

Identifying, Naming and Documenting of Test and Tool Compound Stocks

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Abstract

Handling of chemicals is an often-neglected area of test descriptions. Some important aspects are highlighted here, using methyl-phenyl-tetrahydropyridine (MPTP), ferrous sulfate ($\text{FeSO}_4 \cdot x\text{H}_2\text{O}$) and ciguatoxin as example compounds. These are used to provide some background on aspects of acid-base equilibria, redox state, crystal water, natural compound mixtures, and chemical naming systems. Also, solvents and impurities are addressed, for instance concerning their often high (millimolar range) concentrations in assay buffers and cell culture media. The discussion of these aspects calls for a more standardized preparation of test solutions and a more extensive disclosure of the procedure in publications; it also suggests more flexibility in data mining, as compounds with clearly different identifiers may have been used to produce highly similar or fully identical test conditions. While this short overview is not intended as definitive guidance, it does demand more active involvement of all test developers and performers with these issues, and it calls for more transparent information disclosure concerning the preparation and use of test and control chemical solutions.

1 Anchor story

Assume that a screening assay for neurotoxicants uses ciguatoxin, MPTP and FeSO_4 as positive controls. The protocol suggests that stocks of 5 mg/mL be prepared for all three compounds. In a ring trial, it is observed that the data on the controls differ between participating laboratories. Moreover, retrospective analysis of data is performed in a company known to be using an extremely standardized test system and having all handling steps automated. It is found that there are pronounced variations in the control responses over time. The test managers claim that the assay has been validated and found to be extremely reproducible. Confronted with the troubling news of intra- and inter-laboratory variability, important elements of the test are re-checked (Bal-Price et al., 2018; Schmidt et al., 2017; Leist et al., 2010): test system, analytical

endpoint, prediction model, and exposure scheme. All were very robust and of highest technical and scientific standards.

2 Defining chemicals

What could be the explanation for the variability observed in the above example? The element of a test that is most often neglected is the clear definition of test chemicals and of positive and negative controls. Some of those closely familiar with testing regulations may now claim that this problem has long been solved, as there is clear guidance on chemical definitions. They are right. But as is the case with many complex problems, the opposite is also correct: Daily practice shows that there is a lack of knowledge in the broad community on chemical specifications or a neglect to

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follow available guidance or a hesitance to consider the issue important, or a combination of these. In the published literature, exact definitions of test chemicals are still an exception rather than common practice (Leist and Hengstler, 2018). Even in dedicated methods publications, like the Protocol Exchange database of *Nature Research*¹ or the journal *Nature Methods*, clear chemical identifiers are not commonly included. Guidance on these issues rather is found, e.g., in the Good In Vitro Method Practices (GIVI-MP) document (OECD, 2018) or in the guidance on Good Laboratory Practice (GLP). According to this, a standard operation procedure (SOP) should always define the control chemicals exactly. General guidance on test descriptions, like OECD GD 211 or its more extensive version, the ToxTemp (Krebs et al., 2019), do not give detailed advice. However, the issue is very clearly addressed, e.g., in documents on important conditions for collaborative testing campaigns (Krebs et al., 2020; Moné et al., 2020; Delp et al., 2018, 2019; Nyffeler et al., 2017a,b), for developmental neurotoxicity screening (Masjosthusmann et al., 2020 – Appendix A), and also in the establishment of toxicological *in vitro* testing databases such as ToxCast². The following chapters will make it clear why the names “ciguatoxin”, “MPTP” and “FeSO₄” are not well-suited as chemical identifiers and why, therefore, the positive controls used for testing in our anchor story example may have varied in concentrations or even have contained different substances.

3 Naming of chemicals

Hydroxyethane, CH₃-CH₂OH, EtOH, ethyl alcohol, grain alcohol and ethyl hydroxide are a few of the many ways to name a chemical with the preferred IUPAC name “ethanol”. The redundancy of names of chemicals obviously causes problems for databasing, systematic reviews, etc. Moreover, there are instances where one name may refer to clearly different chemical entities (e.g., glucose may refer to L-glucose or D-glucose, and it might mean the ring-closed pyranose form or the ring-opened aldose). Sometimes, the problem of chemical definition is addressed by making reference to the supplier and by including the respective catalogue number. This approach has some merits (i.e., it is better than no definition at all), but such information is neither universal nor of lasting value: catalogue numbers may change, and they may differ between catalogue versions (in different countries and in different years); most importantly, they are ephemeral, as supplier companies frequently merge or vanish altogether. For this reason, more standardized approaches have been developed.

The best-known naming system is based on the activity of the Chemical Abstracts Service (CAS), which assigns a CAS number (CASN) to all chemicals identified in publications (currently > 160 million annotated compounds). A rapidly-growing alternative, with much additional information linked to the compounds, is PubChem³, with currently > 230 million entries (identified by

a CID number). The advantage of both CASN and CID is that isomers of a given compound receive separate numbers. Also, mixtures (e.g., isomers of one structure or assemblies of different compounds) can have their own IDs. A disadvantage is that the IDs have no semantic value, i.e., they do not describe the underlying chemical. They merely serve as a pointer to a database entry that contains the structural information. For instance, the CASN of ethanol is 64-17-5 and the CID is 702. Such an ID cannot be used directly for *in silico* approaches that require structural knowledge. However, this is not a disadvantage when the main purpose is the clear definition of control and tool compounds for *in vitro* testing.

Other chemical identifiers do contain the full structural information (if combined with canonical background knowledge). A classic example is the full IUPAC name. A version of this with improved machine-readability is, e.g., the International Chemical Identifier (InChI). It is a textual identifier for chemical substances that encodes the molecular structural information. InChIs can be seen as a highly standardized and extremely formalized version of IUPAC names. Their advantage compared to CASN is that they directly encode the chemical structure, that they can be derived for any given structure (whether it is already in a database or not), and that they are mostly human-readable (given some training and practice). The InChI for ethanol is, e.g., 1/C2H6O/c1-2-3/h3H,2H2,1H3.

An alternative naming system that contains structure information is the simplified molecular-input line-entry system (SMILES). The SMILES for ethanol is CCO. Many applications allow a direct conversion of SMILES or InChI into chemical formulae and *vice versa*. The machine-readability makes these systems the preferred basis for cheminformatic and other *in silico* approaches. However, it also must be noted that the chemical universe is so incredibly large and complex that none of these systems is free of flaws in particular cases (e.g., special stereochemistry cases, formation of zwitterionic forms or tautomeric structures).

4 Salt status/aggregate state

A specific issue arises for biochemical, pharmacological and toxicological studies (i.e., when the bioactivity of a chemical structure is studied) from the fact that chemical definition depends to some extent on the aggregate state. Let's take the example of iron sulfate. In the example, a chemical formula of FeSO₄ was given. This means that we are dealing with ferrous iron (oxidation state: 2+) and not with ferric iron (oxidation state: 3+). This can be of massive functional importance. The same applies to the oxidation states, e.g., of manganese, copper and lead ions, and it is astonishing how often such information is missing. For instance, toxicological publications often refer to lead acetate, not specifying whether Pb²⁺ or Pb⁴⁺ is meant.

¹ <https://protocolexchange.researchsquare.com/>

² <https://comptox.epa.gov/dashboard>

³ <https://pubchem.ncbi.nlm.nih.gov/>

For many applications studying the bioactivity of Fe^{2+} , it does not matter whether the sulfate salt is used or whether another anion is present (e.g., phosphate), providing that chelation effects and potential precipitation are excluded. The same applies to the study of other ions, including organic ions, when activity in solutions is of interest. This is the case for most experimental systems based on cell cultures and common laboratory animals. Moreover, it also applies to most studies on humans (considering that their intra- and extracellular spaces can be considered buffered salt solutions with an osmolarity of about 300 mOsM. Figuratively speaking, the Fe^{2+} ion “does not remember which salt it was released from”, and therefore the original counterion is not of importance. Once in solution, Fe^{2+} is simply a hydrated ion, mostly surrounded by water. In all cell culture media and in human extracellular fluids, its major counterion will be chloride, no matter which original salt was used to add Fe^{2+} to the system.

This difference between the solid state (used to produce a stock) and the solution as such (used for functional studies) applies not only to the counterions but also to the crystal water. When Fe^{2+} and SO_4^{2-} ions are combined to form a salt, the crystal structure may include water molecules. For instance, it is most common that for each Fe^{2+} ion, seven water molecules are imbedded in the solid state of FeSO_4 . This compound is called ferrous sulfate heptahydrate or vitriol and has the CID 62662. Its molecular formula is written $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (MW: 278). This differs considerably in its molecular weight (MW) from anhydrous FeSO_4 (CID 24393, MW: 152), but also from iron sulfate hexahydrate ($\text{FeSO}_4 \cdot 6\text{H}_2\text{O}$; CID 9859974), rozenite (iron sulfate tetrahydrate, CID 182426), ferrous sulfate pentahydrate ($\text{FeSO}_4 \cdot 5\text{H}_2\text{O}$; CID 22033958), and several other forms of “ FeSO_4 ”. Due to the up to two-fold difference in MW, the concentration of stock solutions may be far from correct if stocks are produced based on a weight per volume basis, and the crystal water information is not considered. Notably, all these compounds can be used to produce *exactly* the same solution of FeSO_4 , and there is no way to determine any difference among the solutions depending on which salt was used to prepare them. In practice, this means that different starting compounds can be used safely to produce exactly the same test solution if these issues are taken into consideration.

5 Acid-base equilibria

A variation on this theme has to do with the effects of pH. This is exemplified here for carboxylic acids and organic amines. A typical example is valproic acid (iso-octanoic acid, CID: 3121, CASN: 99-66-1). In a physiological buffer, it would be mostly present in its ionized form as valproate (CID: 3549980) (Kisitu et al., 2020). In the presence of sodium ions (always present in large quantities in any physiological buffer or body fluid), it may also be called sodium valproate (CID: 16760703, CASN: 1069-66-5). The relative amounts of valproic acid and valproate will depend *only* on the pH, not on the compound used to produce the solution. This means that the two different compounds (in solid form), valproic acid and sodium valproate will result in *exactly* the same solution if dissolved in a physiological buffer or cell cul-

ture medium and given that the pH is adjusted to the same value. There is no method that could distinguish between a solution produced from CID 3121 or CID 3549980. However, when preparing the solutions, care must be taken to use the same molar amount (weights will differ) and to adjust the pH. If low concentrations of valproate or of valproic acid are added to strong buffers, or if they are present in self-buffering systems (via the CO_2/HCO_3 balance) like human body fluids, there will be no difference whether one starts from the acid or the base form. In this case, the pH does not even need to be adjusted.

The same principle described for valproic acid applies to weak organic bases, e.g., 1-methyl-4-phenyl-tetrahydropyridine (MPTP; CID: 1388; CASN: 28289-54-5; MW: 173) (Schildknecht et al., 2015) (Fig. 1). Such compounds often dissolve poorly in water, and their solubility is increased greatly in acidic solutions as the N-base becomes protonated and thus charges positively. As with the carboxylic acids, it is only the pH that decides on the ratio of the protonated and non-protonated structures. It does not matter which form is originally dissolved: It will always be exactly the same solution of “MPTP” with its protonated and non-protonated variant determined by the solution’s pH. This also implies that the acid used to change the pH does not play any role. It may be HCl, sulfuric acid or acetic acid. In physiological solutions, the major counterion will always be chloride, no matter which acid is used to lower the pH. If the protonated amine is transferred into the solid aggregate state, it is most commonly generated as chloride salt. This may be written generally as amine- H^+/Cl^- . A typical example is ammonium chloride ($\text{NH}_3\text{-H}^+/\text{Cl}^-$, also written as NH_4Cl). For more complex organic amines, it is sometimes not entirely clear where the protonation happens and to which extent this can be seen as a covalent bond. For this reason, the writing convention is to note down the non-protonated form linked with a “·” sign to HCl. Examples are $\text{NH}_3 \cdot \text{HCl}$ (ammonium chloride) or MPTP·HCl. This means that the solid state contains the protonated form of the amine together with chloride ions. Hundreds of medical drugs consist of organic amines (here generically called drug-N). They are frequently produced in form of their protonated version (drug- NH^+), which is usually denoted as drug-N·HCl (e.g., propranolol·HCl). Such solid compounds are obviously different from the crystalized non-protonated form (drug-N). However, the difference is not relevant when the drugs are dissolved. For instance, MPTP·HCl has an own identifier (CID: 161406; CASN: 23007-85-4; MW: 210) and a different MW from MPTP. However, as shown for the hydrates and for the carboxylic acids, once MPTP or MPTP·HCl are dissolved in medium or a physiological buffer, they cannot be distinguished (given the pH is the same). Both solutions produced from MPTP or from MPTP·HCl will contain exactly the same molar amount of the drug substance and have exactly the same ratio of protonated and non-protonated forms. The same applies also to propranolol (a beta-blocker) and hundreds of other drugs. The consequence for chemical testing procedures and for the preparation of control solutions is that it does not matter which compound one starts from. Only the weight difference has to be considered, as MPTP·HCl contains about 18% less of the active compound (Fig. 1). Figuratively speaking, “MPTP needs the addition of a little HCl when it is to be dissolved

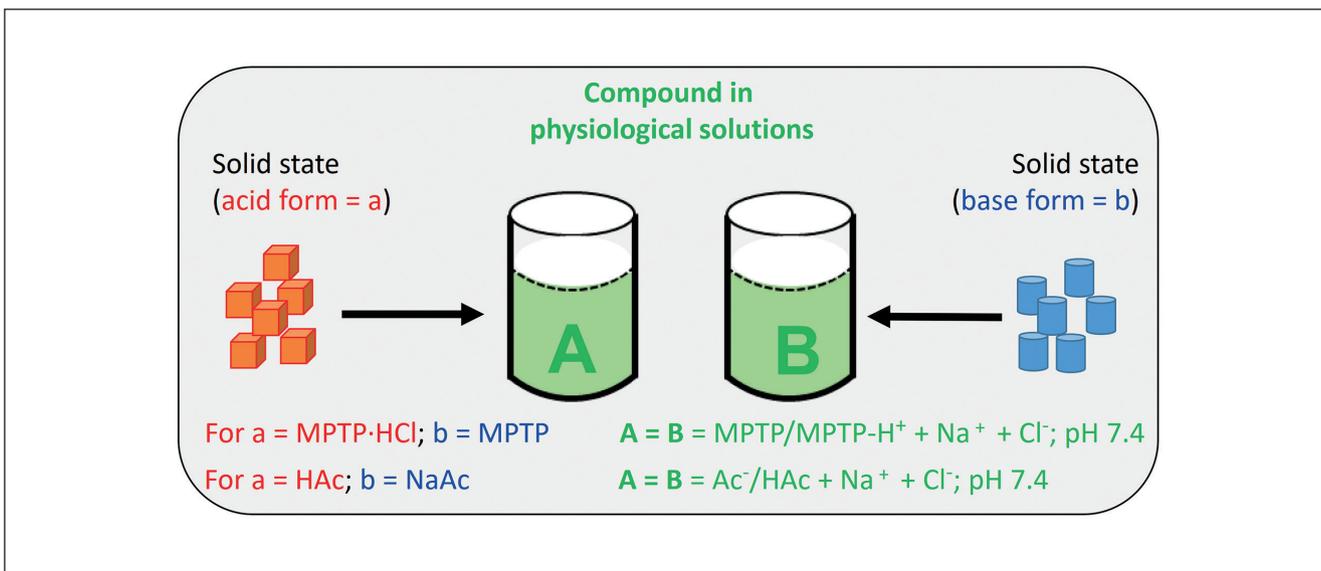


Fig. 1: Role of acid-base equilibria for the solid state and solutions of test compounds

Two exemplary compounds shown here are MPTP and acetic acid (HAc). The base form (non-protonated) of the MPTP molecule is MPTP. The acidic molecular variant (protonated) is MPTP-H⁺. The base form can be generated as a crystalline solid containing only MPTP. The MPTP-H⁺ can be produced as a chloride salt, which is denoted MPTP-HCl and contains an equal amount of Cl⁻ ions and MPTP-H⁺ ions. On a mass basis, solid MPTP-HCl contains 18% less MPTP molecules than the same mass of MPTP powder. The balance is made up mainly by Cl⁻ ions (and to a small extent by the additional proton). Theoretically, the same amount (based on the number of MPTP molecules, independent of their base/acid state) may be dissolved in a given volume of water. To do this, e.g., 1 mg of MPTP or 1.21 mg of MPTP-HCl would need to be dissolved (e.g., in one liter). In this case, the solution produced from MPTP-HCl would be acidic (because of its content of HCl). Both solutions would contain the same total number of MPTP and MPTP-H⁺ molecules, but their ratio would be different, because the ratio of the base and acid form is determined by the pH. Adding a small amount of a base (NaOH) to the solution produced from MPTP-HCl could bring it to exactly the same pH as that of the solution produced from MPTP. As the pH is the *only* factor that determines the ratio of MPTP and MPTP-H⁺, both solutions then would be identical concerning their MPTP content, but the solution produced from pure MPTP would not contain any salt, while the other solution would contain some NaCl. The practical experiment depicted here differs in a small detail from the theoretical experiment described above. Instead of dissolving the compounds in pure water (a non-physiological condition not compatible with the survival of cells or tissues), the solid states would be dissolved in a physiological solution (e.g., cell culture medium or blood plasma). Such a solution differs in two important aspects from water: First, it contains a very high concentration (about 120 mM) of NaCl. Second, it is buffered, i.e., it maintains its pH also when acids and bases are added. The consequence of the first point is that addition of a small amount (e.g., 10–100 μM; a typical highest concentration for compound screens) of NaCl does not make any practical difference (it is below the normal variation arising from experimental errors, water evaporation, etc.). The consequence of the second point is that solutions A and B will have the same pH (some potential minor differences will be eliminated entirely in buffers relying on hydrogen carbonate buffering and pH adjustment via a constant CO₂ gas concentration). In consequence, solutions A and B will be identical for all practical purposes, even though they are produced from compounds with different IDs in their solid state. The same would apply to a typical weak acid like HAc. One can produce the same solution starting from pure HAc or from pure sodium acetate (base form).

in water, while MPTP·HCl contains this HCl already in its solid structure". Once dissolved this way, neither the chloride, nor the amount of MPTP, nor the fraction of protonation will differ. The solutions are identical, whether one starts from the base or the protonated form.

6 Biologics and redox processes

The above paragraphs dealt with special cases of mixtures: In the case of weak bases and acids, this referred to the protonated and non-protonated forms; otherwise, chemical identity was

given. In the case of Fe²⁺, it was briefly mentioned that the oxidation state may play a role. Although ferric and ferrous iron (Fe³⁺/Fe²⁺) are based on one and the same atom, they can have largely different biological activities. The same applies to other redox couples (e.g., reduced and oxidized glutathione: GSH/GSSG). Different from the situation with acids and bases, where the pH determines the composition, the control of redox ratios is not so clear and easy. Moreover, some oxidation processes may be (pseudo-)irreversible (because of complex formation or precipitation) or because of coupled reactions that remove or precipitate as mixed oxide-hydroxide ("rust"), and thus the over-



all iron concentration in a solution may be reduced; GSSG may react further with other thiols or protein-sulfhydryl groups and thus be removed, and, e.g., dopamine may form large polymers and pigments (melanin) in an autoxidation process that removes dopamine (Lotharius et al., 2005). This means that stock solutions may over time change their molecular composition, and thus the concentration of the agent to be tested may be altered. Great care is therefore required for all reagents prone to redox reactions, and sometimes only active measures (e.g., stabilization by chelators or antioxidants) can ensure sufficiently stable working stocks.

Another mixture issue is frequently encountered when compounds isolated from biological sources (here named biologics) are used. Notoriously difficult and complex are lipopolysaccharides or, e.g., heparin. Also, cyclic peptides like microcystins or antimycin A can be complex mixtures of various congeners (Delp et al., 2019; Daneshian et al., 2013). Vendors may offer mixtures of different composition and bioactivity. Large potency differences have, e.g., been found for ciguatoxins, and it is of high importance whether, e.g., ciguatoxin-2 (CID: 76957583) or ciguatoxin-3 (CID: 76966075) is used, as these have the same MW but different activity (Daneshian et al., 2013).

7 Purity

As ciguatoxins are prepared from natural sources (and not by chemical synthesis), different lots of ciguatoxin-2 may also contain other ciguatoxins and additional bioactive molecules. This example indicates the importance of purity information. If the full information is not available, testing may still be performed, but under conditions that ensure consistency (standardization) of the purity level used. The issue does not apply only to complicated biologics, but to virtually all substances. Even a purity of 99.9% of a small molecular drug means that it contains 1 mg of other compounds for each gram of drug. With potencies of test compounds stretching over 5-7 log steps in many assays, such levels of impurities (0.1% level) may be of relevance.

In this context, it is important to understand solvents also as “a type of impurity”, even though these are added intentionally. Often compounds are dissolved in DMSO and then diluted in medium to a final concentration of 0.1% (v/v) DMSO. Such dilutions of DMSO are used in many tests and are often considered negligible. The use of percentage as unit of solvent concentration has many disadvantages (Kisitu et al., 2019), including that it hides the fact that this is a concentration of 14,000 μM (= 14 mM), i.e., easily 3 orders of magnitude higher than that of many test compounds. The same applies to ethanol (0.1% corresponds to 17 mM). As it is illegal in most countries to drive a car with a blood concentration of 0.1% ethanol (because of its narcotic/psychotropic effects), such concentrations are very likely to affect cells. Besides the high concentrations as such, solvents bring along another potential problem. Even if, e.g., the DMSO is 99.9% pure, the remaining impurity (0.1%) may still be in a concentration range that is similar to that of the test compounds (0.1% of 14,000 μM is 14 μM ; many screens are run at

maximum concentrations of 10-50 μM). This problem cannot be entirely avoided, but it can be reduced by using high-quality solvents and by using highly standardized conditions. Such quality control measures do not relate only to the solvent source, but to the entire procedure of producing stock solutions for storage and working. Hardly any academic publication reports on preparation and handling of stocks and on the procedures to generate working solutions and final medium from the stocks. Closing this quality gap may make tests more reproducible when compared over time in one laboratory or when compared between laboratories.

8 Defining chemical identity

The above paragraphs have given an impression of the astonishing complexity and difficulty of knowing what (in terms of chemical composition) is actually tested in a pharmacological, toxicological or biochemical assay. Considering this, one may ask whether absolute and generalizable knowledge on this is possible? The clear and definitive answer is no. However, this is no reason for frustration, as the available knowledge only has to be sufficient for one concrete and defined test situation, and as we can, for many aspects at least, define the limits of uncertainty. In practice, this means that often the information needs of a given test are satisfied by the available knowledge (Hartung et al., 2019; Coecke et al., 2005; Pamies et al., 2017, 2018). For instance, many solvent effects can be controlled, preparation of stock solutions and their further processing can be standardized, compounds can be specified concerning mixture components, crystal water, oxidation state and acid-base equilibria, and several further quality control measures can be taken to control for pH, oxidation and degradation.

In some advanced screening facilities, it has become common practice to ascertain (by analytical methods) for each sample that it does contain the intended chemical, that the concentration is in the expected range, and that degradation products and contaminants do not exceed certain limits. An alternative to classical analytical methods is the functional definition of chemicals by their bioactivity. This allows quality control as per the purpose of a test. If, for instance, a tool compound is used to block a protease or kinase pathway, it is possible to assess the extent of blockage (of kinase target phosphorylation) in the test system and to define acceptance criteria for the use of such a tool compound (Scholz et al., 2018; Gutbier et al., 2018). Such approaches are used more and more commonly in high-quality scientific publications and can only be encouraged. For instance, in the case of MPTP, the acid-base chapter shows that there is no chemical difference whether a test solution is produced from MPTP or from MPTP·HCl. Complementary to this, the functional effects of such solutions may be compared, e.g., concerning formation of the toxic metabolite MPP⁺ or the killing of dopaminergic neurons. Such assays would then indicate the functional/biological identity of test solutions prepared with MPTP or MPTP·HCl from various sources, synthesis methods or batches.



References

- Bal-Price, A., Hogberg, H. T., Crofton, K. M. et al. (2018). Recommendation on test readiness criteria for new approach methods in toxicology: Exemplified for developmental neurotoxicity. *ALTEX* 35, 306-352. doi:10.14573/altex.1712081
- Coecke, S., Balls, M., Bowe, G. et al. (2005). Guidance on good cell culture practice. A report of the second ECVAM task force on good cell culture practice. *Altern Lab Anim* 33, 261-287. doi:10.1177/026119290503300313
- Daneshian, M., Botana, L. M., Dechraoui Bottein, M.-Y. et al. (2013). A roadmap for hazard monitoring and risk assessment of marine biotoxins on the basis of chemical and biological test systems. *ALTEX* 30, 487-545. doi:10.14573/altex.2013.4.487
- Delp, J., Gutbier, S., Klima, S. et al. (2018). A high-throughput approach to identify specific neurotoxicants / developmental toxicants in human neuronal cell function assays. *ALTEX* 35, 235-253. doi:10.14573/altex.1712182
- Delp, J., Funke, M., Rudolf, F. et al. (2019). Development of a neurotoxicity assay that is tuned to detect mitochondrial toxicants. *Arch Toxicol* 93, 1585-1608. doi:10.1007/s00204-019-02473-y
- Gutbier, S., Spreng, A.-S., Delp, J. et al. (2018). Prevention of neuronal apoptosis by astrocytes through thiol-mediated stress response modulation and accelerated recovery from proteotoxic stress. *Cell Death Differ* 25, 2101-2117. doi:10.1038/s41418-018-0229-x
- Hartung, T., De Vries, R., Hoffmann, S. et al. (2019). Toward good in vitro reporting standards. *ALTEX* 36, 3-17. doi:10.14573/altex.1812191
- Kisitu, J., Bennekou, S. H and Leist, M. (2019). Chemical concentrations in cell culture compartments (C5) – Concentration definitions. *ALTEX* 36, 154-160. doi:10.14573/altex.1901031
- Kisitu, J., Hollert, H., Fisher, C. et al. (2020). Chemical concentrations in cell culture compartments (C5) – Free concentrations. *ALTEX* 37, 693-708. doi:10.14573/altex.2008251
- Krebs, A., Waldmann, T., Wilks, M. F. et al. (2019). Template for the description of cell-based toxicological test methods to allow evaluation and regulatory use of the data. *ALTEX* 36, 682-699. doi:10.14573/altex.1909271
- Krebs, A., van Vugt-Lussenburg, B. A., Waldmann, T. et al. (2020). The EU-ToxRisk method documentation, data processing and chemical testing pipeline for the regulatory use of new approach methods. *Arch Toxicol* 94, 2435-2461. doi:10.1007/s00204-020-02802-6
- Leist, M., Efremova, L. and Karreman, C. (2010). Food for thought ... Considerations and guidelines for basic test method descriptions in toxicology. *ALTEX* 27, 309-317. doi:10.14573/altex.2010.4.309
- Leist, M. and Hengstler, J. G. (2018). Essential components of methods papers. *ALTEX* 35, 429-432. doi:10.14573/altex.1807031
- Lotharius, J., Falsig, J., van Beek, J. et al. (2005). Progressive degeneration of human mesencephalic neuron-derived cells triggered by dopamine-dependent oxidative stress is dependent on the mixed-lineage kinase pathway. *J Neurosci* 25, 6329-6342. doi:10.1523/JNEUROSCI.1746-05.2005
- Masjosthusmann, S., Blum, J., Bartmann, K. et al. (2020). Establishment of an a priori protocol for the implementation and interpretation of an in-vitro testing battery for the assessment of developmental neurotoxicity. *EFSA Supporting Publications* 17, 1938E. doi:10.2903/sp.efsa.2020.EN-1938
- Moné, M. J., Pallocca, G., Escher, S. E. et al. (2020). Setting the stage for next-generation risk assessment with non-animal approaches: The EU-ToxRisk project experience. *Arch Toxicol* 94, 3581-3592. doi:10.1007/s00204-020-02866-4
- Nyffeler, J., Dolde, X., Krebs, A. et al. (2017a). Combination of multiple neural crest migration assays to identify environmental toxicants from a proof-of-concept chemical library. *Arch Toxicol* 91, 3613-3632. doi:10.1007/s00204-017-1977
- Nyffeler, J., Karreman, C., Leisner, H. et al. (2017b). Design of a high-throughput human neural crest cell migration assay to indicate potential developmental toxicants. *ALTEX* 34, 75-94. doi:10.14573/altex.1605031
- OECD (2018) Guidance Document on Good In Vitro Method Practices (GIVIMP). *OECD Series on Testing and Assessment, No. 286*. OECD Publishing, Paris. doi:10.1787/20777876
- Pamies, D., Bal-Price, A., Simeonov, A. et al. (2017). Good cell culture practice for stem cells and stem-cell-derived models. *ALTEX* 34, 95-132. doi:10.14573/altex.1607121
- Pamies, D., Bal-Price, A., Chesné, C. et al. (2018). Advanced good cell culture practice for human primary, stem cell-derived and organoid models as well as microphysiological systems. *ALTEX* 35, 353-378. doi:10.14573/altex.1710081
- Schildknecht, S., Pape, R., Meiser, J. et al. (2015). Preferential extracellular generation of the active parkinsonian toxin MPP+ by transporter-independent export of the intermediate MPDP+. *Antioxid Redox Signal* 23, 1001-1016. doi:10.1089/ars.2015.6297
- Schmidt, B. Z., Lehmann, M., Gutbier, S. et al. (2017). In vitro acute and developmental neurotoxicity screening: An overview of cellular platforms and high-throughput technical possibilities. *Arch Toxicol* 91, 1-33. doi:10.1007/s00204-016-1805-9
- Scholz, D., Chernyshova, Y., Ückert, A.-K. et al. (2018). Reduced A β secretion by human neurons under conditions of strongly increased BACE activity. *J Neurochem* 147, 256-274. doi:10.1111/jnc.14467

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