In vitro-to-in vivo extrapolation (IVIVE) for liver safety assessment of anthraquinones

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Abstract

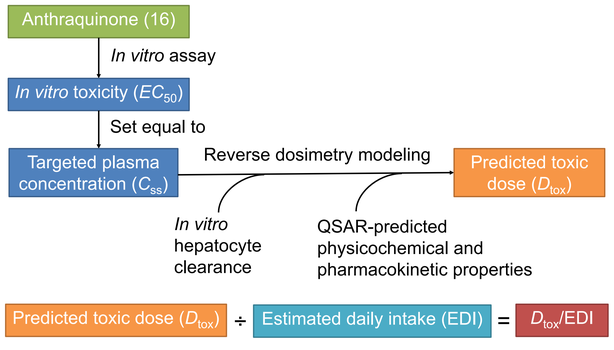
Anthraquinones natural occurrence in botanicals may lead to their presence in foods, dietary supplements and traditional Chinese medicines. Based on this presence, safety concerns such as hepatotoxicity have been raised. Due to a lack of toxicokinetic and liver toxicity data for anthraquinones in animals and humans, there is a need to conduct time- and cost-effective safety assessment to prioritize and select anthraquinones for more in-depth studies. *In vitro-to-in vivo* extrapolation could bridge between *in vitro* toxicity and an *in vivo* dose that causes toxic effects therefore enabling rapid and effective safety evaluation. Here, a combined *in vitro* cytotoxicity and *in silico* reverse dosimetry approach was adopted to evaluate the potential human liver toxicity of 16 anthraquinones and derivatives. First, cytotoxicity (EC_{50}) in two human liver cell lines (HepG2/C3A and HuH-7) was measured under two conditions (single and repeated dosing, 72 hrs). Second, toxic doses (D_{tox}) required to yield plasma steady-state concentrations (C_{ss}) equal to *in vitro* EC_{50} values were predicted by reverse dosimetry simulation using a PBPK model. Finally, D_{tox} was compared to literature-derived estimated daily intake (EDI) of anthraquinones to assess liver safety. Among the 16 anthraquinones, rhein was identified as a potential hepatotoxicant due to a combination of cytotoxicity, plasma concentration, and daily intake level. These *in vitro* and *in silico* findings provide preliminary data and guidance for further animal and clinical studies to confirm liver toxicity of anthraquinones.

Introduction

Anthraquinones are widely distributed in various botanicals such as Rhubarb, Fo-Ti, and Aloe. They have various bioactivities including anti-cancer, laxative, anti-inflammatory effects. Due to their presence in dietary supplements and traditional Chinese medicines, safety concerns such as hepatotoxicity have been raised.

Alternatives to animal testing, namely *in vitro* and *in silico* methods, are emerging as inexpensive and rapid ways to conduct initial safety assessment for large numbers of chemicals with limited toxicological and toxicokinetic information.

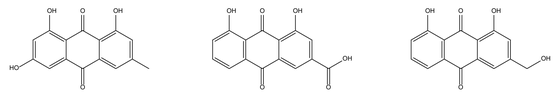
Here, an *in vitro-to-in vivo* extrapolation (IVIVE) approach combining *in vitro* cytotoxicity and *in silico* reverse dosimetry modeling was used to conduct the liver safety assessment for 16 anthraquinones.



Materials and Methods

Anthraquinones and derivatives

Emodin, rhein, aloe-emodin, alizarin, anthranol, casanthranol, chrysazin, chrysophanol, aloe-emodin-8-glucoside, emodin-8-O-beta-D-glucoside, physcion, rubiadin, 1-hydroxyanthraquinone, 1,3-diacetyl aloe-emodin, 3,8-diacetyl aloe-emodin, and triacetyl aloe-emodin.



emodin rhein aloe-emodin

In vitro cytotoxicity assay in HepG2/C3A and HuH-7 cells

HepG2/C3A and HuH-7 cells (cultured for 1 week before treatments) were treated with the 16 anthraquinones and derivatives in triplicates (0.5–1000 µM based on solubility). Cells were treated for 72 hrs at 37°C under two regimens: single dosing or repeated dosing at 24-hr intervals. After 72 hrs, cytotoxicity was determined based on the quantitation of ATP by the CellTiter-Glo assay (Promega, Madison, WI). Vehicle treated cells served as the negative control. EC_{50}, the concentration yielding 50% cytotoxicity, was calculated from the Hill equation.

In vitro clearance assay in primary human hepatocytes

Cryopreserved human hepatocytes (0.5×10^6 viable cells/mL) were thawed and incubated with 1 µM of emodin, rhein, or aloe-emodin in duplicates at 37°C . At 0, 15, 30, 60, and 120 min, aliquots were removed from the incubation mixture and mixed with an equal volume of ice-cold methanol containing an analytical internal standard. Stopped reactions were kept on ice for 10 min and centrifuged to remove precipitated protein. The supernatant was analyzed for the hepatocyte clearance of emodin, rhein, or aloe-emodin by UPLC-MS/MS (Waters Corporation, Milford, MA).

In silico prediction of physicochemical and pharmacokinetic properties

The simplified molecular-input line-entry system (SMILES) strings for emodin, rhein, and aloe-emodin were imported into the ADMET Predictor v9.0 (AP9.0, SimulationsPlus LLC, Lancaster, CA). Physicochemical and pharmacokinetic properties were predicted using quantitative structure-activity relationship (QSAR) models.

In silico prediction of human toxic doses (D_{tox})

The high-throughput pharmacokinetic (HTPK) module in AP9.0 was used to predict the toxic dose (D_{tox}), which was defined as the human dose that would yield a plasma steady state concentration (C_{ss}) equivalent to the *in vitro* cytotoxic concentration (EC_{50}). The lowest EC_{50} values of emodin, rhein, and aloe-emodin among the two cell lines and two treatment regimens were input into the HTPK module. The initial dose estimate was set at 100 mg and dose interval was set at 24 hrs for the simulation.

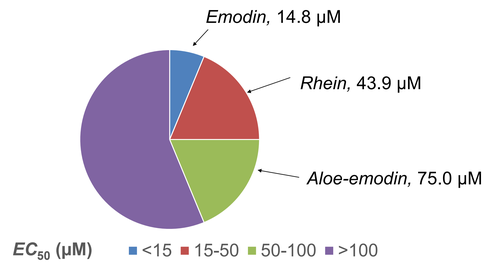
Estimated daily intake and liver safety assessment

A literature search was conducted to survey the levels of emodin, rhein and aloe-emodin in botanicals, along with the botanical daily intake levels. To assess risk in a conservative manner, the estimated daily intake (EDI) of emodin, rhein and aloe-emodin was calculated by multiplying the highest level of each anthraquinone in a botanical by the highest daily intake level of that botanical. A ratio of D_{tox}/EDI was calculated to evaluate its potential to cause liver injury.

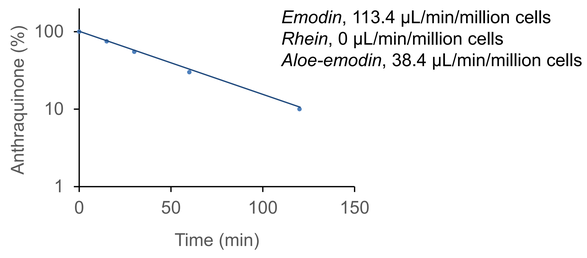
Results and Discussion

In vitro cytotoxicity

Among the 16 anthraquinones and derivatives, 13 compounds were excluded from further evaluation due to high EC_{50} values (> 100 µM) or low abundance in botanicals. Subsequently, emodin, rhein and aloe-emodin were selected for further *in vitro* and *in silico* evaluations.



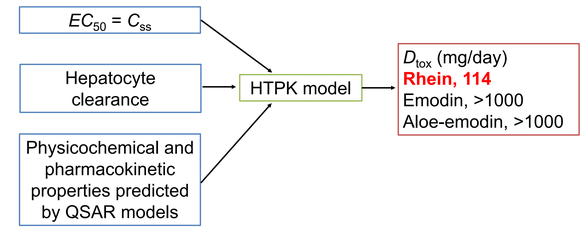
In vitro hepatocyte clearance



In silico QSAR prediction of physicochemical and pharmacokinetic properties

	Emodin	*Rhein*	*Aloe-emodin*
logP	3.06	2.69	2.32
logD at specified pH	2.19	-0.43	2.12
pK_a	10.22	8.89	9.03
Water solubility (mg/mL)	0.10	0.25	0.17
Solubility at specified pH (mg/mL)	0.77	32.32	0.27
Solubility in simulated fasted state gastric fluid (mg/mL)	0.08	0.04	0.17
Solubility in simulated fasted state intestinal fluid (mg/mL)	1.39	1.99	0.82
Solubility in simulated fed state intestinal fluid (mg/mL)	0.89	1.34	0.48
Molecular diffusion coefficient in water ($cm^2/s \times 10^5$)	0.87	0.87	0.87
Effective human jejunal permeability ($cm/s \times 10^4$)	4.35	3.96	4.71
Percent unbound ratio to plasma proteins (%)	6.62	7.05	8.95
Volume of distribution (L/kg)	0.26	0.18	0.87

In vitro-to-in vivo extrapolation



Estimated daily intake

Levels of emodin, rhein and aloe-emodin have been mainly reported in four botanicals, rhubarb, Japanese knotweed, Fo-Ti and aloe, whose daily intake levels in traditional Chinese medicines and dietary supplements were also identified. EDI of emodin, rhein, and aloe-emodin was calculated to be 270, 429, and 231 mg/day, respectively.

Liver safety assessment

D_{tox}/EDI ratios

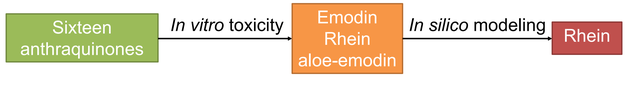
Rhein: 0.3
Emodin: >3
Aloe-emodin: >3

Rhein was identified as potentially hepatotoxic when it was used at maximum prescribed dose in traditional Chinese medicines, which is an extreme exposure compared to common dietary supplements.

Conclusion

This study demonstrated an integrated approach of combining *in vitro* cytotoxicity and *in silico* modeling into the quantitative evaluation of potential liver toxicity of anthraquinones.

This approach enabled rapid hazard identification and compound prioritization for future more in-depth studies, such as *in vivo* animal studies.



FDA mission relevance

This study addresses the FDA's Predictive Toxicology Roadmap of integrating emerging predictive toxicology methods and new technologies into regulatory safety and risk assessments.

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