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An evaluation of chemical photoreactivity and the relationship to phototoxicity

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ABSTRACT

The existing regulatory guidance for photosafety testing of new drug products states that studies are warranted for those chemicals that both absorb light in the range of 290–700 nm, and that are either applied locally/topically, or "reach" (EMEA)/"significantly partition" (FDA) to the skin or eyes. The initial *in vitro* study recommended for the assessment of phototoxic potential is the 3T3 Neutral Red Uptake (NRU) Assay. The current study was undertaken to establish superior triggers for the initiation of biological photosafety testing. In this study, photophysical and photochemical parameters for 40 drug or drug-like molecules were studied. Principal Component Analysis (PCA), Partial Least Squares-Discriminant Analysis (PLS-DA), and a fivefold cross-validation PLS algorithm were used to evaluate the relationship between subsets of photophysical and photochemical parameters with the 3T3 NRU PIF/MPE (Photo Irritation Factor/Mean Photo Effect) results. The parameters most indicative of a 3T3 NRU positive PIF or MPE score were the extent of degradation in solution, the quantum yield of formation of singlet oxygen and the relative formation of superoxide anion. The results demonstrate that while absorption of light is critical to the induction of a light-induced process, it is the resultant events that may be used to predict the 3T3 NRU assay result. It is therefore proposed that the trigger for photosafety testing be revised to include a molecular basis for photoreactivity. From this limited investigation, estimated thresholds leading to 3T3 NRU positive results due to photodegradation, formation of singlet oxygen quantum yield or a relative superoxide anion formation value are proposed.

1. Introduction

The first law of photochemistry states that only light which is absorbed by a system can bring about a photochemical change and the second law of photochemistry, the Stark–Einstein law, states that for each photon of light absorbed by a chemical system, only one molecule is activated for photochemical reaction. Molecules possessing suitable chromophores (moieties capable of absorbing UV or visible light in the range of 290–700 nm such as those with extended conjugation of double bonds or aromatic rings) may be activated photochemically by UV or visible radiation. Consequently, these photoactivated molecules may alter biological systems and if the exposure is sufficient (Epstein, 1983; Parisi and Wong, 1997; ISO, 1999; Diffey, 2002), may elicit harmful effects, including phototoxicity (e.g. erythema/edema, pigmentary alterations, visual impairment/ocular damage), photoallergy or photocarcinogenicity, Notably, there are specific chemical classes of pharmaceuticals, such as the fluoroquinolone antibiotics, that have been associated with a manifestation/exacerbation of these effects (Spielmann et al., 1994a,b, 1998, 2000; Moore, 2002; Jones and King, 2003; Neumann et al., 2005).

1.1. Photosafety testing

The existing regulatory guidance for photosafety testing (including phototoxicity, photoallergy, photogenotoxicity, photocarcinogenicity) of new drug products states that studies are warranted for those chemicals that absorb light in the range of 290–700 nm, and that are either applied locally/topically, or "reach" (EMEA)/"significantly partition" (FDA) to the skin or eyes (EMEA Committee for Proprietary Medicinal Products (CPMP), 2002; FDA, 2003). Where considered necessary, the EMEA guidance recommends a tiered approach to photosafety testing, whereas the FDA guideline recommends a parallel approach. It has been suggested that acute *in vitro* studies for hazard identification should be conducted prior to *in vivo* assessments for risk

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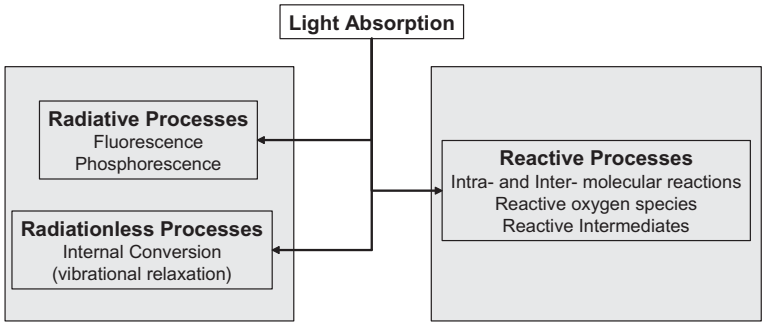

Scheme 1. Typical Modes of Deactivation Following Light Absorption.

characterization. The default *in vitro* study recommended for the assessment of phototoxic potential is the 3T3 Neutral Red Uptake Assay (Balls et al., 1995; OECD, 2004). This test evaluates photocytotoxicity by the relative reduction in the viability of cells exposed to an exogenous chemical in the presence and absence of light. The optimal light source is one that simulates solar sunlight, e.g. the CIE Illuminant D65 [ISO 18909, *which replaced ISO 10977*]. It is assumed that the absence of phototoxicity in the 3T3 NRU assay infers a lack of photoactivity. However, the current regulatory guidances do not allow for a negative result within the *in vitro* 3T3 NRU assay to fully negate the requirement for further photosafety testing.

1.2. Implications of UV–Vis absorption

It is important to consider the nature of the incident light when describing photobiological effects, as the ability of UV or visible radiation to elicit harmful effects depends strongly upon the incident wavelength, which is inversely proportional to energy. Thus, incident light of lower wavelengths (i.e. UV) can provide more energy for chemical or physical transformations compared to light in the visible region of the electromagnetic spectrum. For example, the ability of UV radiation to elicit erythema in human skin varies fourfold between the wavelengths of 250 and 400 nm (Diffey, 2002) with the shorter wavelengths representing the most damaging effects. In fact, at 300 nm, one photon has the energy of approximately 400 kJ/mol which is equivalent to the bond dissociation energy found in many organic molecules (Lowry and Richardson, 1987) and hence, many active pharmaceutical ingredients. As a result, the OECD 432 guidance (OECD, 2004) recommends attenuating the UVB from the light source used within the 3T3 NRU assay. The recommended '*dose*' of light in the 3T3 NRU assay is 5 J/cm^2 in the UVA range and administered at an irradiance of ~1.7 mW/cm^2. This is a much lower UVR dose than that used in confirmatory API and drug product photostability studies as defined by ICH Q1B which entails exposures to 1.2 million lux·hours of visible light and >200 W h/m^2 (~72 J/cm^2) (ICH, 1996).

When a molecule absorbs a photon of UV–Vis light, it gains a specific amount of energy (photoactivation). This additional energy must somehow be dissipated. For an organic molecule, Scheme 1 illustrates the typical modes through which this energy can be released.

Based on current guidances, the initial trigger for chemical photosafety testing is an assessment of a compounds light absorbance between 290 and 700 nm (EMEA Committee for Proprietary Medicinal Products (CPMP), 2002; FDA, 2003). Whilst the absorption of light is a pre-requisite for the initiation of a photochemical/physical event, the energy absorption process does not necessarily lead to a phototoxic reaction or biological consequence. Rather, it is the underlying mechanism of deactivation (how the molecule dissipates energy) that may elicit a photosafety concern. The molar extinction coefficient (MEC, M^{-1} cm^{-1}) is a parameter defined from the Lambert–Beer Law which relates absorbance at a particular wavelength to the concentration of a solution. The MEC is a constant for any given molecule under a specific set of conditions (e.g. solvent, pH and buffering additives, temperature, wavelength and equipment). The MEC reflects the probability with which a molecule may absorb a photon of light of a specific energy (defined by the wavelength) and therefore is an appropriate initial trigger for chemical photosafety testing. The use of MEC, however, as a first step towards possible photosafety testing may also be prone to misinterpretation since it represents a single snapshot (efficiency of light absorption for a single wavelength) instead of absorption across the region of interest (290–700 nm). As such in this study, concentration normalized, integrated areas under the curve (CNAUC, with limits defined by UVB 290–320 nm,[2] UVA 320–400 nm, vis 400–700 nm), which can easily be derived from the UV–Vis spectra, are used. Compared with MEC values, these data are more likely to accurately reflect the absorption characteristics of a test compound and allow comparison of absorption profiles between compounds.

Following light absorption, the main processes for energy release are radiative (light emission via fluorescence or phosphorescence), nonradiative (vibrational and/or rotational relaxation, which results in the release of thermal energy, i.e. heat) and other reactive modes that result in chemical changes (e.g. the generation of free radicals). In cases where all of the light energy is completely released in any combination of emission and/or vibrational/rotational relaxation, no chemical reactivity is expected. Quantum yields are a measure of the efficiency of a specific process relative to the number of photons absorbed. As such, if all (100%) of the absorbed light energy is given off as fluorescence, the fluorescence quantum yield would be 1.0. If half the absorbed light energy is given off as fluorescence and the other half as phosphorescence, then the quantum yield for each would be 0.5. Other key physical parameters include the lifetimes for singlet (typically nanoseconds for organic molecules) and triplet excited states (typically microseconds to milliseconds (μs–ms) for organic molecules). These parameters are often measured by their respective emissions.

A molecule may lose the energy imparted by its photoactivation via any of the processes described above and outlined in Scheme 1. The current study was undertaken to further investigate the link between photochemical and photophysical parameters with phototoxic liability as defined by results from the 3T3 NRU assay, using 40 drug-like compounds including several that have been previously identified as phototoxins. The aim was to provide a more discriminating overview of the processes associated with

[2] ISO and CIE define the break between UVC and UVB at 280 nm. However, regulatory agencies use 290 nm as a cut-off for photosafety considerations because the terrestrial solar radiation cutoff is ~293 nm. Consequently for this analysis, the UVB cutoff is considered to be 290 nm.

phototoxicity liability and thereby establish superior triggers for biological photosafety testing compared with light absorbance measurements or MEC determinations alone. In addition, these studies should improve the basic understanding of chemical phototoxicity liability based on fundamental photochemical and/or photophysical principals.

2. Materials and methods

2.1. Compounds

A series of 40 compounds demonstrating absorbance within the 290–700 nm region of the electromagnetic spectrum, and/or having reported phototoxic liability, were selected primarily from the literature, but also from the internal GSK compound library (Table 1). GSK compounds were chosen as representative drug-like molecules with the potential for photosafety testing due to their UV–Vis absorbance >290 nm. The GSK library compounds were used as supplied. Vitamin B12 (Calbiochem, 99.6%), piroxicam (Biomol International, >99%), riboflavin (ICN Biomedicals, >98%), Furosemide (TCI, 99.7%), dapsone (Sigma–Aldrich, >97.0%), griseofulvin (Sigma–Aldrich, >900 μg/mg), tetracycline (992 μg/mg) and all other compounds studied were sourced from Sigma–Aldrich and possessed purity greater than 98.0%.

Oxybenzone and perinaphthenone (Phenalenone) were chosen for comparison to the other drug-like molecules. Perinaphthenone is well known for generating singlet oxygen very efficiently following photoactivation (Schmidt et al., 1994), whereas oxybenzone is known to proceed by an "energy wasting" mechanism (more specifically, an excited state proton transfer) so that almost all of the light energy absorbed is utilized and expended by this process (Allen et al., 1996; Baughman et al., 2009). The 40 compounds were arbitrarily divided into two subsets, A and B (of 29 and 11 compounds, respectively) while ensuring that the distribution of 3T3 NRU positives to negatives was similar in each group. The initial analysis used subset A as the training set for model construction, and subset B as the test set for model validation. Table 1 shows the subset classification and also highlights references from the literature demonstrating evidence of *in vivo* photosensitivity or phototoxicity. The analytical testing of the 40 compounds was assessed as part of the investigation to determine which parameters were significantly associated with positive outcomes in the 3T3 NRU assay.

2.2. Photochemical/photophysical parameters

2.2.1. Calculated parameters

The following non-photo-related properties of the molecules were calculated using established algorithms (Veber et al., 2002). Molecular weight (MW); Hydrogen bond acceptors (HBA; any heteroatom without a formal positive charge, excluding halogens, pyrrole nitrogen, heteroaromatic oxygen and sulfur, and higher oxidation states of nitrogen, phosphorus, and sulfur but including the oxygen atoms bonded to them), Hydrogen bond donors (HBD; any heteroatom with at least one bonded hydrogen), *c*Log *P* (ACDLabs version 8), *c*Log $D_{7.4}$ (ACDLabs version 8), calculated pK_a (ACDLabs version 8) for acids or bases, the number of rotatable bonds (defined as any single bond, not in a ring, bound to a nonterminal heavy (i.e. non-hydrogen) atom. Excluded from the count were amide C–N bonds because of their high rotational energy barrier, and an estimate of molecular flexibility (integer of (100 * rotatable bonds/total bonds)).

2.2.2. Experimental photophysical measurements

The UV–Vis spectra were taken in methanol or ethanol (to ensure solubility and an aqueous-like environment) at a concentration between 0.8 and 71 μM, while ensuring that the absorbance in the 290–700 nm region was not >1. The area under the curve for each region [UVB (290–320 nm) UVA (320–400 nm), visible (400–700 nm)] of the UV–vis spectrum was integrated using Kaleidagraph 3.6, (Synergy Software). This value was normalized based on the molar concentration of the drug substance in solution so that the final results could be directly compared between molecules. MEC values were determined from the spectra, but not included as part of the analyses because the values for each compound cannot be directly compared quantitatively since the MEC values are taken at peak maxima, which differ between compounds.

Other photophysical measurements included emission lifetimes in ns and μs–ms time ranges, which may relate to fluorescence and phosphorescence, respectively, and may be markers for the lifetime of the singlet or triplet excited states. All emission (fluorescence and phosphorescence) studies were conducted in ethanol or methanol. Fluorescence lifetimes were measured using a time-correlated single photon counting technique, a PicoQuant modular fluorescence lifetime spectrometer (Fluo Time 100) with PicoQuant light sources. Phosphorescence lifetimes were recorded using a Cary Eclipse fluorescence spectrophotometer and off-gated detection in ethanol or methanol at 77 K. The lowest singlet (ambient temperature) or triplet (77 K) energy levels were determined by emission spectroscopy in the same solvents as the spectra. The fluorescence quantum yield was also measured. The fluorescence quantum yield (ϕ) represents the ratio of photons absorbed to photons emitted through fluorescence using the comparative technique outlined by Williams et al. (1983).

2.2.3. Photochemical reactivity assays

For both the loss of active pharmaceutical ingredient and superoxide anion assays, the same irradiation modalities were utilized. In an Atlas Suntest CPS+ Chamber, the sample and an aluminum foil wrapped control sample were exposed to a xenon arc lamp with coated quartz and "window glass" filter (strongly attenuated UV-B between 290 and 320 nm) for 35 min at 250 W/m^2 (Atlas Material Testing Solutions, 2006). The lamp was checked and calibrated by the vendor before and after the study. This corresponds to an irradiance of 1.7 mW/cm^2 (and a radiation dose of 5 J/cm^2) which is identical to the exposure given in the 3T3 NRU assay (OECD, 2004). Solutions to be exposed in the light chamber were made in 20 mM phosphate buffer (pH 7.4) with the addition of various amounts of co-solvents to ensure complete solubilization. For all compounds studied, the co-solvents were ethanol, methanol, acetonitrile (always ⩽25%), or DMSO (⩽18%), except for ciprofloxacin (4% NaOH in water) and GSK260983 (40% acetonitrile).

(i). *The loss of active pharmaceutical ingredient (% degradation)* was measured by HPLC-MS (Agilent HP1100 with diode array and electrospray ionization using a generic gradient method and water/acetonitrile mobile phases). The value was obtained by subtracting the remaining area of the peak for a light exposed sample from the unexposed control sample.

(ii) *Superoxide anion*. The relative amount of superoxide anion was determined according to a UV–vis spectroscopy procedure described by Pathak and Joshi (1984). Samples were evaluated for their potential to induce reduction of nitroblue tetrazolium as measured at 560 nm following UV–vis irradiation.

(iii) *Singlet oxygen* ($^1O_2$). The singlet oxygen quantum yield was measured using the singlet oxygen sensor Green Reagent (GR, *Molecular Probes*), a highly selective technique for $^1O_2$ with no appreciable response to hydroxyl radical (OH) or superoxide anion (O_2^-) by fluorescence spectroscopy (Molecular Probes Inc., 2010). In the presence of singlet oxygen, GR emits a green fluorescence similar to that of fluorescein (excitation/emission maxima ~504/525 nm). Whereas in the absence of $^1O_2$ GR exhibits weak blue

Table 1
List of compounds evaluated, classification of UV–vis absorption (290-700 nm) and 3T3NRU result summary.

Compound	UVB[a] 290-320 nm	UVA[a] 320-400 nm	Vis[a] 400-700 nm	IC_{50}[b] −UV μg/mL (from 3T3 NRU)	IC_{50}[b]+UV μg/mL (from 3T3 NRU)	PIF[c] (from 3T3 NRU)	MPE[d] (from 3T3 NRU)	3T3 NRU PIF classification[e]	3T3 NRU MPE classification[e]	Set A or B[f]	References that describe *in vivo* hototoxicity or photosensitivity for marketed drugs[g]
5-Fluorouracil	x			(316)	(316)	1	−0.03	N	N	A	QM, M, O
5-Methoxypsoralen	X	X		100	0.15	665.2	0.67	P	P	A	HSDB
Acridine	X	X		100	0.17	583.5	0.75	P	P	A	QM, M
Amiodarone	X	x		16.6	0.74	7.1	0.35	P	P	A	QM, M
Carbamazepine	x			(681)	(681)	1	0	N	N	B	QM, M, O
Cetirizine				377.4	417.9	0.9	−0.02	N	N	A	N/A
Chloroquine	x	X		1000	1000	1	0.04	N	N	A	QM, M
Chlorpromazine	X	x		31.34	1.19	26.4	0.43	P	P	A	QM, M, O
Ciprofloxacin	x	X		464	29.3	15.9	0.49	P	P	A	QM, M
Dapsone	X	x		(316)	(316)	1	0.05	N	N	A	QM
Flutamide	X	x		1000	12.18	82.1	0.56	P	P	B	QM
Furosemide	x	X		(316)	(316)	1	0.01	N	N	A	QM, O
Glybenclamide				(316)	(316)	1	0.04	N	N	B	M
Griseofulvin	X	X		(316)	(316)	1	0	N	N	B	QM, M
GSK203815G	X	X		316	164.3	1.9	0.09	N	N	B	N/A
GSK260983A	X			31.6	31.6	1	0.05	N	N	A	N/A
GW848687X	X	x		23.9	1.35	17.7	0.52	P	P	A	N/A
Hydrochlorothiazide	x	X		(1000)	(1000)	1	0.09	N	N	B	QM, M
Indomethacin	x	X	x	(100)	(100)	1	−0.02	N	N	A	O
Ketoprofen	x			316	3.18	99.5	0.58	P	P	A	QM, M, O
Levofloxacin	X	X	x	1000	31	32.2	0.76	P	P	A	DrugDex
Lomefloxacin	X	X		1000	21.51	46.6	0.57	P	P	B	QM, M
Metyrapone	x			1000	39.46	25.4	0.56	P	P	B	None found
Nalidixic acid	X	X		464	52.3	9	0.52	P	P	A	QM, M, O
Naproxen	x	X		316	105.7	3	0.41	e	P	A	QM, M, O
Nifedipine	x	X	x	681	21.5	23.1	0.38	P	P	B	QM
Omeprazole	X	x		316	116.6	2.7	0.36	e	P	A	O
Oxybenzone	X	X		65.8	56.2	1.2	0.06	N	N	A	(Deleo, 2004)
Perinaphthenone (Phenalenone)	X	X	x	681	0.08	8054	0.39	P	P	B	N/A
Piroxicam	X	X	x	(316)	(316)	1	0	N	N	A	QM, M
Promethazine	X	x		136.5	2.93	46.6	0.73	P	P	A	QM, M
Quinidine	x	X		316	38.5	8.2	0.43	P	P	A	QM, M
Riboflavin	X	X	X	100	0.53	190	0.74	P	P	A	N/A
Rose Bengal	x	X	X	2.43	0.09	26.3	0.63	P	P	A	M
SB-271046A	x	x	x	37.1	28.6	1.3	0.01	N	N	A	N/A
Sulfamethoxazole	x			(200)	(200)	1	0.04	N	N	A	QM, M
Terfenadine	x			26.2	16.4	1.6	0.04	N	N	A	QM
Tetracycline	x	X	x	1000	11.6	86.4	0.67	P	P	A	QM,
Trimethoprim	x			(1000)	(1000)	1	0.06	N	N	B	QM, M
Vitamin B12	X	X	X	(1000)	(1000)	1	0.02	N	N	A	N/A

[a] X denotes presence of a peak (lambda max), x denotes presence of a tail of absorption (without discernable peak).
[b] IC_{50} determinations are from the 3T3 NRU assay either in the absence (−UV) or presence (+UV) of 5 J/cm^2 solar simulated light; In cases where the IC50 values are denoted in parentheses, no IC_{50} could be derived due to limited solubility and the values represent the maximum concentrations employed.
[c] PIF = Photo Irritation Factor IC_{50}−UV/IC_{50}+UV:<2 not phototoxic/2–5 probable phototoxin/>5 phototoxic potential.
[d] MPE = Mean Photo Effect. Calculated using ZEBET-Holzhutter algorithm (Holzhutter, 1997).
[e] P = phototoxic, e = possible phototoxin, N = not phototoxic in PIF or MPE analysis.
[f] Set A is the training set, whereas set B is the testing set.
[g] HSDB – Hazardous Substances Data Bank (National Library of Medicine (US), 2009); DrugDex (Micromedix, 2009); M – Moore (2002); O – Onoue and Tsuda (2006); QM – Qunitero and Miranda (2000); N/A – not applicable; none found – no examples found.

fluorescence, with excitation peaks at 372 and 393 nm and emission peaks at 395 and 416 nm. For this assay, solutions were exposed to light for 2 min in each of the following conditions: 254 nm (18 W), 302 nm (VWR Scientific, 80 W), 365 nm (VWR Scientific, 100 W). The sample was 2 cm from the light source. The quantum yield calculation takes into account background emission and is measured relative to a Rose Bengal reference at 525 nm.

2.3. 3T3 NRU in vitro phototoxicity assay

Each compound was tested for phototoxicity as per established guidelines for the *in vitro* 3T3 NRU assay (OECD, 2004). The 3T3 NRU data were reported as an IC_{50} μg/mL in the presence and absence of solar simulated light (5 J/cm^2 as measured in the UVA range, at an irradiance of 1.7 mW/cm^2), and the Photo Irritation Factor (PIF: IC_{50}−UVR/IC_{50}+UVR) and Mean Photo Effect (MPE: ZEBET) parameters (Holzhutter, 1997) were calculated and then classified as follows:

PIF phototoxic classification: <2 no phototoxic potential, 2–5 possible phototoxic potential, >5 phototoxic potential.
MPE phototoxic classification: 0.1< no phototoxic potential, 0.1–0.15 possible phototoxic potential, >0.15 phototoxic potential.

2.4. Data analyses

Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) methods were used to explore the relationship between various subsets of the photochemical reactivity and photophysical parameters, and the 3T3 NRU PIF/MPE results using SIMCA-p v11.5 software (Eriksson et al., 2001). These initial discriminant models were trained on compound subset A and their predictive performance was later evaluated using compound subset B as an independent test set, when data from subset B became available. The final three variable discriminant PLS model to best describe 3T3 NRU phototoxic activity was selected via a fivefold leave-one-out cross-validation PLS algorithm implemented in *R*. The values of percent (%) degradation were log transformed in all statistical models.

3. Results

The 3T3 NRU testing results for all 40 molecules are shown in Table 1. A high concordance was observed between the classification of phototoxic potential using either the PIF or MPE 3T3 NRU grading schemes. Naproxen and omeprazole demonstrated PIF values in the range of 2–5 (i.e. probable phototoxins) and were excluded from the binary classification of positive/negative phototoxic potential in the statistical analyses utilizing PIF classification as the response variable.

3.1. Analysis of photophysical/chemical measurements

Table 2 details the experimentally derived parameters used in the 11-variable PLS-DA analysis. Typical values for the concentration normalized area under the curve (CNAUC) in the UVB and UVA regions were $\sim 10^4$–10^5 M^{-1}, whereas in the vis region values of $<1 \times 10^4$ M^{-1} were more common, with the notable exception of well-acknowledged, strong visible light absorbers including Rose Bengal, riboflavin, and Vitamin B12. These specified model compounds had Vis–CNAUC (for the visible wavelength range) values almost 100-fold those of other absorbers within this range ($\sim 10^6$ M^{-1}). Due to the special standing of UVA and visible light in reference to current guidance a combined UVA + Vis CNAUC was also considered. Note that although not included in the analysis, estimated MEC values have been tabulated in available Supplementary material.

Emission lifetimes varied between <1 and 26 ns (fast) and between <1 and 5 ms (slow). The energy levels were calculated based on the lowest wavelength emission at 77 K. As no further data interpretation was performed, it is not feasible to correlate these lifetimes or energy levels with the identity of excited states (e.g. singlet or triplet), nor with the exact nature of the emission.

Fluorescence quantum yield and loss of active pharmaceutical ingredient (% degradation) spanned the entire range from 0 to 1 and 0% to 100%, respectively. The relative levels of superoxide anion formation also provided a wide range of responses.

3.2. Statistical analysis

An initial PLS-DA analysis of the 29 compounds in subset A revealed that only 11 of the original 27 variables contributed to the classification of phototoxic/non-phototoxic profiles. The relative importance of these 11 parameters is shown in Fig. 1. The 11-variable model suggested that singlet oxygen formation, superoxide anion formation assays, and a measure of the extent of degradation due to light exposure were the three variables with the largest contribution to the discriminant model (Fig. 1). These results were confirmed when the same three variables were selected as best predictors of phototoxicity in the 3T3 NRU model by a PLS algorithm with the fivefold cross-validation loop that was run on the full data set comprising all 40 molecules and all parameters. Although the fluorescence quantum yield was deemed significant based on Fig. 1, inclusion of this parameter did not increase the predictive ability of the model, and thus inclusion of the ϕ_f parameter was deemed redundant to the model.

Fig. 2 visualizes the clustering of the training set compounds (subset A) in the space defined by the first two principal components of the three-variable PLS-DA model for MPE scores. Furosemide, acridine and naproxen were the only compounds misclassified by this model in the training set (subset A). A similar plot was generated for PIF scores.

The three-variable PLS-DA models (log percent degradation, superoxide anion formation and singlet oxygen quantum yield) trained on subset A (29 compounds) correctly classified 8 of the 10 (PIF), or 9 of the 11 (MPE) molecules in the test set (subset B). (The PIF-equivocal compounds were not used in the PIF modeling). The test set (subset B) compounds misclassified in terms of their phototoxic potential were flutamide and glybenclamide in both the PIF and MPE models.

The overall performance (combined subset A and B) of the three-variable PLS-DA model for PIF status resulted in the correct classification of 33/37 molecules, with only flutamide, glybenclamide, acridine and furosemide incorrectly assigned to their phototoxicity activity class.

Similarly, the three-variable PLS-DA model for MPE status resulted in the correct classification of 35/40 molecules, with only flutamide, glybenclamide, acridine, furosemide and naproxen incorrectly assigned to their phototoxicity activity class.

The training set (subset A), test set (subset B), and overall (subsets A and B) sensitivity and specificity of the three-variable PLS-DA models for PIF and MPE are summarized in Fig. 3.

The following classification function was derived from the 3-variable PLS discriminant model predicting 3T3 PIF outcomes:

$$Y = 0.01 + 0.49X_1 + 0.21X_2 + 0.18X_3$$

'*Y*' is the predicted PIF class membership for a given compound (0 – negative to 1 – positive). If $Y < 0.5$, the compound is predicted as negative. If $Y > 0.5$ the compound is predicted to be a positive in the 3T3 NRU assay. The 0.5 cutoff represents the boundary between

Table 2
Summary of parameters in PLS-DA loadings plot (Fig. 1).

Compound	*c* Log $D_{7.4}$	UV-B CNAUC/M^{-1}	UV-A CNAUC/M^{-1}	Vis CNAUC/M^{-1}	Sum CNAUC (UV-A and vis)/M^{-1}	Emission lifetime[a] /ns	Emission lifetime[b]/ ms	Flu ϕ[d]	% Degradation	Relative superoxide	Singlet O_2 ϕ
5 Methoxy psoralen	2	2.88E+05	1.73E+05	<1E03	1.73E+05	1.4	3.9	0.026	24.3	0.160	0.17
5-Fluorouracil	−5.6	7.02E+03	4.87E+03	1.51E+04	2.00E+04	1.7	4.2	0.0003	2.4	0.000	0.11
Acridine	3.4	3.91E+04	2.65E+05	3.43E+04	2.99E+05	4.2	3.4	0.329	0.5	0.980	0.10
Amiodarone	6.9	1.38E+05	6.10E+04	2.55E+03	6.36E+04	3.2	1.0	0.0023	22.1	0.210	1.0
Carbamazepine	2.7	3.47E+04	4.92E+03	7.60E+02	5.68E+03	4.6	0.4	0.002	1.5	0.005	0.00
Cetirizine	−1.1	1.98E+04	2.66E+04	8.00E+02	2.74E+04	2.1	<0.1	0.001	0	0.002	0.06
Chloroquine	1.9	8.99E+04	2.60E+05	2.28E+03	2.62E+05	0.7	3.6	0.0075	1.9	0.000	0.00
Chlorpromazine (CPZ)	3.2	9.35E+04	5.16E+04	8.53E+03	6.02E+04	6.6	4.1	0.0021	81.4	0.000	0.19
Ciprofloxacin	−0.8	2.34E+05	2.88E+05	2.07E+03	2.90E+05	25.7	1.9	0.0019	11.7	0.000	0.25
Dapsone (4-aminophenyl sulfone)	0.9	5.29E+05	1.05E+04	2.08E+02	1.07E+04	1.4	3.7	0.018	0	0.007	0.24
Flutamide	3.7	2.20E+05	1.92E+05	1.45E+02	1.92E+05	3.0	0.4	0.002	1.7	0.000	0.00
Furosemide	−0.1	6.93E+04	1.35E+05	2.49E+03	1.38E+05	2.8	3.8	0.0045	49.4	0.102	0.14
Glybenclanide (Glyburide)	1.9	<1E+03	<1E+03	<1E+03	<1E+03	9.0	0.4	0.12	24.2	0.205	0.00
Griseofulvin	3.5	2.48E+05	9.19E+04	<1E+03	9.19E+04	1.5	3.5	0.193	1.5	0.001	0.00
GSK203815G	2.5	9.10E+04	3.62E+04	3.25E+03	3.95E+04	9.5	0.2	0.039	1.0	0.176	0.00
GSK260983A	3.3	1.08E+05	<1E+03	<1E+03	<1E+03	3.6	0.5	0.0025	2.3	0.000	0.12
GW848687X	3.3	2.56E+05	9.45E+04	1.40E+05	2.34E+05	1.7	<0.1	0.004	71	0.353	0.29
Hydrochlorothiazide	−0.1	3.63E+04	3.74E+04	4.38E+04	8.12E+04	6.5	0.3	<0.0003	1.8	0.005	0.00
Indomethacin	−0.2	1.39E+05	1.81E+05	3.82E+04	2.20E+05	2.0	0.6	0.0025	0	0.120	0.00
Ketoprofen	−0.2	3.33E+04	9.45E+03	4.70E+03	1.42E+04	1.5	3.5	0.0033	99.6	0.037	0.30
levofloxacin	−0.7	2.42E+05	2.30E+05	<1E+03	2.30E+05	6.7	3.9	0.183	6.9	1.07	0.26
Lomefloxacin HCl	0.4	3.65E+05	2.32E+05	<1E+03	2.32E+05	2.2	0.4	0.301	77	0.288	0.00
Metyrapone	1.2	1.34E+04	2.03E+04	3.05E+04	5.08E+04	6.6	0.5	0.002	70.2	0.000	0.00
Nalidixic acid	−0.4	8.15E+04	8.11E+04	2.63E+03	8.38E+04	4.5	5.0	0.017	14.6	0.593	0.25
Naproxen	0.5	3.39E+04	3.28E+04	<1E+03	3.28E+04	0.3	4.6	0.19	0	0.029	0.40
Nifedipine	3.0	5.80E+04	1.32E+05	1.30E+04	1.45E+05	7.3	0.3	0.001	99.9	0.000	0.06
Omeprazole	2.1	3.53E+05	2.91E+04	1.02E+04	3.93E+04	3.5	0.8	0.057	17.6	0.027	0.29
Oxybenzone	3.9	2.64E+05	2.59E+05	<1E+03	2.59E+05	1.3	3.8	0.0029	0.3	0.001	0.03
Phenalenone (Perinaphthenone)	3.5	1.01E+05	6.30E+05	1.73E+05	8.03E+05	0.37	0.4	0.001	3.5	0.005	1.0
Piroxicam	−1.1	1.91E+05	6.61E+05	4.66E+04	7.07E+05	3.0	4.3	0.021	0.8	0.102	0.09
Promethazine	3.2	4.37E+04	1.10E+04	2.26E+03	1.33E+04	1.1	4.0	0.0112	17.6	0.001	1.0
Quinidine	1.6	9.33E+04	9.69E+04	<1E+03	9.69E+04	1.1	3.5	0.011	1.7	0.063	1.0
Riboflavin	−4	6.50E+04	5.49E+05	8.17E+05	1.37E+06	4.2	5.0	0.323	30.1	1.19	1.0
Rose bengal	4.8	9.95E+04	1.22E+05	1.42E+06	1.54E+06	0.74	0.4	0.03	0.8	2.46	0.76
SB-271046-A	3.3	6.35E+04	1.71E+04	<1E+03	1.62E+04	1.4	0.5	0.0002	1.3	0.001	0.17
Sulfamethoxazole	−0.6	9.48E+04	8.29E+01	3.36E+03	3.44E+03	2.7	4.0	0.0127	0	0.001	0.27
Terfenadine	4.4	2.95E+03	4.42E+03	9.49E+03	1.39E+04	3.3	4.3	0.021	0	0.024	0.12
Tetracycline	−4.3	1.27E+05	3.11E+05	1.56E+04	3.27E+05	0.4	4.1	0.0014	75.4	0.22	0.14
Trimethoprim	0.6	2.82E+04	3.83E+03	3.22E+03	7.05E+03	7.6	0.3	0.015	1.6	0.000	0.00
Vitamin B12	−6.9[c]	2.26E+05	7.94E+05	8.18E+05	1.61E+06	2.3	2.2	0.0002	0	0.000	0.20

[a] Fast radiative deactivation pathway observed at ambient temperature.
[b] Slow radiative deactivation pathway taken at 77 K.
[c] Calculated without phosphorylated side-chain due to limitation of prediction program (number of atoms).
[d] Fluorescence quantum yield.

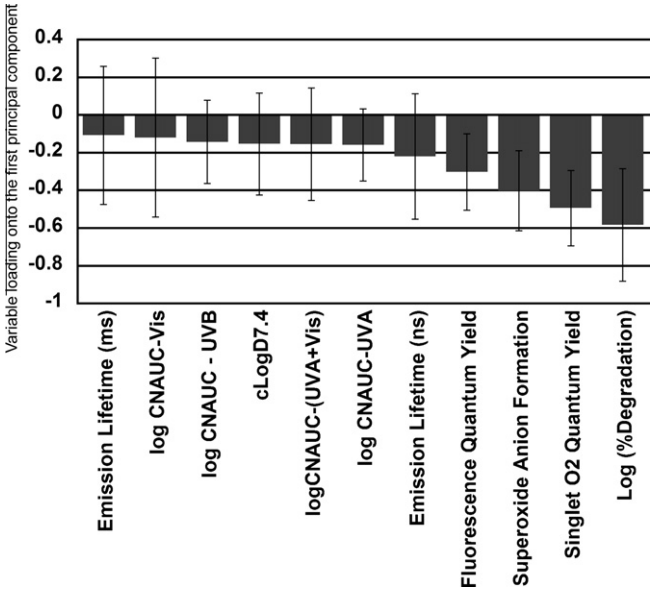

Fig. 1. PLS-DA loadings plot with 95% confidence intervals. The Y-axis shows the loadings of each of the 11 original variables onto the first principal component of the PLS-DA model for PIF status. Variables with high positive or negative loadings with confidence interval not containing 0 are important discriminatory variables for PIF status in the 3T3 assay.

0 and 1 and is somewhat arbitrary. The closer the prediction is to 0 or 1, the higher the confidence in the negative or positive class membership, respectively.

X_1 = log 10 (%degradation + 1)
X_2 = relative superoxide anion assay result
X_3 = singlet oxygen assay result

The equivalent classification function derived using the 3T3 MPE outcome is:

$$Y = 0.05 + 0.47X_1 + 0.19X_2 + 0.18X_3$$

'*Y*' is the predicted MPE class membership for a given compound. If $Y < 0.5$, the compound is predicted as negative. If $Y > 0.5$ the compound is predicted to be a positive in the 3T3 NRU assay. X_1, X_2, and X_3 are the same as above.

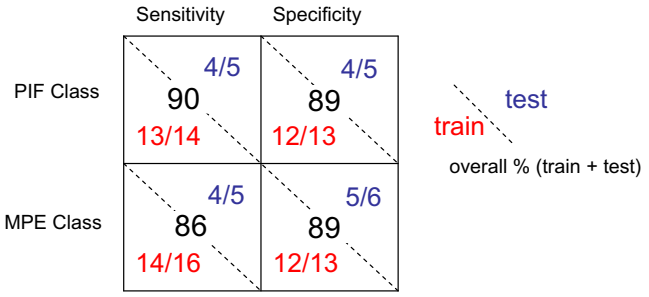

Fig. 3. Training, test, overall specificity and sensitivity of the 3-variable PLS-DA models for PIF and MPE status. Both models were trained on the training set only (subset A).

4. Discussion

The measured parameters for photochemical reactivity employed within this investigation were clearly capable of discriminating between those compounds that were regarded as phototoxic within the 3T3 NRU assay, and those which demonstrated no phototoxic potential. Furthermore, the ability to classify molecules on the basis of their phototoxic potential was independent of the grading scheme used to summarize the 3T3 NRU response, i.e. Photo Irritation Factor (PIF), or Mean Photo Effect (MPE).

4.1. Impact of photophysical properties on the photocytoxicity response in the 3T3 NRU assay

Since light is a form of energy, in order for a photo-induced process to be initiated, a molecule must absorb light. Interestingly, the wavelength of molecular absorption appeared to have little bearing on the phototoxic potential as the variables defining absorption within the UVB/UVA/visible light regions of the electromagnetic spectrum did not contribute significantly to the discriminant classification function. From this perspective, it appears that it is not the absorption of light that directly correlates to the observed 3T3 NRU results. It is worthwhile to note that although this analysis utilized the CNAUC values to afford comparison between drug-like molecules in this study, MEC threshold values have been suggested by others as a primary step in the assessment of phototoxicity (Henry et al., 2009). The data generated in this study also afforded an estimate of the MEC values from a single spectral data

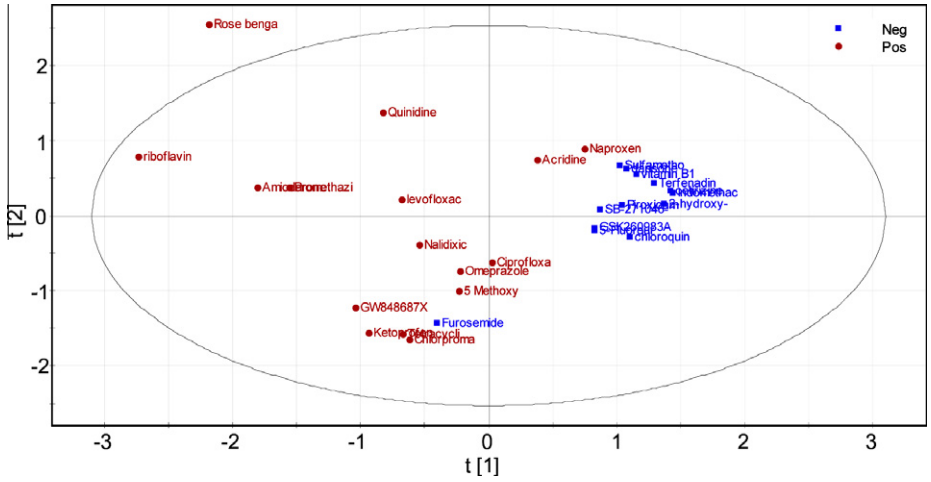

Fig. 2. Three variable PLS-DA scores plot (MPE) visualizing the clustering of the training set compounds (subset A) in the space defined by the first two principal components of the model. Furosemide, Acridine and Naproxen were the only misclassified compounds in subset A.

point (for absorbance maxima of peaks between 290 and 700 nm, see Supplementary material). All 3T3 NRU positive compounds with at least one absorbance maximum in the UV–vis spectrum between 290 and 700 nm produced an MEC value greater than 1000 L mol^{-1} cm^{-1}. This is consistent with the proposed threshold trigger for photosafety testing as defined by the International Workshop on Genotoxicity Testing (IWGT, which took place in Basel, August 2009, see http://www.iaems.net/iwgt.asp).

In terms of emission parameters, the emission lifetime assays suggested a negligible correlation to positive 3T3 NRU results. In contrast, higher fluorescence quantum yields were found to be indicative of positive 3T3 NRU outcomes. While shorter excited state lifetimes, or higher fluorescence quantum yields, could be thought of as reducing the likelihood of a potential photocytotoxic response since the deactivation process is via a non-reactive physical nature, this was not borne out in the results. This may be due to the fact that although molecules may exhibit some form of emission, a small amount of energy may be used in other mechanisms that lead to photoreactivity and positive 3T3 NRU responses. As a consequence, molecules with higher fluorescence quantum yields are possibly manifesting a photocytotoxic response in the assay via one of the other non-photophysical mechanisms.

4.2. Impact of Photochemical reactivity on the photocytotoxicity response in the 3T3 NRU assay

Three measures of photochemical reactivity were included in this study – a measure of the extent of chemical degradation, the quantum yield of singlet oxygen formation and the relative amount of superoxide anion formation.

Singlet oxygen is typically formed via an interaction between ground state oxygen (triplet ground state) and an excited triplet state. This is an energy transfer process which leads to the formation of a ground state molecule and singlet oxygen. Consequently, the energy of light absorption is being used to create reactive singlet oxygen. Superoxide anion is formed via an electron transfer process from an excited singlet or triplet state to ground state O_2. Similar to singlet oxygen, the resultant radical cation and O_2 radical anion (superoxide anion) may each go on to react with other endogenous molecules in the system.

There are many mechanisms of photodegradation (Albini and Fasani, 2004). It should be noted that the photostability assessment (% degradation) employed within this study utilized a radiant UV exposure of 5 J/cm^2 (which is equivalent to the irradiant dose given in the 3T3 assay). This value is considerably lower than the UV exposure prescribed in ICH Q1B for a confirmatory photostability study (200 W h/m^2) which approximately corresponds to 1–2 days of sunlight derived UVA exposure through window glass (Anderson, 1996).

Each of these mechanisms of photoreactivity is initiated by light absorption and the subsequent formation of an (photoactivated) excited state. Molecules may react differently (e.g. via a carbocation, carbanion, carbene, radical cation or anion, etc.), but regardless of the nature of the reactive intermediate that is formed, the subsequent process consumes the energy imparted by the photons. Oxybenzone provides an excellent case study. As mentioned earlier, it is well known that almost all of the absorbed light energy is utilized via an excited state proton transfer mechanism. This is borne out by a PIF score of close to 1 (MPE = 0.06) even though there is very significant UV-B and UV-A absorption.

In contrast to a photophysical response to photon absorption, each of the three photochemical responses studied here (superoxide anion, singlet oxygen generation and loss of active pharmaceutical ingredient/degradation) result in reactive species which may go on to interact in the local biological environment. Perinaphthenone, which has a singlet oxygen generation quantum yield of almost unity and provides an excellent example of demonstrating the link with chemical photoreactivity and positive 3T3 NRU results as it had the highest PIF score.

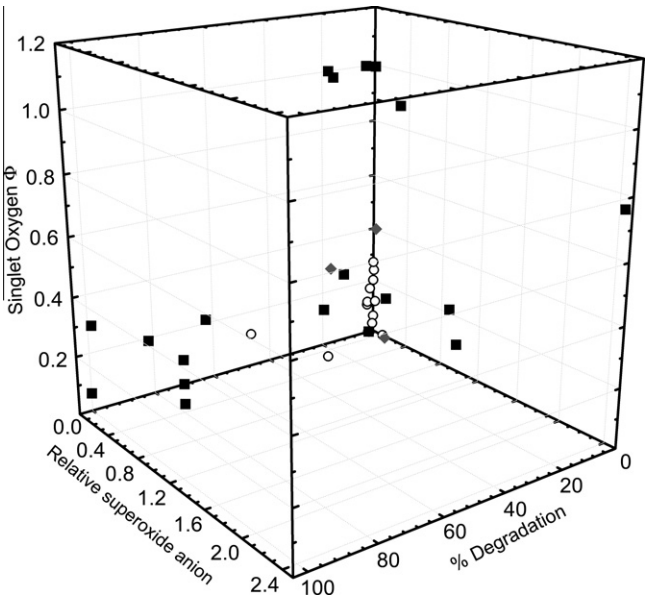

Fig. 4. PIF score three dimensional representation in the space defined by the 3 variables selected by PLS-DA modeling. 3T3 PIF scores denoted as positive (■), equivocal (◆) and negative (○).

The experimentally determined measurements of singlet oxygen and superoxide anion production, as well as photodegradation, were seemingly themselves capable of '*predicting*' the 3T3 NRU assay result. This is consistent with the findings of other authors who have postulated that these pathways are important in clinical phototoxicity reactions (Johnson and Ferguson, 1990; Moore, 2002; Ferguson, 2002; Onoue and Tsuda, 2006). In another example, Gocke et al. (2000) postulated that photostability by itself is insufficient to classify molecules on the basis of their photogenotoxic potential, however a combined analysis of the multiple photochemical properties studied within this analysis was not undertaken (Gocke et al., 2000).

In the current study, the formation of various reactive species was evaluated, i.e. photodegradation in solution, the quantum yield of formation of singlet oxygen and the relative formation of superoxide anion. It is the combination of these three highlighted properties which together give rise to the predictive value seen with the statistical model employed. While loss of the starting active pharmaceutical ingredient is easy to discern since it arises from reactivity, other examples exist, e.g. methylene blue, which are photostable and are able to photosensitize other molecules in their immediate surroundings. In fact, this behavior is employed in photodynamic therapy because of its ability to generate singlet oxygen following light absorption (Tardivo et al., 2005).

5. Conclusion

In conclusion, all of the 3T3 NRU phototoxic positives reviewed in the 40 compound test set were associated with underlying photochemical mechanisms which we consider to explain the outcome of the assay in terms of photoreactivity. Therefore, while absorption of light initiates the photoactivity, it is not the act of absorbing the light energy that results in a positive 3T3 NRU result. Rather, it is the subsequent steps in the energy cascade (how the energy imparted to the molecule from light absorption is dissipated) that result in phototoxicity as measured by a positive 3T3 NRU response.

In terms of analytical requirements for photochemical assessment, each of the assays for the three key discriminators (photodegradation, singlet oxygen and superoxide anion formation) require very limited capital investment, little resource to run, and have well-established methodologies. They can be made to be very robust and ultimately will be quicker and less expensive to run relative to the 3T3 NRU assay. It is therefore proposed that a molecular basis for photoreactivity (i.e. singlet oxygen, superoxide anion and photodegradation) be included as one of the triggers for photosafety testing in addition to UV–Vis absorption and tissue distribution. Based on the results of the current study, it would appear that if under the light exposure conditions used in this study a compound demonstrated one or more of the following: (a) $\geqslant$2.5% degradation; (b) a singlet oxygen quantum yield of $\geqslant$0.35; or (c) relative superoxide anion value of $\geqslant$0.3 then the compound would have a high probability of being positive in the 3T3 NRU assay (Fig. 4).

Conversely, the absence of any photoreactive signal, or a signal below the thresholds stated above, would indicate a lack of phototoxic potential and thus no biological investigations would be warranted. Finally, the photochemical reactivity concordance with the *in vitro* phototoxic result profile obtained within the 3T3 NRU assay supports the utility of this test to detect phototoxic hazard as no 'false' phototoxic responses were observed. However, extrapolation of these data to *in vivo* phototoxic risk warrants further investigation because of the high percentage of compounds eliciting a positive response in the 3T3 NRU assay that are negative in animal studies or in the clinic (Ferguson, 2002; Lynch and Wilcox, 2010). This may be due to factors such as lack of significant photoactivating light penetrating through tissue, or a lack of systemic distribution of drug into light exposed tissues.

Conflict of interest statement

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yrtph.2010.06.013.

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