The use of AcutoX, a human-relevant animal product-free *in vitro* assay, to predict acute oral toxicity of surfactants

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Introduction

Acute oral toxicity is currently assessed using the rodent LD_{50} test following OECD test guidelines 420, 423 and 425. These tests are widely criticized on scientific and ethical grounds, as they lack reproducibility (Hoffman, 2010) and relevance to human exposure. Previously, we have shown that it is possible to predict acute oral toxicity with the *in vitro*, cell-based AcutoX assay (Ward *et al.*, ALTEX, 2025).
This assay offers a protective prediction (correct or higher classification) for EPA and GHS hazard categories in 90.2% and 93.4% of cases, respectively. The AcutoX assay was developed using a curated library of 70 chemicals, with cytotoxicity calculated in the presence and absence of a human metabolic component. Surfactants are often mis-classified in *in vitro* assays, as they disrupt the membrane of cells grown in 2D and 3D cultures, resulting in over-prediction of toxicity classifications. Here, we examine a library of surfactants using the AcutoX assay and assess the use of the current prediction model for these chemistries.

Methods

Eighteen surfactants, representing a range of chemistries and toxicity classifications were selected in collaboration with industry partners (Table 1).
These test chemicals were first assessed to confirm their solubility in tissue culture media. In 96-well plates, adult human dermal fibroblasts (HDFa; Figure 1), grown in animal-product free media containing human serum, were exposed to a 10-fold dilution series of soluble test chemicals in the presence or absence of a pooled human liver extract (human S9) for 3- or 24-hours, respectively. At 24 hours post dose the viability was assessed using the neutral red uptake (NRU) assay and an EC_{50} was calculated used to determine a narrower range of concentrations to be tested in the main assay.
In the main test, HDFa cells were subsequently exposed to a 2-fold dilution of test chemicals in the presence or absence of a pooled human liver extract (human S9) for 3- or 24-hours, respectively, and viability assessed by both NRU (a measure of cell membrane integrity) and MTT (a measure of cellular metabolic activity) assay. EC_{50} values were calculated for each endpoint. The AcutoX prediction models that assign “Highly Toxic” or “Low Toxicity” predictions from EC_{50} values were applied to the data, and further refinement to predict EPA and GHS classifications performed. The performance of the prediction models with these chemicals was assessed against the known acute oral toxicity classifications of the surfactants.

Figure 1. Human Dermal Fibroblasts

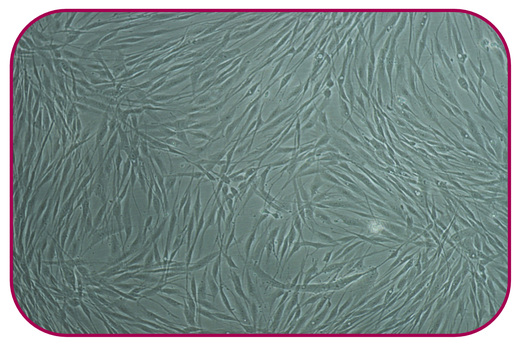

Table 1. List of Surfactants

Chemical	CAS	Type	Category
Tween 20	9005-64-5	Non-ionic	polyoxyethylene sorbitol ester
Triton X-100	9036-19-5	Non-ionic	octyl phenol ethoxylate ether
Sodium Dodecyl Sulfate	151-21-3	Anionic	Sulfate-based surfactant
Pureact Gluco L*	110615-47-9	Non-ionic	Sugar-based nonionic surfactant
Span 80- Sorbitan Monooleate	1338-43-8	Non-ionic	Sorbitan-based surfactant- low HLB
Empigen BAC-50	68424-85-1	Non-ionic	Cocamidopropylamine
Cola®Teric COAB-UP	147170-44-3	Amphoteric	Betaine-type amphoteric surfactant
Nansa HS 90/S	68411-30-3	Anionic	anionic surfactant
Empigen BS/H50	61789-40-0	Non-ionic	Cocamidopropylamine
Suga®Det L*	110615-47-9	Non-ionic	Sugar-based nonionic surfactant
Dodecylbenzenesulfonic acid solution	27176-87-0	Anionic	Sulfonate-based surfactant
(1-Hexadecyl)trimethylammonium chloride, 96%	112-02-7	Cationic	Quaternary ammonium compound
N-Dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate	14933-08-5	Cationic	Quaternary ammonium compound
Sodium Lauryl Ether Sulfate	68891-38-3	Anionic	Sulfate ether-based surfactant
Didecyldimethylammonium chloride	7173-51-5	Cationic	Quaternary ammonium compound
Cola®Mate LA-40 MB	26838-05-1	Anionic	sulfosuccinate surfactant
Cola®Lux CAO-35	68155-09-09	Amphoteric	Amidoamine oxide surfactant
N,N,N-Trimethyldocosan-1-aminium chloride	17301-53-0	Cationic	Quaternary ammonium compound

Table 2. Summary of Test Method

PRELIMINARY TESTING	Establish solubility limit in testing media.
DOSE RANGE FINDING (DRF) EXPERIMENT	Dose cells for 3 h +S9 and 24 h –S9 with highest soluble concentration and 10-fold dilution. Perform NRU assay and use resulting EC_{50} to determine top concentration for main test.
MAIN TEST	Dose cells for 3 h +S9 and 24 h –S9 using the concentration from DRF and a 2-fold dilution. Perform NRU and MTT assay to calculate specific EC_{50}. Repeat experiment for n=3.
ANALYSIS	Apply the prediction model to the results and determine likely GHS or EPA category. Xcellr8 category provided alongside comparison chemicals with similar toxicity.

Results

The EC_{50} values from the NRU experiment correlated well with those from the MTT assay in the absence and presence of metabolism (R^2 = >0.99), suggesting good reproducibility across the assays (Figure 2A) . All surfactants examined were over-classified for EPA hazard category with 17/18 surfactants predicted as EPA category 1 and the remainder as EPA category 2. The GHS hazard category prediction was similarly over-classified with 14/18 surfactants predicted as GHS category 1, a further 3 surfactants were predicted as GHS category 2 and the remainder as GHS category 3. However, a clear, stepwise increase in EC_{50} was observed with increasing hazard category.
For example, in the EPA classification system, the mean EC_{50} for NRU assays performed in the absence of liver metabolism were 0.004 mg/mL, 0.05 mg/mL and 1 mg/mL for EPA categories 2, 3 and 4, respectively. For the same endpoint, the mean EC_{50} were 0.04 mg/mL and 0.7 mg/mL for GHS categories 4 and 5, respectively (Figure 2B & 2C).
By comparing EC_{50} values from this experiment with historic LD_{50} values, a surfactant specific prediction model was developed to categorize each test chemical into both the EPA and GHS acute toxicity classifications. In the first stage, precision was prioritized producing rates of 76% and 75% for NRU assays performed in the absence and presence of liver metabolism respectively. Both results are assessed, and the higher toxicity category is selected to produce a more protective prediction. The prediction model has a precision of 100%, 90.9% and 60% for EPA hazard categories 2, 3 and 4, respectively. For the GHS hazard categories 4 and 5, precision of 100% and 22% are achieved while offering a protective prediction (correct or higher category). These data are presented for the EPA and GHS systems in Table 3 and Table 4.

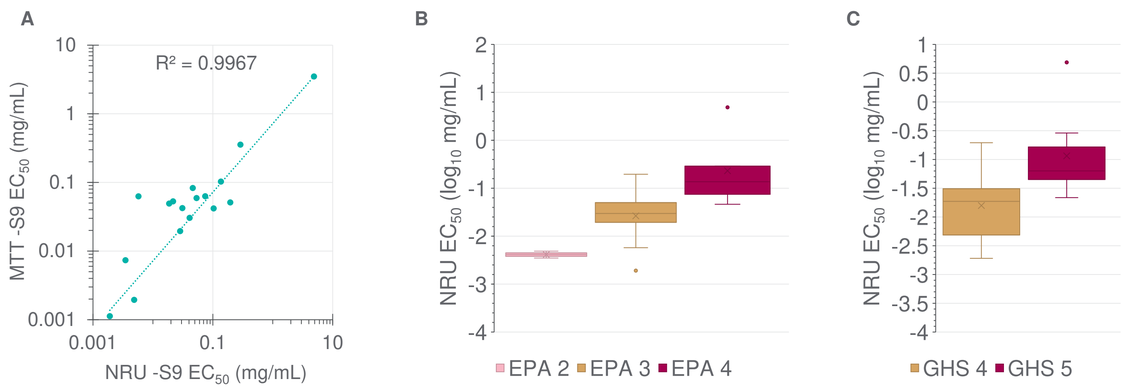

Figure 2.
A. Correlation between NRU -S9 and MTT -S9 EC_{50} results.
B. NRU –S9 EC50 results plotted against EPA classification.
C. NRU –S9 EC50 results plotted against GHS classification.

Table 3. AcutoX prediction for EPA and GHS category

Name	CASN	Hazard category		NRU -S9 AcutoX prediction		NRU +S9 AcutoX prediction	
		EPA	GHS	EPA	GHS	EPA	GHS
Tween 20	9005-64-5	4	5	4	5	4	5
Triton X-100	9036-19-5	3	4	3	4	3	4
Sodium Dodecyl Sulfate	151-21-3	3	4	3	4	3	4
Pureact Gluco L*	110615-47-9	4	5	3	4	3	4
Span 80- Sorbitan Monooleate	1338-43-8	4	5	4	5	4	5
Empigen BAC-50	68424-85-1	2	4	2	4	2	4
Cola®Teric COAB-UP	147170-44-3	3	5	3	4	4	5
Nansa HS 90/S	68411-30-3	3	5	4	5	3	4
Empigen BS/H50	61789-40-0	3	5	3	4	3	4
Suga®Det L*	110615-47-9	4	5	4	5	3	4
Dodecylbenzenesulfonic acid solution	27176-87-0	3	4	3	4	3	4
(1-Hexadecyl)trimethylammonium chloride, 96%	112-02-7	3	4	2	4	2	4
N-Dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate	14933-08-5	3	4	4	5	3	4
Sodium Lauryl Ether Sulfate	68891-38-3	4	5	4	5	4	4
Didecyldimethylammonium chloride	7173-51-5	2	4	2	4	2	4
Cola®Mate LA-40 MB	26838-05-1	3	4	4	5	3	4
Cola®Lux CAO-35	68155-09-9	3	4	3	4	3	4
N,N,N-Trimethyldocosan-1-aminium chloride	17301-53-0	3	5	NR	NR	3	4

Table 4. AcutoX prediction performance for EPA and GHS categories

AcutoX Surfactant Prediction						Precision (%)	Over-categorised (%)	Protective (%)
EPA Category		1	2	3	4			
	2	0	2	0	0	100.0%	0.0%	100.0%
	3	0	1	10	0	90.9%	9.1%	100.0%
	4	0	0	2	3	60.0%	40.0%	100.0%

AcutoX Surfactant Prediction							Precision (%)	Over-categorised (%)	Protective (%)
GHS Category		1	2	3	4	5			
	4	0	0	0	9	0	100.0%	0.0%	100.0%
	5	0	0	0	7	2	22.2%	77.8%	100.0%

Discussion

The AcutoX assay is scientifically and ethically robust screening alternative to the rodent LD_{50} assay and these proof of principle data suggest that, while the original AcutoX prediction model is not suitable for surfactants, it is possible to develop a prediction model to address specific chemistries. Indeed, we are able to differentiate between EPA and GHS categories using this assay with precision, over-categorisation and under-categorisation rates of of 83%, 17% and 0% respectively for EPA classification, and 61%, 39% and 0%, for GHS classification, demonstrating a protective effect.
The prediction model for surfactants is preliminary at this stage based on a small dataset. Further development and validation of a prediction model for these chemistries is ongoing.
The AcutoX assay could be run alongside *in silico* approaches such as the CATMoS collaborative acute toxicity modeling suite computational model (Mansouri, 2021), to improve the prediction and is considered to be suitable for inclusion in a weight of evidence approach for acute oral toxicity classification
The AcutoX assay contributes to a registrant’s 3Rs initiative by replacing aspects of *in vivo* acute oral toxicity testing and offers a novel animal product-free and metabolically relevant, human *in vitro* approach to acute toxicity assessment.

References

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The authors have stated that there are no conflicts of interest. CT, HG, TAW, IF and KH are XCellR8 employees.