50} values indicate a higher potential for cardiotoxic effects. For instance, cannabidiol (CBD), a major cannabinoid, has been shown to inhibit hERG potassium channels with an IC_{50} value of 2.07 μM at room temperature, suggesting a significant risk of cardiotoxicity at certain concentrations (Orvos et al., 2020). Understanding the IC_{50} values of SCs is essential, as these substances are often used recreationally, sometimes in high doses, increasing the risk of adverse cardiac events. In general, IC_{50} represents the concentration of

a compound required to inhibit 50% of the hERG channel's activity (Xia et al., 2011). Compounds with IC_{50} values below 10 μM are generally considered to have a high risk of cardiotoxicity due to significant hERG inhibition, which can lead to cardiac arrhythmias, including torsade de pointes. The IC_{50} values for UR-144 (19.6 μM), 5F-UR-144 (22.7 μM), and XLR-12 (18.8 μM) are above this critical threshold, suggesting a moderate potential for hERG inhibition. However, these values still warrant attention, as moderate inhibition can pose risks, especially under conditions of high exposure or in individuals with pre-existing cardiac conditions. However, it should be noted that the RIs for UR-144 (0.17) and 5F-UR-144 (0.26) are low, indicating that these predictions should be interpreted with caution and ideally confirmed through experimental studies. XLR-12 has a slightly higher RI of 0.30, but this is still considered borderline. In addition to the IC_{50} values discussed earlier, the table presents further predictive data concerning the likelihood of hERG channel inhibition for UR-144, 5F-UR-144 (XLR-11), and XLR-12, evaluated using various *in silico* methods (Percepta and Admetlab 3.0).

The second parameter assessed is the probability of a compound acting as a hERG inhibitor with Ki < 10 μM, based on patch-clamp data predicted using Percepta. For UR-144, the probability of hERG inhibition was 0.25, with a RI of 0.17, indicating low confidence in this prediction. For 5F-UR-144 (XLR-11), the probability was 0.30, with an RI of 0.26, again suggesting limited reliability. XLR-12 displayed the highest inhibition probability of 0.49, with a borderline reliability index of 0.30. These probabilities suggest that XLR-12 may exhibit a slightly higher potential for hERG inhibition compared to the other two compounds (however, there is a low RI values). The third parameter evaluates the probability of hERG inhibition at Ki < 40 μM, utilizing Admetlab 3.0. This broader concentration range offers a more lenient assessment of potential cardiotoxicity. Here, UR-144 showed a high probability of inhibition at 72.9%, while 5F-UR-144 (XLR-11) and XLR-12 had slightly lower probabilities of 65.3% and 65.6%, respectively. Although this method does not provide reliability indices, the consistently high probabilities suggest that all three compounds exhibit a moderate to significant potential for hERG inhibition within this broader concentration range.

The obtained results indicate that while the IC_{50} values suggest moderate risk, the predicted probabilities of hERG inhibition (especially at Ki < 40 μM) highlight a concerning trend.

UR-144, in particular, demonstrates the highest probability for inhibition in the broader concentration spectrum, suggesting that it could pose a more pronounced cardiotoxic risk at higher exposure levels. Considering that synthetic cannabinoids like UR-144 and its analogs are frequently used in uncontrolled doses, the risk of hERG inhibition becomes more pertinent.

One example is JWH-030 which acts as an inhibitor of hERG with relatively low affinity (Yun et al., 2016). Due to Admetlab 3.0 3.0 hERG inhibitors results were obtained at 65.3–72.9% probability that the tested compounds could act as potassium channel inhibitors (Table 8). Unfortunately, as mentioned above, mainly street formulations may contain unidentified synthetic cannabinoids, the patterns of which are still evolving as new compounds emerge. This makes preclinical toxicological analysis difficult. With in silico studies that can be used early in the evaluation process, they are well suited for the initial evaluation of new compounds with limited availability (Hancox et al., 2023). All obtained results are summarized in Table 8.

Table 8
Results of cardiotoxicity prediction for investigated cyclopropylindole-based synthetic cannabinoid: UR-144, 5F-UR-144 (XLR-11) and XLR-12 received with various *in silico* methods.

Cardiotoxicity parameters	***In silico* method**	**UR-144**		**5F-UR-144 (XLR-11)**		**XLR-12**	
		Reliability	**Probability**	**Reliability**	**Probability**	**Reliability**	**Probability**
hERG half-maximal inhibitory concentration	Percepta	NA	IC50: 19.6 μM	NA	IC50: 22.7 μM	NA	IC50: 18.8 μM
hERG inhibitor (K_i < 10 μM, patch-clamp)	Percepta	Not Reliable (RI = 0.17)	0.25	Not Reliable (RI = 0.26)	0.30	Borderline (RI = 0.30)	0.49
hERG inhibitor (K_i < 40 μM, patch-clamp) 10 μm	Admetlab 3.0	NA	72.9	NA	65.3	NA	65.6
RI – Reliability Index; NA – Not applicable							

4. Conclusions

The successful application of integrated *in silico* methodology enabled the comprehensive prediction of key toxicological parameters for the problematic and atypical cyclopropylindole-based SCs (UR-144, 5F-UR-144 (XLR-11), and XLR-12), offering valuable insights into their potential toxicological risks and contributing to a deeper understanding of their clinical and forensic implications. Given the complexity of the study, involving three SCs and the evaluation of different parameters within a single article, the conclusions have been limited to the most critical findings and observations. The most important conclusions are schematically summarized in Fig. 3.

In terms of acute toxicity, 5F-UR-144 exhibited the highest oral toxicity (t-LD50: 21–66 mg/kg), followed by XLR-12 (220–750 mg/kg) and UR-144 (470–970 mg/kg). All compounds showed a high probability of inhalation toxicity (65–71%) and were classified as non-toxic for dermal exposure, although 5F-UR-144 presented a higher probability (80%). The presence of fluorinated alkyl chains notably increased the overall toxicity, confirming that fluorination enhances bioavailability and membrane penetration.

The assessment of organ toxicity highlighted the lungs, blood, kidneys, and cardiovascular system as key targets. Lung toxicity probabilities were highest for UR-144 and 5F-UR-144 (89% each) and slightly lower for XLR-12 (80%). Blood toxicity was also high across all compounds (82–86%), with XLR-12 showing the greatest risk. Nephrotoxicity predictions ranged from 70% for UR-144 to 76% for XLR-12, with fluorinated structures contributing to higher risk. Cardiovascular toxicity showed moderate predictions, with XLR-12 at 72%, indicating its greater potential to disrupt cardiac function.

The genotoxicity assessment (Ames test) showed moderate predictions in Admetlab 3.0

(58–61%) but lower probabilities in admetSAR (9–18%). VEGA QSAR classified UR-144 and 5F-UR-144 as mutagenic, while XLR-12 was non-mutagenic. Notably, experimental data for XLR-11 revealed DNA damage mechanisms beyond point mutations, indicating that the Ames test may underestimate genotoxic risk.

For eye irritation, UR-144 exhibited the highest predicted risk (up to 88.3%), while

XLR-11 and XLR-12 showed lower potentials (42.1–49.9%). In contrast, all compounds were classified as non-irritants regarding skin irritation, although UR-144 presented slightly higher probabilities.

In the context of cardiotoxicity, IC_{50} values for hERG inhibition were moderate across all compounds (18.8–22.7 μM), suggesting moderate cardiotoxic potential. However, broader inhibition predictions (Ki < 40 μM) showed high probabilities, particularly for UR-144 (72.9%), highlighting potential risks, especially under high exposure conditions.

The integrated *in silico* methodology provided critical insights into the toxicological profiles of investigated SCs, with significant implications for both clinical and forensic toxicology. Clinically, early identification of acute toxicity, organ toxicity, and cardiotoxicity risks enables timely intervention, particularly in cases of ingestion or inhalation. The high toxicity potential of fluorinated compounds like 5F-UR-144 necessitates close monitoring of respiratory, renal, and cardiac functions. Additionally, genotoxic risks, particularly for XLR-11, underscore the need for long-term health monitoring, while variations in dermal and ocular irritation call for specific protective measures. From a forensic perspective, detailed toxicological profiling aids in accurately determining causes of death related to SCs exposure, with particular emphasis on organ and cardiac damage. The identification of hazardous toxicophores, such as fluorinated side chains, assists in differentiating compounds in forensic investigations and highlights the need for regulatory scrutiny. Furthermore, focusing post-mortem analyses on target organs like lungs and kidneys enhances the accuracy of forensic conclusions.

The obtained *in silico* studies presented in this manuscript provide valuable preliminary insights into the toxicological profiles of problematic and atypical cyclopropylindole-based synthetic cannabinoids, serving as an important foundation for further research. Given the identified toxicity risks, future studies should focus on understanding the ADME (Absorption, Distribution, Metabolism, and Excretion) profiles of these compounds. Understanding how these substances are processed within the body should be required and crucial step for accurately assessing their toxicological impact.

Declarations

Conflict of interest

All the authors declare that they have no conflicts of interest.

Ethics approval

The manuscript does not contain newly generated clinical studies or patient data.

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Author Contribution

KJ: Conceptualisation, Data curation, Supervision, Writing - review and editing, Formal analysis and investigation, Writing - original draft preparation, Writing - review and editing, Visualisation; OF: Writing - original draft preparation, Writing - review and editing; KK: Writing - original draft preparation, Writing - review and editing; EN: Writing - original draft preparation, Writing - review and editing. All the authors have read and approved the manuscript.

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Data Availability

All the data generated or analysed during this study are included in this published article.

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Tables

Tables 1, 2, 4, 6 and 7 are available in the Supplementary Files section.

Figures

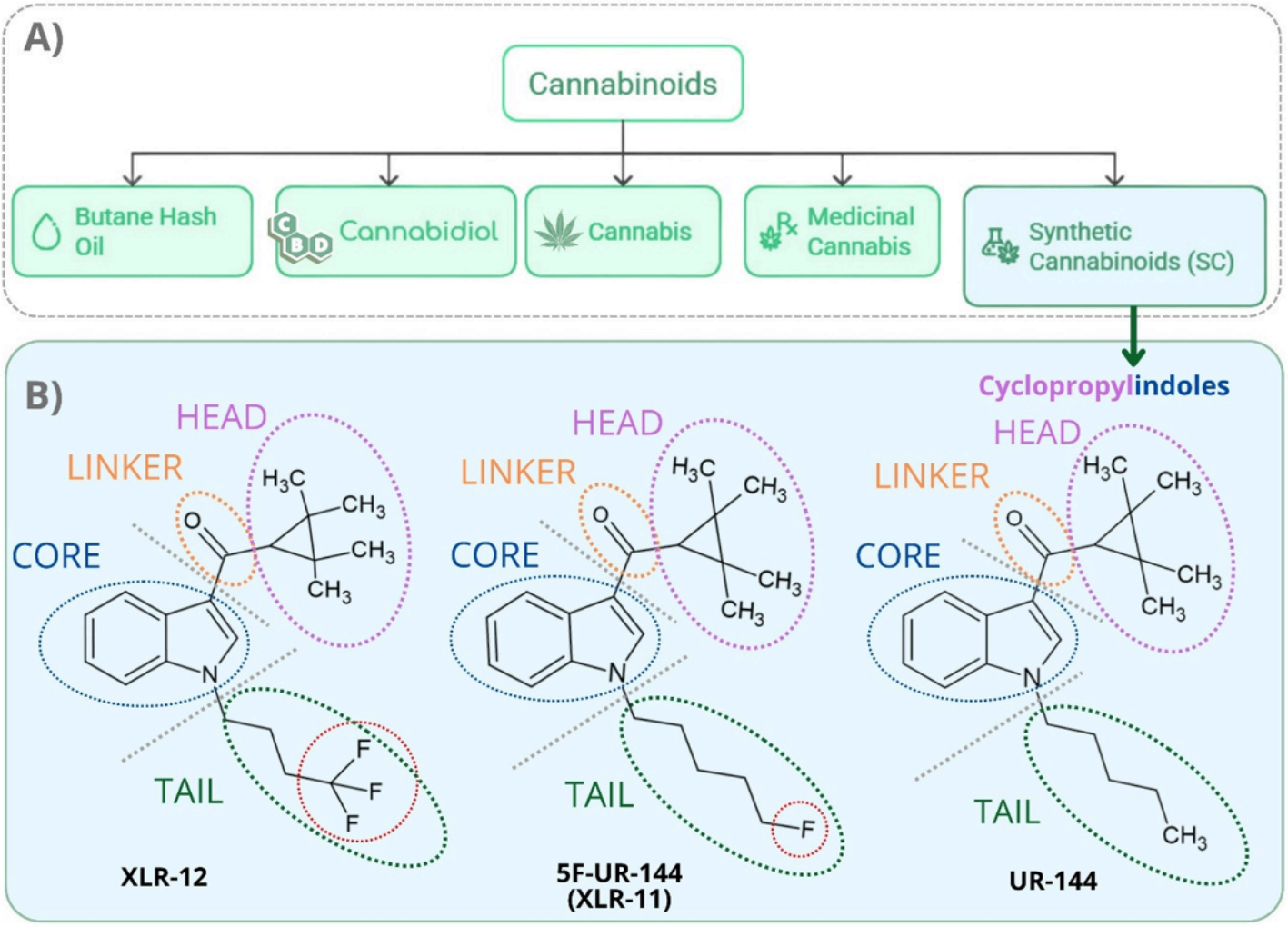

Figure 1

A) Classification of cannabinoid; B) Structure and nomenclature for three atypical and problematic cyclopropylindole-based synthetic cannabinoids: UR-144, 5F-UR-144 (XLR-11) and XLR-12.

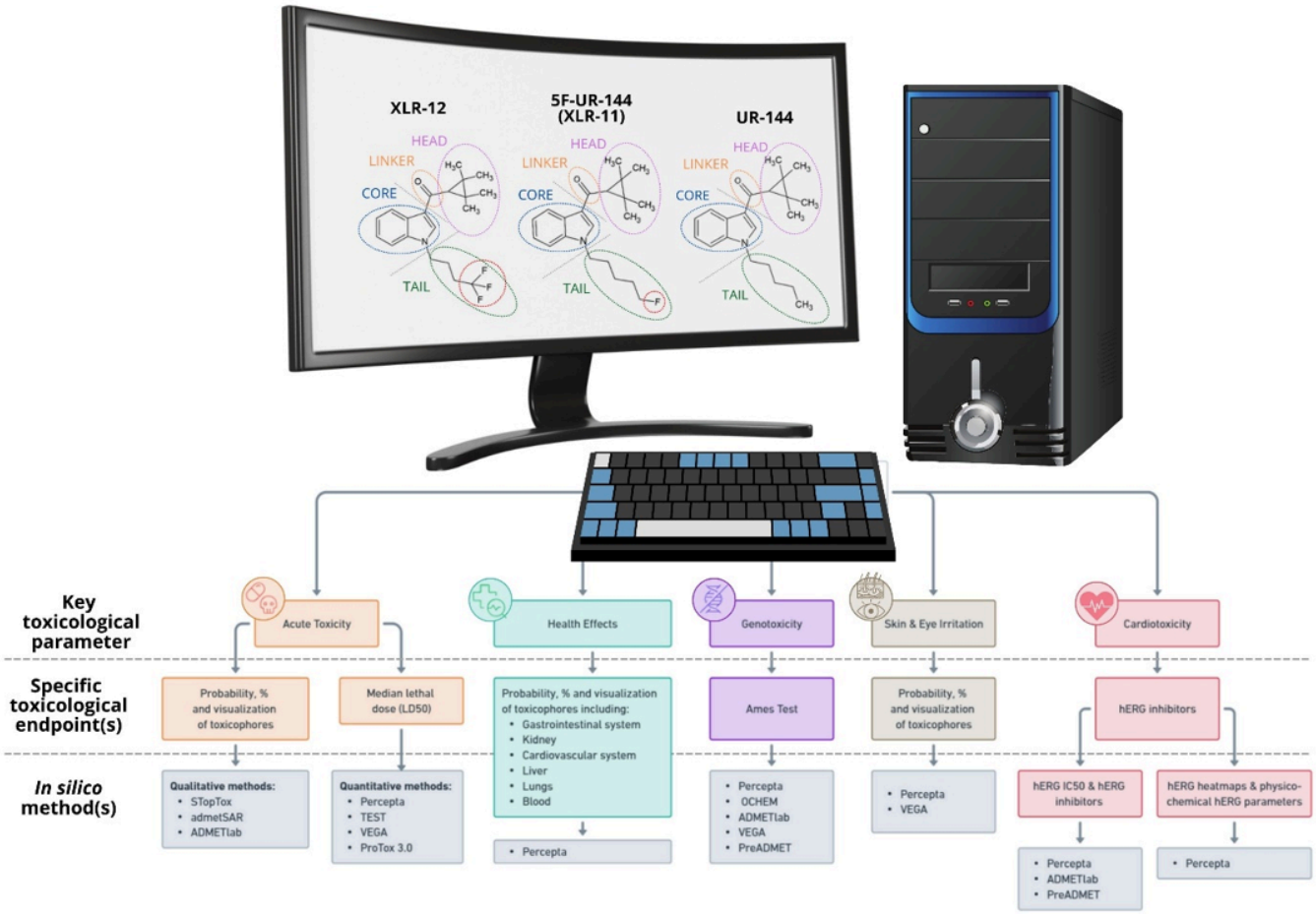

Figure 2

The workflow of conducted *in silico* studies for cyclopropylindole-based SCs: UR-144, 5F-UR-144 (XLR-11) and XLR-12 according to key toxicological endpoints using different qualitative and quantitative *in silico* methods.

Figure 3

Integrated toxicological assessment and comparative analysis of cyclopropylindole-based synthetic cannabinoids (UR-144, 5F-UR-144, XLR-12): acute toxicity, health effects, genotoxicity, irritation potential, and cardiotoxicity.

Supplementary Files

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