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Toxicity Prediction of Photosensitizers Bearing Carboxylic Acid Groups by ECOSAR and Toxtree

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ABSTRACT

Tetrapyrrolic macrocycles bearing carboxylic acid groups have received considerable interest as photosensitizing agent for Photodynamic Therapy (PDT). It is necessary to consider the toxic potency of those compounds. The present study was designed to predict the toxicities of tetrapyrrolic compounds using Ecological Structure Activity Relationships (ECOSAR) and Toxtree, for further use in design and synthesis of photosensitizers. The ECOSAR prediction showed that tetrapyrrolic macrocycles with more carboxylic acid groups or hydroxyl groups showed lower toxic potency than those with fewer carboxylic acid groups or hydroxyl groups. Toxicities estimation using Toxtree to human and based on the Cramer rules, Verhaar, Structural Alerts for Reactivity in Toxtree (START) biodegradability, eye irritation/corrosion and skin irritation/corrosion, all of the compounds fell into class 3, 5, 2, 1 and 1, respectively. Application of the Benigni-Bossa method showed that all studied compounds had structural alerts for genotoxic carcinogenicity. Cytochrome P-450 mediated drug metabolism was positive for all site of metabolism except for PPIX-1OH and PPIX-2OH. Most of the studied tetrapyrrolic compounds fell into unreactive group of compounds by Michael addition classification, except for purpurin 7 and rhodin $_{g7}$. Skin sensitization evaluation of all compounds were alert to Michael acceptor, except for BPhe a-OH. Moreover, Kroes Threshold of Toxicological Concern (TTC) decision tree had negligible risk for all compounds.

Key words: Tetrapyrrolic macrocycle, carboxylic acid group, photodynamic therapy, ECOSAR, Toxtree

INTRODUCTION

Porphyrins, chlorins, bacteriochlorins and other closely related tetrapyrrolic macrocycles occur widely in nature with significant roles in various biological processes. Protoporphyrin IX (PPIX), the immediate precursor of heme; chlorophylls which play important roles in photosynthesis and vitamin B_{12}, are representatives of those molecules.

Many tetrapyrrolic macrocycles are effective photosensitizers in Photodynamic Therapy (PDT) (Maiya, 2000; Miah, 2002a; Castano *et al*., 2004; Tjahjono, 2006). Tetrapyrrole molecules are also fluorescent compounds, thus its imaging capability can be applied in photodynamic detection (Shahbazi-Gahrouei and Khodamoradi, 2007; Miah, 2002b). Moreover, a photo insectiside activities has also been reported for tetrapyrrole derivatives (El-Tayeb *et al*., 2011).

PDT is a treatment technique for cancer and for certain benign conditions which use a photosensitizing drug and light at a specific wavelength to produce reactive oxygen species in cells (Castano *et al*., 2004). This two-part procedure makes it possible to avoid the toxicity which associated with drug-based therapy, because only the light-activated sensitizer will yield a cytotoxic effect to target cell. However, it is necessary to consider the long-term dark toxicity of those compounds.

Prior to labor-intensive and expensive laboratory toxicity assays, a toxicity predictions by (Quantitative) Structure-activity Relationships ((Q)SARs) models have been used to support in hypothesis and prioritizing further experimental studies. A large number of relationships have been reported that the biological effect, mathematically determined as the output of the model, was defined in relation to some chemical parameters, identified as the model inputs (Cronin and Livingstone, 2004).

Recently, we have focused our interest on 1-hydroxyethyl derivative of tetrapyrrolic macrocycles bearing carboxylic acid groups. Tetrapyrrolic macrocycles with conjugated substituents bearing carboxylic acid group have red-shift absorption compared to non-substituted tetrapyrrolic macrocycles. The red-most absorption is ideal criteria for PDT as the light can penetrate deep into tissues (Eriksson and Eriksson, 2011). Naghavi and Baygi (2009) have developed a model that is able to predict the depth of tissue necrosis during PDT. The present of 1-hydroxyethyl substituent is expected to increase the hydrophilicity, an advantage when the drug is formulated as parenteral dosage form.

In the present study, we investigated the influence of carboxylic acid group and 1-hydroxyethyl side chain (substituted vinyl groups) on the toxic potency of porphyrin, chlorin and bacteriochlorin. The aim of this study was to predict the toxicities of some tetrapyrrolic macrocycles bearing carboxylic acid groups, i.e., ecotoxicity and one for human toxicological endpoints. The results of the study may serve as a useful considerant in the development of more effective photosensitizers.

MATERIALS AND METHODS

ECOSAR: ECOSAR is an easy-to-use computer program which are developed and routinely applied by the US EPA for predicting aquatic toxicity to fish, aquatic invertebrates (daphnids) and green algae. The program's latest version (v.1.00a February, 2009) is freely available from the EPA website (ECOSAR, 2009). The input data is the SMILES notation of the substance and the related log K_{ow} value. If the experimental log K_{ow} is not available, the log K_{ow} is then calculated by ECOSAR using the sub-routines Kow Win and or CLOGP. However, predicted values will be eliminated from the dataset if ECOSAR has indicated that the log K_{ow} of the substance was too high for correct prediction (log K_{ow} cut off).

TOXTREE: Toxtree was developed by IDEA consult. Ltd. (Sofia, Bulgaria). It has been made available as a free download (Toxtree, 2010). The current version (v.2.2.0 October, 2010) includes decision trees for predicting Cramer rules, Cramer rules with extensions, Verhaar scheme, START biodegradability, eye irritation and corrosion, structure alerts for the *in vivo* micronucleus assay in rodents, Michael acceptors, Benigni/Bossa rulebase (for mutagenicity and carcinogenicity), skin irritation/skin corrosion, cytochrome P450-mediated drug metabolism, skin sensitisation alerts and Kroes TTC decision tree. The main window of Toxtree is classified into three different areas: the compound properties area, used to resume the available information on the current compound; the

compound structure diagram area which shows a picture of the current compound and provides an easy way to navigate through the list of compounds in the currently opened file and the classification area which provides access to the classification output for the current compound.

To generate predictions with Toxtree, user-defined molecular structures can be input as SMILES codes or using the built-in 2D structure diagram editor. The software can also be used to perform batch processing of large numbers of compounds by importing datasets of various file types.

Kow Win: The log K_{ow} (Kow Win) program estimates the log octanol/water partition coefficient of organic chemicals using an atom/fragment contribution method developed at the Syracuse Research Corporation. The log K_{ow} values generated by Kow Win are then integrated into the ECOSAR model.

SMILES: The Simplified Molecular Input Line Entry System (SMILES) is a chemical notation system used to represent a molecular structure by a linear string of symbols. SMILES notations are comprised of atoms (designated by atomic symbols), bonds, parentheses (used to show branching) and numbers (used to designate ring opening and closing positions) (Weininger, 1988). The PBT-Profiler program has been used to generate SMILES notations for all investigated compounds that did not have known CAS numbers. Chemical structures are drawn using the Structure Drawing Interface portion of the program and the SMILES notation of the investigated compound is generated. The generated SMILES notation is then input into the ECOSAR and Toxtree model.

RESULTS AND DISCUSSION

The structure of three free-base porphyrin, ten chlorin and two bacteriochlorin (Fig. 1, Table 1) were inputted into the ECOSAR and Toxtree software. The studied porphyrins were

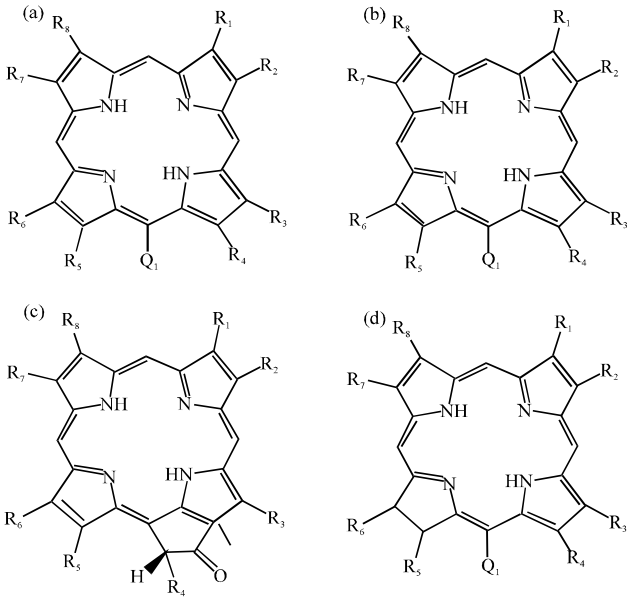

Fig. 1(a-d): Chemical structure of (a) Porphyrin (b, c) Chlorin and (d) Bacteriochlorin

Table 1: Porphyrins, chlorins and bacteriochlorins studied

Compounds	Structure[1]	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	Q_1
Porphyrin										
PPIX	a	CH_3	C_2H_3	CH_3	CH_2CH_2COOH	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	
PPIX-1OH	a	C_2H_3	CH_3	CH_3	CH_2CH_2COOH	CH_2CH_2COOH	CH_3	C_2H_3	$CH(OH)CH_3$	
PPIX-2OH	a	CH(OH)	CH_3	CH_3	CH_3CH_2COOH	CH_2CH_2COOH	CH_3	C_2H_3	$CH(OH)CH_3$	
Chlorin										
Chlorin e_6	b	CH_3	C_2H_5	CH_3	COOH	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	CH_2COOH
Rhodochlorin	b	CH_3	C_2H_5	CH_3	COOH	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	H
Purpurin 7	b	CH_3	C_2H_5	CH_3	COOH	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	COCOOH
Rhodin g_7	b	CHO	C_2H_5	CH_3	COOH	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	CH_2COOH
Pyrophe a	c	CH_3	C_2H_5	CH_3	H	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	
Chlorin e_4	b	CH_3	C_2H_5	CH_3	COOH	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	CH_3
Chlorin p_6		CH_3	C_2H_5	CH_3	COOH	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	COOH
Isochlorin e_4	b	CH_3	C_2H_5	CH_3	CH_2COOH	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	CH_2COOH
Phe a	c	CH_3	C_2H_5	CH_3	$COOCH_3$	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	
Phe a-OH	c	CH_3	C_2H_5	CH_3	$COOCH_3$	CH_2CH_2COOH	CH_3	CH_3	$CH(OH)CH_3$	
1	b	CH_3	C_2H_5	CH_3	H	COOH	CH_3	CH_3	C_2H_3	H
2	b	CH_3	C_2H_5	CH_3	H	CH_2COOH	CH_3	CH_3	C_2H_3	H
3	b	CH_3	C_2H_5	CH_3	H	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	H
4	b	CH_3	C_2H_5	CH_3	H	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	CH_3
5	b	CH_3	C_2H_5	CH_3	H	COOH	CH_3	CH_3	C_2H_3	COOH
6	b	CH_3	C_2H_5	CH_3	H	CH_2COOH	CH_3	CH_3	C_2H_3	COOH
7	b	CH_3	C_2H_5	CH_3	H	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	COOH
8	b	CH_3	C_2H_5	CH_3	H	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	CH_2COOH
9	b	CH_3	C_2H_5	CH_3	COOH	COOH	CH_3	CH_3	C_2H_3	COOH
10	b	CH_3	C_2H_5	CH_3	COOH	CH_2COOH	CH_3	CH_3	C_2H_3	COOH
11	b	CH_3	C_2H_5	CH_3	COOH	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	COOH
12	b	CH_3	C_2H_5	CH_3	COOH	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	CH_2COOH
Bacteriochlorin										
BPhe a	d	CH_3	C_2H_5	CH_3	$COOCH_3$	CH_2CH_2COOH	CH_3	CH_3	C_2H_3	
BPhe a- OH	d	CH_3	C_2H_5	CH_3	$COOCH_3$	CH_2CH_2COOH	CH_3	CH_3	$CH(OH)CH_3$	

[1]The structure (a, b, c and d) refer to Fig. 1

include 7,12-diethenyl-3,8,13,17-tetramethyl-21H,23H-porphine-2,18-dipropanoic acid (PPIX); 7-(1-hydroxyethyl)-12-ethenyl-3,8,13,17-tetramethyl-21H,23H-porphine-2,18-dipropanoic acid (PPIX-1OH) and 7,12-bis(1-hydroxyethyl)-3,8,13,17-tetramethyl-21H,23H-porphine-2,18-dipropanoic acid (PPIX-2OH). The studied chlorins were include chlorin e_6, rhodochlorin, purpurin 7, rhodin g_7, pyropheophorbide a (pyrophe a), chlorin e_4, chlorin p_6, isochlorin e_4, pheophorbide a (Phe a) and 3-(1-hydroxyethyl) pheophorbide a or Phe a-OH while the studied bacteriochlorin derivatives were bacteriopheophorbide a (BPhe a) and 3-(1-hydroxyethyl)-bacteriopheophorbide a (BPhe a-OH).

The ECOSAR program was capable to generating LC_{50} or EC_{50} of the studied compounds. The results are presented in Table 2 and 3.

All of the studied compounds may not be soluble enough to measure this predicted effect. Nevertheless, these results can be reasonable to compare the toxic potential of the test compounds each other. In examining the toxic potency data, several observations are apparent. Firstly, comparing toxic potency for PPIX, PPIX-1OH and PPIX-2OH, it demonstrated that toxic potency decrease with increasing the number of hydroxyl substituents. This is due to the hydroxyl groups

Table 2: Baseline toxicity (neutral organic SAR) of some tetrapyrrolic macrocycles as predicted by ECOSAR

				Neutral organic SAR (baseline toxicity) (mg L^{-1})		
Compounds	MW (g mol^{-1})	Water solubility (mg L^{-1}) Wskow Win estimate	Log K_{ow} (Kow Win estimate)	Fish LC_{50}, 96 h	Daphnid LC_{50}, 48 h	Green algae EC_{50}, 96 h
Porphyrin						
PPIX	562.67	3.714×10^{-6}	6.80	0.037	0.039	0.119
PPIX-1OH	580.69	4.462×10^{-5}	5.40	0.592	0.515	0.897
PPIX-2OH	598.70	2.409×10^{-4}	4.41	4.245	3.276	3.791
Chlorin						
Chlorin e_6	596.69	6.163×10^{-6}	6.29	0.109	0.105	0.263
Rhodochlorin	538.65	3.144×10^{-6}	7.07	0.021	0.023	0.078
Purpurin 7	610.67	3.865×10^{-5}	5.24	0.845	0.722	1.178
Rhodin g_7	610.67	9.334×10^{-5}	4.80	2.024	1.638	2.224
Chlorin e_4	552.68	8.694×10^{-7}	7.62	0.008	0.009	0.037
Chlorin p_6	582.66	2.000×10^{-5}	5.79	0.276	0.252	0.516
Isochlorin e_4	552.68	9.454×10^{-6}	6.40	0.080	0.078	0.206
Pyrophe a	534.66	5.531×10^{-6}	6.81	0.035	0.036	0.112
Phe a	592.70	6.61×10^{-6}	6.28	0.109	0.105	0.263
Phe a-OH	610.72	7.93×10^{-5}	4.88	1.724	1.408	1.979
Bacteriochlorin						
BPhe a	594.72	6.99×10^{-6}	6.24	0.119	0.115	0.281
BPhe a-OH	612.73	8.393×10^{-5}	4.83	1.885	1.533	2.114

MW: Molecular weight, SAR: Structure-activity relationship, K_{ow}: Octanol-water partition coefficient

the same reason was proposed for Phe a and Phe a-OH, as well as for BPhe a and BPhe a-OH. Second, toxic potency of most of chlorin derivatives were not significantly different, except rhodin g_7 (less toxic). In rhodin g_7 the aldehyde group contribute in decreasing toxicity. Third, toxic potency of bacteriochlorin (i.e., BPhe a) was lower than that of chlorin (i.e., Phe a). It is an advantageous because bacteriochlorin are promising candidates for PDT as they display the red-most absorption (Q_x) at longer wavelengths and its absorptivity is also stronger compared to those of corresponding chlorin.

ECOSAR requires an estimated or measured value of log K_{ow}. Toxicity values for new chemicals is then calculated by inserting the estimated K_{ow} into the regression equation and correcting the resultant value for the molecular weight of the compound. The toxicity value for a substance increases as the solubility of the compound in water increases (Table 2, Fig. 2).

Based on the SMILES notation, each substance was allocated to a chemical class as defined by ECOSAR. It might happen however, that due to its molecular structure, a substance is assigned to more than one chemical class. This was allowed in the current evaluation as it was attempted to get information on the prediction capability with respect to the individual ECOSAR chemical classes and the corresponding SARs. When an acid moiety was found in a molecule, ECOSAR multiplied the predicted values which were calculated from the SARs of the specific class, e.g. neutral organics, by a factor of 10. The chemical was then allocated to the same class but with the acid moiety, e.g. pyrazoles/pyrrole acid (Reuschenbach *et al.*, 2008). Table 3 shows the results of toxicity prediction in fish of a specific ECOSAR class.

Tetrapyrrolic macrocycles with fewer carboxylic acid groups showed higher toxic potency than those with more carboxylic acid groups and greater toxicity was observed with increasing molecular weight (Table 4, Fig. 3). These results were consistent with previous research (Frank *et al.*, 2009).

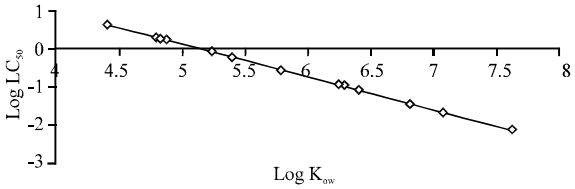

Fig. 2: Plot of log LC_{50} (fish, 96 h) against log K_{ow} for some tetrapyrrolic macrocycles as predicted by ECOSAR

Table 3: Toxicity based on ECOSAR class as predicted by ECOSAR

Compounds	ECOSAR classes	LC_{50}[1]	Compounds	ECOSAR classes	LC_{50}[1]
PPIX	Aliphatic Amines-acid	1.274	chlorin p_6	Aliphatic Amines-acid	5.336
	Pyrazoles/Pyrroles-acid	0.084		Pyrazoles/Pyrroles-acid	0.315
	Vinyl/Allyl Amines-acid	5.85×10^{-4}		Vinyl/Allyl Amines-acid	0.004
PPIX-OH	Aliphatic Amines-acid	9.167	isochlorin e_4	Aliphatic Amines-acid	2.175
	Vinyl/Allyl Alcohols-acid	46.186		Pyrazoles/Pyrroles-acid	0.137
	Pyrazoles/Pyrroles -acid	0.519		Vinyl/Allyl Amines-acid	1.25×10^{-3}
	Vinyl/Allyl Amines-acid	0.009	pyrophe a	Aliphatic Amines-acid	1.194
PPIX-2OH	Aliphatic Amines-acid	37.532		Vinyl/Allyl Ketones-acid	1.473
	Benzyl Alcohols-acid	38.356		Pyrazoles/Pyrroles-acid	0.079
	Vinyl/Allyl Alcohols-acid	62.998		Vinyl/Allyl Amines-acid	5.45×10^{-4}
	Pyrazoles/Pyrroles -acid	1.905	Phe a	Aliphatic Amines-acid	2.768
	Vinyl/Allyl Amines-acid	0.066		Esters-acid	2.877
Chlorin e_6	Aliphatic Amines-acid	2.768		Pyrazoles/Pyrroles-acid	0.173
	Pyrazoles/Pyrroles-acid	0.173		Vinyl/Allyl Amines-acid	1.7×10^{-3}
	Vinyl/Allyl Amines-acid	1.7×10^{-3}	Phe a-OH	Aliphatic Amines-acid	19.888
Rhodochlorin	Aliphatic Amines-acid	0.842		Esters-acid	18.766
	Pyrazoles/Pyrroles-acid	0.057		Vinyl/Allyl Alcohols-acid	56.263
	Vinyl/Allyl Amines-acid	3.32×10^{-4}		Pyrazoles/Pyrroles -acid	1.063
Purpurin 7	Aliphatic Amines-acid	11.981		Vinyl/Allyl Amines-acid	0.027
	Pyrazoles/Pyrrole acid	0.667	BPhe a	Aliphatic Amines-acid	2.954
	Vinyl/Allyl Amines-acid	0.013		Esters-acid	3.060
Rhodin g_7	Aliphatic Amines-acid	22.297		Pyrazoles/Pyrroles -acid	0.183
	Vinyl/Allyl Aldehydes-acid	4.798		Vinyl/Allyl Amines-acid	0.00187
	Pyrazoles/Pyrroles-acid	1.181	BPhe a-OH	Aliphatic Amines-acid	21.220
	Vinyl/Allyl Amines-acid	0.032		Esters-acid	19.961
Chlorin e_4	Aliphatic Amines-acid	0.405		Vinyl/Allyl Alcohols-acid	57.158
	Pyrazoles/Pyrroles-acid	0.029		Pyrazoles/Pyrroles -acid	1.129
	Vinyl/Allyl Amines-acid	1.17×10^{-4}		Vinyl/Allyl Amines-acid	0.030

[1]Fish LC_{50} 96 h is the dose (mg L^{-1}) required to kill half the members of fish after 96 h

Table 4: Baseline toxicity (neutral organic SAR) of some tetrapyrrolic macrocycles with one, two and three carboxylic acid groups as predicted by ECOSAR

Compounds	MW (g mol^{-1})	LC_{50}[1]	Compounds	MW (g mol^{-1})	LC_{50}[1]
1	466.59	0.099	7	538.65	0.203
2	480.61	0.039	8	552.68	0.080
3	494.64	0.015	9	554.61	1.781
4	508.67	0.005	10	568.63	0.701
5	510.60	1.302	11	582.66	0.276
6	524.62	0.514	12	586.69	0.109

[1]Fish LC_{50} 96 h (mg L^{-1}) required to kill half the members of fish after 96 h

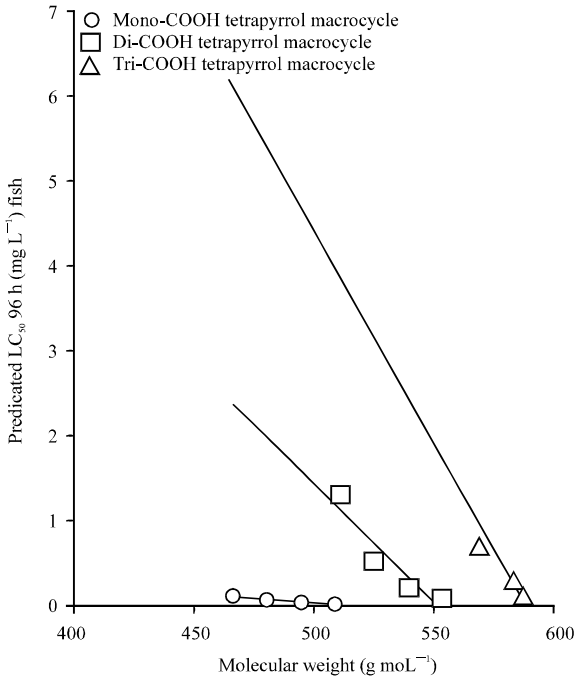

Fig. 3: Predicted toxicity against molecular weight of some tetrapyrrolic macrocycles with 1,2 and 3 carboxylic acid groups as predicted by ECOSAR

Table 5: Toxtree plugins and classes

Toxtree Plugins	Classes
Cramer rules	1: Low
	2: Intermediate
	3: High
Verhaar scheme	1: Narcosis or baseline toxicity
	2: Less inert compounds
	3: Unspecific reactivity
	4: Compounds and groups of compounds acting by a specific mechanism
	5: Not possible to classify according to these rules
START biodegradability	1: Easily biodegradable chemical
	2: Persistent chemical
	3: Unknown biodegradability
Eye irritation and corrosion	1: Not skin corrosion R34 or R35
	2: Not lesions R34, R35, R36 or R41
	3: Not eye irritation R41
	4: Not eye irritation R36
	5: Not corrosion R34, R35 or R41
	6: Not lesions R34, R35 or R36
	7: Not eye irritation R36 or R41
	8: Serious lesions to the eye
	9: Moderate reversible irritation to the eye
	10: Skin corrosion
	11: Unknown
Structure alerts for the *in vivo*	1: At least one positive structural alerts for the micronucleus assay

Table 5: Continue

Toxtree plugins	Classes
micronucleus assay in rodents	2: No alerts for the micronucleus assay
Michael acceptors	1: Michael acceptor
	2: Not reactive via Michael addition
Benigni /Bossa rulebase	1: Structural alert for genotoxic carcinogenicity
(for mutagenicity and carcinogenicity)	2: Structural alert for non-genotoxic carcinogenicity
	3: Potential *Salmonella* Typhimurium TA100 mutagen based on QSAR
	4: Unlikely to be a *Salmonella* Typhimurium TA100 mutagen based on QSAR
	5: Potential carcinogen based on QSAR
	6: Unlikely to be a carcinogen based on QSAR
	7: For a better assessment a QSAR calculation could be applied
	8: Negative for genotoxic carcinogenicity
	9: Negative for nongenotoxic carcinogenicity
	10: Error when applying the decision tree
Skin irritation/skin corrosion	1: Not corrosive to skin
	2: Not irritating or corrosive to skin
	3: Not irritating
	4: Irritating to skin
	5: Corrosive to skin
	6: Irritating or corrosive to skin
	7: Unknown
Cytochrome P450-mediated drug	1: SMARTCyp.Rank1.sites
metabolism	2: SMARTCyp.Rank2.sites
	3: SMARTCyp.Rank3.sites
	4: SMARTCyp.Rank=.sites
Skin sensitisation alerts	1: Alert for SNAr identified
	2: Alert for Schiff base formation identified
	3: Alert for Michael acceptor identified
	4: Alert for acyl transfer agent identified
	5: Alert for SN2 identified
	6: No skin sensitisation alerts identified
Kroes TTC decision tree	1: Substance would not be expected to be a safety concern
	2: Negligible risk (low probability of a life time cancer risk greater than 1 in 10^6)
	3: Risk assessment requires compound-specific toxicity data

The enhanced strength of the electronic charge which induced by the increase of number of carboxylic acid in a structure could impair uptake by a cellular membrane, consequently reducing toxicity. Such evidences were observed when investigating the impact of ionization on the toxicity of aliphatic carboxylic acids (Seward and Schultz, 1999).

The majority of the data input into the ECOSAR model's database did not contain information regarding to the pH of test solutions. Therefore, all solutions were assumed to have a pH of 7 and as a result, the model was not able to accurately predict the toxicity of ionic compounds at pH other than 7.

Toxtree is able to estimate toxic hazard by applying a decision tree approach. The decision tree approaches and classes were then used to predict the toxicities (Table 5, 6).

Table 6: Toxic hazard classification as predicted by Toxtree

Compounds	Cramer rules	Verhaar scheme	START biodegrad-ability*	Eye irritation/ corrosion	Alerts for mn#	Michael acceptors	Benigni/ bossa rulebase†	Skin irritation/ corrosion	CYPs‡	Skin sensitisation	Kroes TTC~
PPIX	3	5	2	1	1	2	1,9	1	1,2,3,4	3	2
PPIX-1OH	3	5	2	1	1	2	1,9	1	1,2,4	3	2
PPIX-2OH	3	5	2	1	1	2	1,9	1	1, 3,4	3	2
Chlorin e_6	3	5	2	1	1	2	1,9	1	1,2,3,4	3	2
Rhodochlorin	3	5	2	1	1	2	1,9	1	1,2,3,4	3	2
Purpurin 7	3	5	2	1	1	1	1,9	1	1,2,3,4	3	2
Rhodin g_7	3	5	2	1	1	1	1,9	1	1,2,3,4	3	2
Chlorin e_4	3	5	2	1	1	2	1,9	1	1,2,3,4	3	2
Chlorin p_6	3	5	2	1	1	2	1,9	1	1,2,3,4	3	2
Isochlorin e_4	3	5	2	1	1	2	1,9	1	1,2,3,4	3	2
Pyrophe a	3	5	2	1	1	2	1,9	1	1,2,3,4	3	2
Phe a	3	5	2	1	1	2	1,9	1	1,2,3,4	3	2
Phe a-OH	3	5	2	1	1	2	1,9	1	1,2,3,4	3	2
BPhe a	3	5	2	1	1	2	1,9	1	1,2,3,4	3	2
BPhe a-OH	3	5	2	1	1	2	1,9	1	1,2,3,4	6	2

The classification numbers refer to Table 5.*Structure alerts for reactivity in Toxtree (START) biodegradability, #Structure alerts for the *in vivo* micronucleus assay in rodents, †Benigni/Bossa rule base for mutagenicity and carcinogenicity, ‡Cytochrome P450-Mediated drug Metabolism, ~Kroes threshold of toxicological concern (TTC)

Cramer rules classify chemicals into three structural classes based on a decision tree. Questions asses different features include structural features (functional groups, ring substituents, etc.), propensity of reaction, natural occurrence in body and in traditional foods and the logic of tree relies primarily on knowledge of common metabolic pathways (Patlewicz *et al*., 2008). As shown in Table 6, all of the studied compounds fell into class 3, indicating that the compounds are substances that permit no strong initial impression of safety and may even suggest a significant toxicity. This result probably misclassified as porphyrins, chlorins and other closely related macrocyclic tetrapyrrole occur widely in nature with significant roles in various biological processes. In terms of the Cramer classification rules, the studied tetrapyrrolic macrocycles are naturally present in the life system and therefore, proposed to be Class 1 by virtue of a positive response to question 1. The list of normal components in the life system could be extended within Toxtree to include the compounds as another example. All of the test compounds fell into class 3 by Cramer rules because a negative response to question 1 due to heterocyclic rings with complex substituents in the compounds.

Potential mechanisms of toxic action were identified for these compounds through application of the Verhaar scheme. A number of mechanisms have been identified that can lead to aquatic toxicity, with the majority of industrial chemicals exerting their toxic influence via two non-covalent mechanisms; polar narcosis and non-polar narcosis. According to the Verhaar scheme, all of the compounds fell into class 5 (Table 6). Compounds which cannot be classified as belonging to classes 1, 2 or 3 and that are not known to be compounds acting by a specific mechanism can only be classified as 'not possible to be classified according to these rules'. Verhaar scheme needs a number of improvements. One possible scheme would be one which identifies reactive chemicals first, using the wealth of knowledge currently available. In addition, sub-classes are required within Verhaar classes 3 and 4 to reflect the number of differing mechanisms of action that have been identified since the original Verhaar publication in 1992 (Enoch *et al*., 2008).

START biodegradation and persistence is a compilation of structural alerts for environmental persistence and biodegradability. These structural alerts are molecular functional groups or substructures that are known to be linked to the environmental persistence or biodegradability of chemicals. According to START biodegradability, all of the compounds fell into class 2 because they have two or more rings (Table 6).

Skin irritation and skin corrosion refer to localized toxic effects resulting from a topical exposure of the skin to a substance. There is strong evidence that chemicals which are corrosive to the skin should also be classified as being corrosive to the eye, especially if the assessment is made from knowledge of acidity and alkalinity. In particular, in the EU and OECD classification schemes, chemicals that have been found to be corrosive to the skin are automatically considered to be corrosive to the eye. Toxtree implements the BfR rules (The German Federal Institute for Risk Assessment) for predicting skin/eye irritation and corrosion. The system is based on the combined use of two predictive approaches: exclusion rules based on physicochemical cut-off values to identify chemicals that do not exhibit a certain hazard (e.g., skin irritation/corrosion) and inclusion rules based on structural alerts to identify chemicals that do show a particular toxic potential. According to eye irritation/corrosion and skin irritation/corrosion methods, all of the compounds were classified as not skin corrosion R34 or R35 and not corrosive to skin, respectively (Table 6). These results indicated that the compounds were likely safe as photosensitizer for skin cancer treatment.

Structure alerts for the micronucleus assay in rodent resulted in a classification of class 1 for all compounds (Table 6). It means that all of compounds have at least one positive alerts for the micronucleus assay. This plugin provides a list of structural alerts for a preliminary screening of potential *in vivo* mutagens. These structural alerts are molecular functional groups or substructures that are known to be linked to a positive *in vivo* micronucleus assay. Molecular functional groups or substructures that are known to be linked to a positive *in vivo* micronucleus assay are heterocyclic polycyclic aromatic hydrocarbons and molecules which can form non-covalent interactions with protein or DNA. Such interaction, as in the case of DNA intercalation or groove binding, is potentially genotoxic. The negative *in vitro* results in genotoxicity testing are usually considered sufficient to indicate lack of mutagenicity, whereas a positive result is not considered sufficient to indicate that the chemical represents a mutagenic hazard (i.e.,. it could be a false positive). The micronucleus test in rodents is the most widely used as a follow-up to positive *in vitro* mutagenicity results.

A study published by Benigni *et al.* (2010) showed analyses and considerations relative to the role of the *in vivo* micronucleus assay in the pre-screening of chemical carcinogenicity. The positive predictivity (i.e., probability for a chemical with structure alert to be positive) by this method is 0.33. As shown in Table 6, application of the Benigni-Bossa method showed that all of the compounds had structural alerts for genotoxic carcinogenicity. Those need to be verificated through experimental assay.

Michael acceptors resulted in a classification of class 2 for all compounds except for purpurin 7 and rhodin g_7 (class 1). Purpurin 7 and rhodin g $_7$have structural alerts for Michael acceptors (Table 6) due to their **α-β**-unsaturated aldehyde or ketone. Michael type addition can lead to the molecular initiating event of protein alteration which results in the formation of covalent adduct at a soft electro (nucleo) philic center without expulsion of a leaving group in the molecule. Electrophiles acting in this manner are typically organic molecules that contain olefinic π-bonds polarized by a neighboring electron-withdrawing substituent. Schultz *et al.* (2007) have observed that **α-β**-unsaturated carbonyl compounds:

- Acetylenic-substituted derivatives are more reactive than their corresponding olefinic-substituted analogues
- Vinyl-substituted derivatives are more reactive than their vinylene-substituted analogue
- Methyl substitution on a vinyl C atom diminishes reactivity with methyl substitution on the α-C atom which resulting in a larger reduction
- Vinyl-substituted derivatives are more reactive than aldehydes
- Having an additional unsaturated groups increases reactivity

Moreover, reactive toxicity (the irreversible interaction of a xenobiotic chemical with endogenous molecules including specific sites on proteins) has been identified as the major gap to predict key hazards such as skin sensitization accurately (Schultz *et al*., 2007).

Toxtree can identify potential mechanism of toxic action for skin sensitization. It was observed that all compounds have alerts for skin sensitization by Michael type addition, except for BPhe a-OH (no skin sensitization alerts identified). These results were different to those of Michael acceptors method. It is possible because the structure alerts also include alerts for precursors of Michael reaction acceptor.

Cytochrome P450-mediated drug metabolism resulted in positive for all site of metabolism except for PPIX-1OH (negative in SMARTCyp.Rank3.sites) and for PPIX-2OH (negative in SMARTCyp.Rank2.sites). SMARTCyp is an *in silico* method that predicts the sites of metabolism for drug metabolites mediated by cytochrome P450 3A4 isoform. The idea behind SMARTCyp is that activation energies of CYPs reacting with ligand fragments computed by quantum chemical methods are the best possible reference for the reactivity of a fragment. Those results indicated that the compounds were likely easily metabolized.

TTC is a pragmatic risk assessment tool which is based on the principle of establishing a human exposure threshold value for all chemicals, below which there is a very low probability of an appreciable risk to human health. Kroes TTC decision tree resulted in negligible risk for all compounds (Table 6).

CONCLUSION

The correlation between similarity of structure and similarity of biological response is the basic to making prediction on toxicological properties. Toxic estimation software can reduce animal testing, time and cost. ECOSAR predict the aquatic toxicity based on Structure-Activity Relationship (SAR) while Toxtree predicts different type of toxicological hazard and modes of action by applying decision tree approaches and SAR. Thus, both packages can be used for initial toxicity assessments of designed or existing molecules. The ECOSAR prediction showed that tetrapyrrolic macrocycles with more carboxylic acid groups or hydroxyl groups showed lower toxic potency than those with fewer carboxylic acid groups or hydroxyl groups. Bacteriochlorin has lower toxic potency than that of chlorin. Based on the prediction by Toxtree, the studied compounds could be categorized as negligible risk (by Kroes TTC) and not corrosive to skin. Most compounds are likely easily metabolized. However, they were classified as persistent chemical and they showed, at least, one positive alerts for micronucleus assay.

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