

Review

# Has the Toxicity of Therapeutic Deep Eutectic Systems Been Assessed?

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**Featured Application:** As emerging therapeutic systems, the toxicity of THEDES at different levels should be assessed prior to using them clinically.

**Abstract:** Therapeutic Deep Eutectic Systems (THEDESs) are a mixture of components, including an active pharmaceutical ingredient, that have recently emerged because of their interesting properties for drug therapies. In general, they have been recognized to increase the solubility and permeability of some drugs, and consequently, their bioavailability. Moreover, they have also been used for novel formulations of pharmaceuticals. Despite the potential benefits of THEDESs, concerns about their safety and toxicity remain. In this review, we summarize previous studies that have investigated the toxicity of THEDESs. These studies evaluate the toxicity of THEDESs using various methods, including cell cultures, animal models, and human trials. The results of previous findings suggest that THEDESs are generally well-tolerated and have low toxicity. However, further research is needed to fully understand the long-term effects of these systems on human health and to identify any potential adverse effects.

**Keywords:** eutectic; cytotoxicity; cell viability; gene expression; animal models; human therapeutics



**Citation:** García, C.B.; Concha, J.; Culleré, L.; Lomba, L.; Sangüesa, E.; Ribate, M.P. Has the Toxicity of Therapeutic Deep Eutectic Systems Been Assessed? *Appl. Sci.* **2023**, *13*, 5980. <https://doi.org/10.3390/app13105980>

Academic Editor: Ángel J. Gutiérrez Fernández

Received: 31 March 2023

Revised: 5 May 2023

Accepted: 11 May 2023

Published: 12 May 2023



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## 1. Introduction

The solubility of drugs in an aqueous medium is a major problem in the pharmaceutical industry. More than 50% of newly developed drug molecules suffer from low aqueous solubility [1–3]. The Biopharmaceutics Classification System (BCS) is a classification system that categorizes drugs based on their solubility and permeability properties, which are important determinants of drug absorption and bioavailability. The BCS is a classification system that categorizes drugs into four classes (I–IV) based on their solubility and permeability properties [4]. Class I drugs have high solubility and high permeability, while Class II drugs have low solubility and high permeability. Class III drugs have high solubility and low permeability, and Class IV drugs have low solubility and low permeability [5]. The poor solubility of drugs is a common problem, particularly for Class II and IV drugs [6].

There are several techniques available to improve the solubility of poorly soluble drugs, and this is important because drug solubility affects many pharmaceutical parameters such as pharmacokinetics or pharmacodynamics [7]. Some of the commonly used techniques to enhance drug solubility include physical modifications such as particle size reduction by micronization and nanosuspension, alteration of pH, manipulation of solid state, and complexation [8–10]. Other techniques for solubility enhancement include chemical modifications, such as salt formation, hydrotrophy, and precipitation inhibitors, as well as the use of surfactants, self-emulsifying drug delivery systems, microemulsions, and liquid solid methods [9,11].

During the last few years, a trend has emerged to improve drug solubilization techniques, and that is the use of Deep Eutectic Solvents (DESs) for increasing drug solubility [12,13]. These mixtures are formed by the hydrogen bond acceptor (HBA) and hydrogen

bond donor (HBD), which, at a particular molar composition, become liquid at room temperature. Their liquefaction is reached only by the interaction between their components, not involving any chemical reaction. However, the stability of DESs is a crucial factor that must be considered when evaluating their potential uses, and it was found that the hydrogen bond plays an important role in its thermostability [14,15]. As it can be possible to choose the components, they are usually non-toxic, biodegradable, and biocompatible molecules [16]. The most used components are chemicals such as sugars, alcohols, urea, natural metabolites, organic acids, and choline chloride (ChCl), among others [17,18]. When one of these components is an active pharmaceutical ingredient (API), these systems are renamed THEDESs (Therapeutic Deep Eutectic Systems). The first THEDES was reported by Stott and co-workers in 1998 by mixing ibuprofen as an API and seven skin penetration enhancers to obtain eutectic systems [19]. In a THEDES, an API can be served both as an HBA and HBD. For example, aspirin can be used as the hydrogen bond donor to form DES with ChCl [20], while lidocaine and atropine can be used as the hydrogen bond acceptor to construct DES with carboxylic acid [21].

They offer a wide range of advantages when combined. In several pharmaceutical fields, DESs have been useful in order to increase the drug solubility, permeation, and absorption [22]. Compounds such as paracetamol, aspirin, and salicylic acid are very poorly soluble in water. However, by using a DES as a solvent, for example, mixtures of urea, ChCl could dissolve benzoic acid, danazol and itraconazole [23]. Duarte et al. prepared different THEDESs based on menthol complexed with different APIs (ibuprofen, phenylacetic acid, and benzoic acid). The solubility and permeability of the systems in an isotonic solution was evaluated and compared with the pure APIs [24]. The authors concluded that with the exception of the system containing phenylacetic acid, for the rest of the mixtures, the solubility of the APIs when they are in the THEDES can be improved up to 12-fold, and the permeability can be improved up to 3-fold in comparison with the pure APIs. Therefore, this work demonstrates the efficiency of a THEDES as a new formulation in order to improve the bioavailability of APIs by changing the physical state of the molecules from a solid dosage to a liquid system. However, only a few studies study the combination of two APIs as counterparts to obtain a THEDES. This is the case of the paper published by Yin et al., who constructed a new THEDES based on two APIs derived from natural products, osthole and paeonol. Osthole has a wide variety of pharmacological functions, such as antimicrobial, antioxidant, and anticancer functions, and paeonol is known as a great anti-inflammatory, antioxidant, and antibacterial agent [25].

DESs can have a variety of applications including human health [26]. Biomedical potential applications of hydrophobic THEDESs based in menthol and saturated fatty acids with different chain lengths are studied by different authors, and for example, Silva et al. found a huge versatility of THEDESs as an approach toward the development of many effective therapies [27]. THEDESs are used as an alternative to enhance the therapeutic action of certain antibiotics [28], and also to increase the antibacterial action [29]. Pereira et al. [30] showed that the production of new THEDESs involves combining terpenes with anticancer properties, such as safranal, menthol, and linalool, with nonsteroidal anti-inflammatory drugs, such as ibuprofen and ketoprofen. To evaluate the therapeutic potential of these THEDESs, it is necessary to carry out the evaluation of their physicochemical properties and bioactivity. Moreover, Pereira et al. observed that the combination of limonene with ibuprofen as a THEDES formulation presented anticancer activities, associated with increased ibuprofen solubility [31]. Different THEDESs of ibuprofen with several terpenes such as thymol and menthol were already reported. Menthol/ibuprofen (3:1) is able to increase Ibuprofen solubility, while THEDESs with thymol did not increase its solubility. Moreover, menthol and stearic acid were used to formulate a THEDES for wound healing [27]. Interestingly, it is rather promising to develop API-NADESs (Natural Deep Eutectic Solvents) formulations based on various components from Chinese herbal medicines to improve their targeted properties, such as solubility, bioavailability, stability, or fast drug delivery capacity to the target site [32].

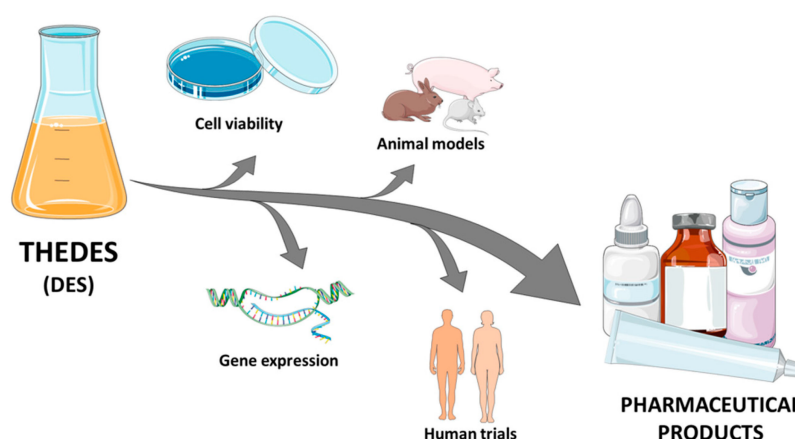
THEDESs have been also used in the formulation of pharmaceuticals, including creams and suppositories, due to their ability to improve skin permeability or drug absorption, or even to provide controlled drug release or as an alternative strategy for drugs' delivery [33–37]. In some other cases, the formulation could also improve the stability of APIs and even promote a controlled release to achieve the therapeutic effect. In the work from Mano et al. (2017) [38], the THEDES formed by ChCl-mandelic acid (1:2) and encapsulated in gelatin showed a fast-dissolving release profile in phosphate-buffered saline without cytotoxicity. The formulation also maintained the antibacterial effect of mandelic acid on both Gram-positive and -negative bacteria. Additionally, one THEDES based on a NADES (ChCl/ascorbic acid) and sunitinib malate as a drug was used to obtain an ion-gel system that could be used as a novel carrier for this anticancer drug. In addition, no cytotoxicity was found for the NADES mixture toward HN-5 cells [39]. The same NADES (ChCl/ascorbic acid) was also used to solubilize dexamethasone and a supercritical fluid sintering process was followed to obtain a controlled drug delivery system for this glucocorticoid with low water solubility. The authors highlighted the potential applications of this THEDES in biomaterials science beyond the pharmaceutical industry involving drugs [40].

To sum up, the potential advantages and main applications of THEDESs are collected in Table 1.

**Table 1.** Potential advantages and applications of THEDESs.

Potential Advantages	Applications
Higher API solubility Higher API permeability Improved API absorption Improved API bioavailability	Enhancement of therapeutic action of API Faster drug delivery Controlled drug release

The main objective of this paper is to gather different studies regarding the toxicity of THEDESs and to check if there is enough supporting information to confirm their safety to be used for human therapies. Previously, Lomba et al. carried out an exhaustive review of similar information regarding DESs [41]. Therefore, the most attention was paid on the toxicity results of THEDESs including cell lines, animal models, and human trials, as shown in Figure 1.



**Figure 1.** Focus of the review about toxicity studies developed with THEDESs.

## 2. Cell Lines

### 2.1. Cell Viability

The analysis of DESs' cytotoxicity has been an objective since they were firstly described and potential applications were proposed [26]. In fact, each time that a new mixture is characterized, and the goodness of its properties is demonstrated, its toxicity should be

also checked. Despite being a mixture of components that are not harmful, toxicological behavior of the final resulting DES can change the expected result, taking into account the components separately. For example, Hayyan et al. demonstrated a low cytotoxicity compared to ionic liquids, and they pointed that due to this fact, they could be useful for therapeutic applications. They examined the cytotoxicity of six different concentrations of several ammonium-based DESs toward seven cell lines (PC3, A375, HepG2, HT29, MCF-7, OKF6, and H413), most of them being cancer cell types, using MTT cell viability assay. When comparing the cytotoxicity results of DESs with their individual components, a synergistic effect was supposed to play a role, since the results could not be explained by the individual behavior of each one alone. Additionally, some components of the DES can show a higher toxicity, and even the effect in different cell lines can be variable [42]. One of the components that was pointed out as a potential factor that increases the cytotoxicity of DESs is oxalic acid, when fish (CCO) and human (MCF-7) cells are exposed to a eutectic mixture with ChCl. Nevertheless, the sensitivity of the cell lines was also different to this DES [43]. The HBA was considered as the main responsible factor of the cytotoxicity found by Macario et al. in their study involving several THEDESs toward two cell lines [44]. When compared to ionic liquids, NADESs have also been recognized with a low cytotoxicity profile, although some constituents, such as tartaric acid, could affect the metabolism of cells [45]. Similarly, one study about the cytotoxicity of NADESs showed that the cytotoxicity was lower than in the former version, DESs, at the same time that it highlighted the importance of several factors such as their composition (e.g., presence of organic acids) or physical properties (e.g., viscosity) influencing it [46].

Sometimes, the toxicity of DESs is searched to destroy bacteria. Hayyan et al. found that some phosphonium-based DESs were effective in the growth inhibition of both kinds of bacteria, Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) [47]. Zhao et al. characterized different ChCl-based DESs and suggested that those DESs that are acid-based could be more harmful to bacteria [48]. If the attempt of confirming the antibacterial activity of DESs is successful, DESs can even enhance the antibacterial activity of drugs used for this purpose.

Occasionally, DESs are not used to solubilize any chemotherapy agent or to enhance their activity, but they show their own cytotoxic usefulness for destroying cancer cells. In such cases, selectivity is the main property that must be verified in order to avoid side-effects [42]. Moreover, DESs have been also used as reaction media for the preparation of an anticancer compound, such as in the case of a derivate of a quinazoline. The final product was also tested using MTT assay with three cell lines (MCF-7, A549, and MCF-10A) in order to find a higher cytotoxic effect on the cancer cell lines than in the non-malignant one (MCF-10A) [49]. Other studies concerning NADESs as potential anticancer agents evaluated their cytotoxicity toward six hepatic cell lines. As expected, it was found that there were different toxicity profiles depending on the composition of NADESs as well as the tested cell lines. The metabolic pathway of each component was specifically highlighted as a possible cause of the differences [50].

Finally, DESs proposed to be used as solvents of some APIs need to be analyzed to demonstrate that they are safe enough for their latter potential therapeutic use. For instance, in the case of lamotrigine (antiepileptic agent) that is effectively solubilized by DESs consisting of ChCl and urea/ethylene glycol or glycerol, the cytotoxicity of such DESs was tested. Using an MTT cell viability assay, a moderate toxicity was found for the A549 lung cell line when exposed to three different combinations of those THEDESs [51].

As DESs are being used as part of THEDESs, it is necessary for new cytotoxicity assessments to be developed to check the possible synergistic effect of the final product, similarly to DESs [31,40,52]. They have been compiled according to the applications of THEDESs.

There are some drugs that are better solubilized with DESs, such as coumarin. Different final THEDESs were obtained, and low toxicity was found for them toward a cancer cell line [53].

The use of anti-inflammatory drugs is widely spread among the general population. Many drugs with this application are not easily dissolved, and THEDESs have risen as a good alternative to solve the problem. In fact, DESs have also been used to solubilize ibuprofen and the absence of cytotoxicity in xylitol; ChCl:water toward HaCaT (immortalized human epidermal keratinocytes) and HepG2 (hepatocellular carcinoma) cell lines using a PrestoBlue cell viability assay was demonstrated [54]. Ibuprofen is one of the most extended drugs within THEDESs, and cytotoxicity assays were developed to test if such combinations are not cytotoxic [30,55]. Nevertheless, there is one study that was performed with dexamethasone that was successfully solubilized with ChCl and ascorbic acid. At some higher concentrations, cytotoxicity was found, while it was not shown at lower ones. Interestingly, a synergistic effect was found inside the components of THEDESs combining the antioxidant activity of ascorbic acid with the activity of dexamethasone [40].

Bacterial toxicity was an outcome explored with the use of several THEDESs, while cytotoxicity toward other cell lines was also explored. Olivares et al. analyzed the potential cytotoxic effect of THEDES including betaine/urea with imipenem. They found that at the same time that betaine/urea could serve as an effective stabilizer for imipenem, it also enhanced the antimicrobial activity toward some bacteria. Moreover, its cytotoxicity was low for human fibroblasts and even when using human skin explants with the absence of histopathological changes. In this case, as the cytotoxicity profile was tested with human fibroblasts and skin explants, the authors suggested that it could be used for topical applications [56]. Silva et al. combined menthol, a terpene which generally increases the permeation of other components, with three different acids, namely, stearic acid, myristic acid, and lauric acid. The assessment of cytotoxicity was performed toward keratinocytes cells because they were searching a specific application for wound healing. The antibacterial activity of the THEDESs was maintained, and relevant cytotoxic effects were not found in the analyzed cell line [27]. Pedro et al. found a reduced toxicity profile of ciprofloxacin within THEDES combinations (with proline/urea/malonic acid or citric acid/xylitol) when compared to the cytotoxicity of ciprofloxacin alone, while the therapeutic action against bacteria was maintained. They used immortalized human epidermal keratinocytes (HaCaT) as cell models [28,57]. In general, some THEDESs show good properties when the bacterial toxicity is the objective, while the low cytotoxicity of the other exposed cell lines is maintained. A similar situation was found when amoebae was the focus of the antimicrobial activity of the THEDESs [58].

As cancer remains as a challenging disease for effective therapies, some THEDESs have also checked for this therapeutic application. Pereira et al. explored the possibility of including limonene within a THEDES while trying to decrease its intrinsic toxicity for normal cells. Mixtures of limonene with capric acid or menthol did not seem to decrease its toxicity, whereas combinations of limonene with ibuprofen (1:4) did. Moreover, a synergistic effect of this latter THEDES was found that overcomes the simple mixture of its individual components, as previously shown with the DES. Even a different mechanism of action is followed by the THEDESs compared to the isolated ibuprofen and limonene, but their advantages for cancer therapy are preserved [31]. Notably, in a review, Oliveira et al. suggested to understand the cytotoxicity process of THEDESs when affecting different kinds of cell lines, malignant and non-malignant, in order to take advantage of this knowledge to design proper therapies for specific cancer types, such as colorectal cancer [59]. Later on, Pereira et al. evaluated the usefulness of different THEDES including terpenes and nonsteroidal anti-inflammatory drugs. They found that a specific mixture containing menthol/ibuprofen (3:1) was selectively cytotoxic toward cancer cells [30].

In diabetes mellitus, chlorpropamide and tolbutamide were solubilized with tromethamine and a proportion of water after trying other conformers. In the study developed by Sarraguça et al., these THEDESs showed a low toxicity profile in two cell lines involving murine fibroblasts and human cancer cells [60].

The treatment of tuberculosis is another field where THEDESs are being analyzed. Santos et al. combined citric acid with L-arginine or ethambutol, and the cytotoxicity was

assessed using the Caco-2 cell line. Citric acid was found to be more toxic than ethambutol for this specific cell line. The acidity of the media was pointed out as a relevant factor that could increase the toxicity of THEDESs, as seen with previous studies with DESs [61].

In relation to the development of new drug delivery systems, Mano et al. found that the cell proliferation of fibroblast was not inhibited by the exposure to gelatin fibers with encapsulated THEDESs composed of ChCl and mandelic acid. At the same time, fibers impregnated with THEDES maintained the expected antibacterial activity [38]. In another study, a liquid L-arginine-based THEDES was encapsulated in a lipidic matrix. When checking its cytotoxicity toward a mouse fibroblast cell line, no toxicity was found [62]. In addition, Pedro et al. explored the cytotoxicity profile of a THEDES including ibuprofen in the form of hydrogel for transdermal administration. The main action of the ibuprofen was maintained while arginine/glycerol (1:4) was used to solubilize it. Human abdominal skin samples were used to check the enhancement of drug permeation. Additionally, murine macrophages were analyzed in relation to the cytotoxicity, and no toxicity was found [55].

A summary of the cytotoxicity studies carried out with THEDESs is gathered in Table 2.

**Table 2.** Cytotoxicity studies of THEDESs using different cell lines ordered by their therapeutic areas and dates from oldest to newest.

Therapeutic Area	Components	Cell Lines	Cytotoxicity Assay	Reference
Anticoagulation	ChCl: urea/ethylene glycol/glycerol + coumarin	Melanoma skin cell line	MTT cell viability assay	[53]
Anti-inflammation	ChCl: ascorbic acid + dexamethasone	Immortalized mouse lung fibroblasts cell line (L929)	MTS cell viability assay	[40]
Anti-inflammation	Arginine: glycerol + ibuprofen	Murine raw 264.7 macrophages	Resazurin cell viability assay	[55]
Antimicrobial	ChCl + mandelic acid	Immortalized mouse lung fibroblasts cell line (L929)	MTS cell viability assay	[38]
Antimicrobial	Menthol + stearic acid/myristic acid/lauric acid	Immortalized human epidermal keratinocytes (HaCaT)	MTS cell viability assay	[27]
Antimicrobial	Betaine: urea + imipenem	Human primary dermal fibroblasts	CellTiter-Blue® Cell Viability Assay (resazurin)	[56]
Antimicrobial	Proline: urea: malonic acid/citric acid: xylitol + ciprofloxacin	Immortalized human epidermal keratinocytes cell line (HaCaT)	MTT cell viability assay	[28]
Antimicrobial	Triethylphosphine/trihexylamine/trioctylamine + malonic acid/salicylic acid	Henrietta Lacks cervical cancer cell line (HeLa)	LDH cytotoxicity assay	[58]
Cancer	Menthol/capric acid/ibuprofen + limonene	Human colon adenocarcinoma cell lines (Caco-2 and HT29)	MTS cell viability assay (and antiproliferative assay)	[31]
Cancer	Safranal/menthol/linalool + ibuprofen/ketoprofen/flurbiprofen	Human colon adenocarcinoma cell lines (Caco-2 and HT29)	MTS cell viability assay (and antiproliferative assay)	[30]
Diabetes mellitus	Tromethamide: water + clorpropamide/tolbutamide	Immortalized mouse lung fibroblasts cell line (L929) and human colon adenocarcinoma cell line (Caco-2)	MTT cell viability assay	[60]
Tuberculosis	Citric acid + L-arginine/ethambutol	Human colon adenocarcinoma cell line (Caco-2)	MTS cell viability assay	[61]
Tuberculosis	Citric acid + L-arginine	Immortalized mouse lung fibroblasts cell line (L929)	MTS cell viability assay	[62]

## 2.2. Gene Expression

Although the above findings classify THEDESs as generally safe, little is known about their interaction at the cellular and molecular level, and how they can affect DNA structure and gene expression [63].

Considering the little evidence about genotoxicity, similar strategies to those carried out in the studies with NADESs could be followed. They must be focused on genes related to the mechanism of action of drugs whose solubility is intended to be increased.

The evidence to date for the genotoxicity of DESs/NADESs is more extensive, having been tested in different cell lines and methods. Between them, gene expression or DNA damage are measured. Grozdanova et al. (2020) [64] carried out a comet assay, a single-cell gel electrophoresis for measuring DNA strand breaks, to assess the genotoxicity of tested NADESs applied for the extraction of two medicinal plants as follows: ChCl: glycerol/*Sideritis scardica*, ChCl: glycerol/*Plantago major*, Citric acid-1,2—propanediol 1:4 (CAPD)/*Sideritis scardica* and CAPD/*Plantago major*. All tested NADESs were harmless for CCL1 cells, and the genotoxicity was concentration-dependent [64]. Ammonium-based DESs with glycerin, ethylene glycol, triethylene glycol, or urea were tested on MCF-7 cells to evaluate DNA integrity. The results obtained via DNA fragmentation analysis on agarose gel show that the cell deaths were not caused by DNA damage [42].

Trans-resveratrol is a natural polyphenol widely used in cosmetics and the pharmaceutical industry, both in its topical and oral forms (e.g., antioxidants and hypolipidemic supplements). When exposed to UV light, it is isomerized to trans-1,4,6-trihydroxyphenanthrene (THP), with genotoxic effects. Proline/glycerol NADES showed a photo-protective effect under UV exposure, inhibiting the isomerization of resveratrol and THP formation [65]. Moreover, another study evaluated resveratrol, improving its bioavailability via lipid conjugation. Endothelial permeability protection was evaluated by MMP-9 inhibition, which plays an important role in the inflammation and healing processes. Resveratrol-linoleic acid dissolved in NADESs, composed by 1,2-propanediol:ChCl:water, showed the highest MMP-9 inhibitory effect in THP-1 tested cells [66].

A study carried out by Szél et al. (2019) [67] tested the protective effect to hyperosmotic stress produced by sorbitol addition of glycerol and xylitol in keratinocyte HaCaT cells. The inflammatory cytokines' expression was measured to evaluate stress cell levels. The results show that both polyols decreased IL-1 $\alpha$  expression, while only glycerol affects IL-1 $\beta$  and NFAT5 expression, despite having similar chemical structures.

Lee et al. (2022) [68] tested a fermented NADES ginger extract on HTC-116/R cells (colorectal cancer model). The NADES ginger extract has a higher phenolic content and antioxidant activity than organic solvent extract. The NADESs were composed of a mixture of betaine/lactate/water. The fermented NADES extract were shown to inhibit the expression of NF- $\kappa$ B (via activity assay) and CXC chemokine receptor 4 (CXCR4) (using Real time PCR) genes, enhancing the therapeutic effects in oxipalatin-resistant CRC cells.

Likewise, similar studies, such as Wang et al. (2019) [69], were carried out in ionic liquids, demonstrating their influence on the expression of p53 and Bcl-2 family genes, and showing NADESs' cytotoxic and apoptotic effect on hepatocellular carcinoma (HepG2) cells treated with 1-dodecyl-3-methylimidazolium chloride.

To the best of our knowledge, the only study really considered as a THEDES formed by betaine-urea DESs containing imipenem was tested in monocyte cells, showing that this formulation increases therapeutic efficacy. The immunogenic response of the THP-1 cells was measured by the proinflammatory cytokines gene expressions (IL-1, IL-6, and TNF- $\alpha$ ). This THEDES showed no immunogenic effect, suggesting its potential as a formulation [56].

## 3. Animal Models

Regarding the toxicity of DESs, to date, these compounds have been tested mainly in invertebrates and animal models (rodents).

Studies in invertebrate animals included *Daphnia magna*, *Artemia salina*, and *Hydra sinensis* with mixed results in the toxicity of the DES use. In reference to the *Daphnia magna*

toxicity test, only two studies evaluated different DESs [70] or NADESs [71], and none of the tested combinations induced significant toxicity toward this model. Hayyan et al. tested different DESs in *Artemia salina* to determine the toxicity and the mortality. The results of the assays indicated that the toxicity of DESs was higher than their individual components, suggesting that the toxicity can be influenced by the composition of DESs by varying the salt/HBD combination and molar ratio [42,47]. The study of *Hydra sinensis* concluded that the survival times of *Hydra sinensis* were significantly shortened by exposure to a DES-containing aqueous solution, suggesting that the DES exhibited a toxic effect on the hydras. It was suspected that depending on the type of HBD of the eutectic mixtures, the charge will be delocalized in the HBA and modify the toxicity [72].

Regarding more complex animal models, Hayyan et al. also tested the toxic potential of ammonium-based DESs in vivo in a murine model. As in *Artemia salina* had shown previously, in mice tests, DESs were relatively more toxic in comparison to their individual components [38]. Consequently, Mbous et al. tested the toxicity of DESs and NADESs in mice, showing that NADESs presented higher toxicity than DESs, and induced liver failure due to their viscosity, which made the circulation of the mixture difficult [50]. In the same line, Benlebna et al. administered phenolic NADES extract from green coffee beans to Wistar rats, and its oral administration induced mortality in two rats, and short-term side effects such as excessive water consumption, weight loss, hepatomegaly, and plasma oxidative stress associated with hyperlipemia [73].

On the other hand, more recent articles observed less toxicity on the DESs studied. Nurunnabi et al. investigated the toxicity of oral CAGE (Choline bicarbonate and Geranic acid) administration in rats treated for 30 days. The results obtained indicate that the CAGE formulation was well tolerated, that no changes were found in the red blood cells and platelet count of treated rats compared with the controls [74]. Zhao et al. used metabolites of *Coptidis rhizoma* extract to artificially prepare the NADES. Then, the toxicity in the mice was evaluated, showing that after the oral administration of the NADES, the mice became irritable and presented dyskinesia, which means that the NADES itself showed an acute toxicity to the mice. However, the mice that were given 10% dilution of the NADES in water showed no toxicity, and none died after oral administration [75].

Different NADESs/DESs were used to improve the bioavailability and/or biocompatibility of different compounds [36,76–80]. Xiao et al. developed a novel hydrogel system incorporating an amino acid-based DES. The DESs were integrated into Carbomer® 940 hydrogels for the skin delivery of “sanwujiawwan” extract, a Chinese herbal medicine. Sanwujiawwan is composed of six ingredients, some of them with a certain degree of toxicity. By preparing the DES extract complex, it demonstrated good dissolution and skin permeability of the ingredients in the extracts. The authors used a collagen-induced-arthritis rat model, and the hydrogel with the DES extract complex exerted an enhanced therapeutic effect that reduced the inflammatory response with systemic toxicity of the extracts [32].

Due to their high stabilization and solubilization power, DESs offer the ability to tune the solubility, permeation, and absorption of APIs. For that, APIs with low solubility or permeability could be improved by including these solvents on their formulation in in vivo studies. The toxicity of THEDESs has been tested primarily on animals such as mice, rats, or pigs focused on the preclinical angle. Previous studies are shown according to their routes of administration and are summarized in Table 3.

**Table 3.** Animal model studies with API dissolved in DESs.

Route of Administration	Components	Animal Model	Reference
Injection: intravenous (in vivo)	Choline bicarbonate: oleic acid (CODES) + verteporfin	Balb/c female mice	[81]
Injection: subcutaneous (in vivo) + skin from dorsal side (ex vivo)	CAGE + Apomorphine	Wistar rats and pigs	[82]



Table 3. Cont.

Route of Administration	Components	Animal Model	Reference
Injection: transdermal (in vivo) + porcine skin (ex vivo)	CAGE + insulin	Male Wistar rats and Porcine skin	[83]
Injection: transdermal skin (ex vivo)	CAGE + insulin	Porcine skin	[84]
Injection: subcutaneous (in vivo)	Choline glycolate, acetylcholine glycolate, choline lactate, acetyl-choline lactate, choline propionate, acetylcholine propionate, choline hexenoate, acetylcholine hexenoate, CAGE, and acetylcholine geranate (aCAGE) + insulin.	Balb/c female mice	[85]
Injection: transdermal iontophoresis (in vivo)	ChCl/urea, ChCl/glycerol, and ChCl/ethylene glycol + insulin	Diabetic rats	[86]
Injection: skin topical (in vivo)	Capric acid: Risperidone	Male rodents	[87]
Injection: intraperitoneal (in vivo)	3-(4-(4-(bis(2chloroethyl)amino)phenyl)butanoyloxy)-N,N,N-trimethyl propane-1-aminium chloride (CABAL), 1,4-butanediol + doxorubicin	Adult female Sprague Dawley rats	[76]
Injection: topical and subcutaneous (in vivo)	Arginine (Arg)-citric acid (CA) + Methotrexate	Male Wistar rats	[88]
Oral (in vivo)	Betaine, Mandelic acid (Bet-Man NADES) + RA-XII	Healthy male Sprague Dawley rats	[89]
Oral (in vivo)	Fumaric acid + Diacerein 2,4-dihydroxybenzoic acid + Diacerein	Healthy Sprague Dawley rats	[90,91]
Oral (in vivo)	Mixtures of sugars (glucose, xylitol, sorbitol), amino acids (glutamic acid, proline), organic acids (citric, malic, oxalic, and tartaric acid), and other nitrogen-containing compounds (urea, ChCl, acetylcarnitine and carnitine) + Berberine	Balb/c female mice	[36]
Oral (in vivo)	Malic acid + Berberine	Grade II ICR mice (male and female)	[75]
Oral (in vivo)	ChCl/glycerol + salvianolic acid B	Mice (male and female) and Sprague Dawley male rats	[92]
Oral (in vivo)	CAGE + insulin	Adult nondiabetic male Wistar rats	[93]
Oral (in vivo) + porcine buccal tissue (ex vivo)	CAGE + chitosan + insulin	Nondiabetic adult male Wistar rats and Yorkshire pigs	[94]
Nasal (in vivo)	ChCl and malic acid (CM-DES) + insulin	Male Sprague Dawley rats	[95]

### 3.1. Injection Routes: Transdermal/Subcutaneous/Intravenous Administration

Injection administrations require the drug to be delivered through the skin with a needle (subcutaneous, intravenous, or transdermal). Some drugs are delivered body-wide through a patch on the skin. These drugs are sometimes mixed with chemical compounds, as DESs, that enhances penetration through the skin into the bloodstream without any injection. According to transdermal skin delivery, it is difficult to assess the skin permeability of materials using only in vivo experiments. Consequently, numerous ex vivo models are frequently employed to assess drug skin permeation profiles and kinetic parameters [96].

Kim et al. used DESs to formulate a nanocomplex for the systemic administration of verteporfin, a chemotherapeutic drug. They investigated the efficacy of the nanocomplex

administered intravenously in female mice bearing a 4T1 tumor in the mammary pad. The authors observed a better drug penetration into the tumor tissue, probably due to the DESs' contribution to the fluidization of the tissue through the interaction between the DESs and membrane lipids. In addition, greater drug sensitivity and effective inhibition of orthotopic tumor growth was shown [81]. The same authors reported a deep eutectic-based formulation that slows the release of apomorphine after subcutaneous injection. In this case, CAGE1:2 and a mixture with the drug was prepared, which is called SEAPORT formulation. The skin from the dorsal side of Wistar rats was used to study apomorphine release from SEAPORT *ex vivo* and it was observed that CAGE 1:2 plays a critical role in its release. Moreover, *in vivo* pharmacokinetic of the apomorphine from subcutaneously injected SEAPORT was measured in the rats and pigs and showed a prolonged duration of a higher apomorphine concentration in plasma in comparison to the clinical comparator (reference drug formulation) [82].

Other studies also synthesized CAGE, as DESs, and included them in drug formulations for the application in *ex vivo* and *in vivo* models, mainly with insulin, because of its difficult transdermal delivery by conventional chemical permeation enhancers. Banerjee et al. reported that CAGE can deliver insulin across porcine skin and significantly reduce blood glucose levels in rats when insulin-CAGE is topically administered. The *ex vivo* penetration into the skin samples was measured using porcine skin; to measure the insulin-CAGE penetration, Fluorescein isothiocyanate-labeled insulin (FITIC-insulin) was added to CAGE and applied to the porcine skin. *In vivo* experiments were performed in male rats applying transdermal insulin in CAGE [83]. Tanner et al. synthesized variants of CAGE by controlling the ratio of a range of 1:4 to 2:1. In this case, an *ex vivo* pig's skin model demonstrated that the 1:2 and 1:4 molar ratio variants were capable of transporting FITIC-insulin through to the stratum corneum, epidermis, and dermis (transdermal transport) [84]. Recently, Curreri et al. developed a new tool toward improving subcutaneous formulation using biocompatible deep eutectics. The DESs were synthesized using a salt metathesis reaction at the cation/anion ratios of 1:2 and were then formulated with solubilized 100 U/mL of regular insulin. Pharmacokinetics of subcutaneous insulin administration using deep eutectics (SPADES-insulin) was compared against the equivalent dose of the clinical gold standard of the fast-acting insulin analog (Humalog) in male Wistar non-fasting rats and improved the systemic absorption of subcutaneously injected insulin faster than Humalog. The toxicity was analyzed in healthy female Balb/c mice receiving a single subcutaneous injection of DES solutions and demonstrated that SPADES is a safe and non-toxic formulation [85]. In a new paper, DESs were introduced as novel chemical penetration enhancers for transdermal iontophoresis of insulin across rat skin. The results of the *in vivo* studies indicated a reduction in the blood glucose level in diabetic rats using ChCl/ethylene glycol and ChCl/glycerol in the iontophoresis delivery of insulin [86].

Al-Akayleh et al. investigated the skin permeability of risperidone, an antipsychotic drug, using eutectic systems. The prepared THEDES mixture of risperidone and capric acid was spread on the skin of male rodents and was found to be tolerable, less irritant, and safe for application to the skin compared with the control untreated skin. A histopathological study was performed that did not show changes in the state of the skin and morphological changes [87].

Rajan et al. synthesized DESs, which influenced the formation of folic acid (FA)-tagged  $\gamma$ -beta-alanine-co-Poly  $\epsilon$ -caprolactone (PCL) polymer (DES@FA- $\gamma$ - $\beta$ -alanine-co-PCL polymeric system) to encapsulate the doxorubicin drug. The enhanced anticancer potential of doxorubicin-loaded polymeric micelle was studied in an *in vivo* breast cancer model. The advantage of this polymeric system is that it could increase the bioavailability of hydrophobic doxorubicin drugs. The intraperitoneal injection of doxorubicin-loaded polymeric micelle significantly reduced the body weight and tumor size when compared with dimethylbenzanthracene-induced mammary carcinoma-bearing rats and polymeric micelle-treated rats [76].

Recently, Li et al. reported a transdermal delivery strategy using a silica nanoparticle matrix (MSN) encapsulated with methotrexate and impregnated in DES hydrogels for the topical management of rheumatoid arthritis. This formulated DES-MSN hydrogel system was applied to the skin of rats to test the skin irritation and the *in vivo* toxicity of this system. They observed a sustained penetration and accumulation of MSNs at subcutaneous inflammation sites [88].

### 3.2. Oral Administration

Regarding *in vivo* pharmacokinetic studies, Liu et al. administered RA-XII dissolved in 5–20% *v/v* Betaine and Mandelic acid (NADES) solution in rats. The authors observed that the oral absorption of RA-XII dissolved in 20% NADES solution was significantly increased. They concluded that NADES solutions were effective approaches for improving solubility, permeability, and oral bioavailability of the anti-tumor candidate RA-XII [89]. Patel et al. prepared a novel eutectic of Diacerein with fumaric acid (FMA) as a cofomer in various molar ratios. Diacerein and its active metabolite, called rhein, are anthraquinone derivatives used for treating osteoarthritis. Healthy Sprague Dawley rats were distributed in two groups and administered pure Diacerein and prepared eutectic suspension orally, respectively. Blood samples were collected, and pharmacokinetic parameters were analyzed after oral administration. The results of the pharmacokinetic evaluation suggested greater oral bioavailability of the prepared eutectic versus pure Diacerein [90]. These authors repeated the same study but changed the fumaric acid for 2,4- dihydroxybenzoic acid (DHA) as cofomer, and again, the pharmacokinetic study in the rats proved higher oral bioavailability of the prepared eutectic [91]. Berberine is a quaternary benzylisoquinoline alkaloid with huge properties as an antihypertensive, antiarrhythmic, anti-inflammatory, and hypolipidemic, among other properties. Despite its therapeutic properties, berberine is poorly absorbed after oral administration and its plasma concentration in pharmacokinetic studies is extremely low. In this study, several NADESs were prepared and used to solubilize berberine. The NADESs selected were urea or organic acids (malic and lactic acid), and proline. The berberine solubilized in the selected eutectics mixtures were used to evaluate their pharmacokinetic properties in the female Balb/c mice and were compared with berberine. The plasma levels were determined up to 6 h after oral administration via gavage in the mice. The administration of NADESs with berberine yielded in a significant increase in the plasma level of berberine [36]. These authors were in concordance with another recent report that observed that the pharmacokinetic parameters of berberine were improved in the liver and indicated that NADES dilutions promoted the intestinal absorption of oral berberine by increasing solubility in mice. The authors suggested that NADESs may help to promote its use in oral drug delivery [75].

Another natural compound, the salvianolic acid B with anti-inflammatory and antioxidant properties, was studied, adding the model drug in ChCl/glycerol with the common molar ratio (ChCl/glycerol 1:2) as a drug vehicle for this poorly soluble compound. The results obtained demonstrated that the oral administration using gavage of ChCl/glycerol (1:2) was non-toxic with LD<sub>50</sub> 7733 mg/kg in mice. The results indicated that ChCl/glycerol (1:2) could accelerate the absorption and elimination of salvianolic acid B, maybe because the eutectic system could enhance the membrane penetration ability of drugs [92].

Barnejee et al. also studied the efficacy of insulin-CAGE administered through the oral route. For the oral efficacy in *in vivo* studies, enterically coated capsules were administered via oral gavage to adult nondiabetic male rats. *In vivo*, when 10 U/kg insulin-CAGE was administered, a 45% decrease in blood glucose was observed, and by delivering insulin-CAGE, the authors evade gastric degradation of insulin and enhance its intestinal permeability [93]. Other authors reported the fabrication of a biodegradable polymeric patch for the buccal delivery of insulin using CAGE, as DESs, to transport facilitator and chitosan as mucoadhesive matrix. They applied *ex vivo* and *in vivo* studies. The *ex vivo* permeation was performed using porcine buccal tissue to study the extent of drug transport. The results show that CAGE induced an increase in insulin transport and enhanced insulin

penetration through buccal tissue. To test in vivo the performance of insulin delivered by the patches, the patches were placed in the pouches of the nondiabetic rats in the buccal cavity of the rats. The authors concluded that the CAGE/insulin patches placed in the rat buccal pouch lowered the blood glucose levels, and non-tissue damage was shown [94].

### 3.3. Nasal Administration

Nasal administration was proposed as an alternative administration route for insulin. Like the transdermal and oral routes, this route must be evaluated to determine if there is toxicity and to determine if it is a good route of administration. Li et al. developed a biocompatible DES as a carrier for improving the nasal delivery of insulin. Nasal toxicity was evaluated in vivo using rats, after intranasal epithelia administration of the solution. The authors concluded that the in vivo studies demonstrated that DES caused no evident toxicity to rat nasal epithelia and could improve the permeability of nasal epithelia, because DESs absorb water to facilitate the disassociation of the structure and the release of drugs upon contact with the nasal mucus [95].

Regarding delivery methods, the studies mentioned focused on oral, buccal mucosa, transdermal, intravenous injection, subcutaneous injection, and topical delivery with very good results and low toxicity. However, other potential routes require investigation, such as ocular, sublingual, or vaginal/anal drug delivery.

## 4. Human Trials

The properties of DESs have been widely demonstrated at different levels, yet there are only few studies carried out directly in humans, and none of them are considered as conventional THEDESs. The antimicrobial properties of DESs and their ability to deliver drugs transdermally have generated great interest in the dermatology industry, mainly in [33,55]. One of the first human trials focuses on the treatment of rosacea. The study of Ko et al. showed that DES with a composition of CAGE is a good candidate to improve the effectiveness of rosacea treatment due to the good results of in vitro studies with this ionic liquid/DES [97,98]. Its high ability to penetrate into the deep layers of the skin allows for the treatment of pathogens at this level [83]. The CGB-400 gel, with a CAGE1:2 composition, has managed to complete the “seven steps of translation”, reaching the final step of human studies for the treatment of rosacea. The high antibacterial activity of CAGE against *Propionibacterium acnes*, associated with the biological origin of rosacea, has also been demonstrated [98]. This clinical trial was conducted in 52 patients with rosacea, and a 34.6% reduction in the mean papules and pustules typical of this disorder was observed after only two weeks of therapy with CGB-400 gel. At 12 weeks, the reduction observed was much greater, specifically, 71.9%. This study and the process to obtain the final product open a door to other products based on the properties that have been demonstrated for CAGE. The use of CGB-400 for the reduction in facial redness and bumps and blemishes are still in phase one of clinical trials (<https://www.cdek.liu.edu/api/119951/#trials>, accessed on 24 March 2023) [99].

Human tissues were also used for ex vivo analysis of toxicity. For example, abdominal skin samples were obtained to carry out a permeation study of risperidone. Al-Akayleh et al. concluded that the amount of this drug permeated as part of a THEDES including capric acid was high [83]. Pedro et al. also performed a permeation assay of an alginate-based hydrogel containing ibuprofen using human abdominal skin as an ex vivo model. The authors showed that the permeation of ibuprofen was improved compared to that obtained with the hydrogel without the DES [51].

## 5. Conclusions

Despite being recognized as promising solvents with many potential biomedical applications, THEDESs have not been fully analyzed on their toxicity profile, although there are some studies about this topic. There is a lack of comprehensive toxicity studies available in the literature. DESs have received more attention about their toxic profile,

but combinations with APIs are not always recognized as THEDESs, and further research is needed to fully assess the potential risks associated with their use. As soon as a new THEDES arrives, its toxicity should be assessed, especially if it is going to be used for therapeutics. It is suggested that toxicity should be checked systematically since these kinds of data are scarce. As the complexity of the models increased, a smaller number of studies were developed. Interestingly, some authors have found a synergistic effect when mixing original compounds of the THEDESs, and toxicity can change in both directions, so it is necessary to test any new combination.

Since each THEDES has its own composition with unique properties, it is difficult to establish a universal toxicity testing protocol that would be applicable to all THEDESs. However, the development of such model would aid in the safe and effective use of THEDESs in their various applications. As THEDESs are tailored to be used for specific therapeutic areas, the models used to carry out the toxicity assessment should vary. Notably, an effort must be made to maintain the individual bioactivity of the components of the THEDESs, whereas some additional advantages could be found. Regarding cell cultures and gene expression, cell lines and genes must be selected in relation to the intended application. This should be the first step before using more complex animal models or even human trials. In animal models, a variable toxicity was found, with injection being the most tested route of administration of the compounds.

This manuscript is focused on the toxicity of THEDESs specifically, but it is also essential to investigate their degradation products to ensure they are non-toxic as well. THEDESs can break down into various substances, some of which may have different toxic properties from the original compound. Therefore, the investigation of the toxicity of THEDESs' degradation products is also crucial to ensure their safe use.

**Author Contributions:** Conceptualization, C.B.G. and L.L.; writing—original draft preparation, C.B.G., J.C., L.C., E.S. and M.P.R.; writing—review and editing, C.B.G., L.L. and E.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was partially funded by Departamento de Ciencia, Universidad y Sociedad del Conocimiento, from the Gobierno de Aragón (Spain) (Research Group B58\_23D).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Abbreviations

API	Active Pharmaceutical Ingredient
BCS	Biopharmaceutics Classification System
CAGE	Choline bicarbonate and Geranic acid
CAPD	Citric acid-1,2—propanediol 1:4
ChCl	Choline Chloride
DES	Deep Eutectic Solvents
DHA	2,4-Dihydroxybenzoic Acid
FA	Folic Acid
FMA	Fumaric Acid
FITIC-insulin	Fluorescein Isothiocyanate-Labeled Insulin
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
MSN	Silica Nanoparticle Matrix
NADES	Natural Deep Eutectic Solvents
PCL	Poly $\epsilon$ -Caprolactone
THEDES	Therapeutic Deep Eutectic Systems
THP	Trans-1,4,6-Trihydroxyphenanthrene

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