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Phototoxic properties of systemically applied pharmaceuticals may be the cause of serious adverse drug reactions. Therefore, a reliable preclinical photosafety assessment strategy, combining in vitro and in vivo approaches in a quantitative manner, is important and has not been described so far. Here, we report the establishment of an optimized modified murine local lymph node assay (LLNA), adapted for phototoxicity assessment of systemically applied compounds, as well as the test results for 34 drug candidates in this in vivo photo-LLNA. The drug candidates were selected based on their ability to absorb ultraviolet/visible light and the photo irritation factors (PIFs) determined in the well-established in vitro 3T3 neutral red uptake phototoxicity test. An in vivo phototoxic potential was identified for 13 of these drug candidates. The use of multiple dose levels in the described murine in vivo phototoxicity studies enabled the establishment of no- and/or lowest-observed-adverse-effect levels (NOAELs/LOAELs), also supporting human photosafety assessment. An in vitro-in vivo correlation demonstrated that a drug candidate classified as "phototoxic" in vitro is not necessarily phototoxic in vivo. However, the probability for a drug candidate to cause phototoxicity in vivo clearly correlated with the magnitude of the phototoxicity identified in vitro.

Key words: phototoxicity; local lymph node assay; photoirritation factor; photosafety.

Phototoxicity of pharmaceutical products may cause serious adverse drug reactions. This does not only apply to topically applied chemicals absorbing ultraviolet (UV) and/or visible (vis) light, but also to those that reach light-exposed tissues such as skin or eyes following systemic exposure (for review see Drucker and Rosen, 2011; Ferguson, 2002; Moore, 2002). The contact phototoxic potential of topically applied pharmaceuticals is typically assessed preclinically using in vivo phototoxicity assays. These include monitoring of skin reactions in topically treated guinea pigs or the murine local lymph node assay (LLNA) in albino mice, including its nonradioactive modifications (for these, the term “modified LLNA” is commonly used), with quantification of skin and lymph node (LN) reactions (Homey et al., 1998; Neumann et al., 2005; Ulrich et al., 2001; Vohr et al., 2001). However, for systemically applied pharmaceuticals, an integrated preclinical photosafety assessment strategy has not been established so far.

The standard preclinical in vitro assay for phototoxicity assessment is the "in vitro 3T3 neutral red uptake (NRU) phototoxicity test" (OECD, 2004), which may be considered for compounds showing relevant light absorption in the range of natural sunlight (290–700 nm; Bauer et al., 2014). Neumann et al. (2005) and Vohr et al. (2001) reported the testing of selected systemically applied reference compounds in different preclinical in vitro and/or in vivo assays, including an "integrated model for the differentiation of skin reactions" (IMDS) based on a modified murine LLNA with endpoints limited to ear thickness, local LN weight, and cell counts.

Here, we report the establishment of a further optimized and extended modified murine LLNA, adapted for phototoxicity assessment of systemically applied compounds (photo-LLNA), the correlation between the in vitro and in vivo photosafety testing of 34 drug candidates in this system, and the relevance to preclinical photosafety assessment.

The reference compounds included sparfloxacin (Dawe et al., 2003; Hamanaka et al., 1998; Lipsky et al., 1999a,b; Pierfitte et al., 2000), enoxacin (Dawe et al., 2003; Izu et al., 1992; Kang et al., 1993), lomefloxacin (Cohen and Bergstresser, 1994; Correia and Delgado, 1994; Man et al., 1999), doxycycline (Bjellerup and Ljunggren, 1994; Blank et al., 1968; Frost et al., 1972; Layton and Cunliffe, 1993), ketoprofen (Bagheri et al., 2000; Foti et al., 2011), and 8-methoxypsoralen (8-MOP), the latter also being used as an orally administered photoactive drug together with UVA irradiation in photochemotherapy ("PUVA," psoralen + UVA) of severe psoriasis (for review see Lapolla et al., 2011). For completeness, previously reported results with the reference compound vemurafenib (Boudon et al., 2014) are included as well.

In addition to clinically relevant reference compounds, 34 systemically applied drug candidates were tested at three dose levels in this in vivo assay. The following major optimizations compared with the described IMDS for systemically applied phototoxic reference compounds (Neumann et al., 2005; Vohr et al., 2001) were done: (1) systematic monitoring of erythema...
formation at least twice daily using a defined scoring system, (2) determination of ear biopsy weights instead of ear thickness, i.e., exclusion of a subjective component associated with the measurement of ear thickness using a micrometer (Ulrich and Vohr, 2012), (3) inclusion of histopathological analysis of the retina due to residual absorption of visible light at wavelengths that reach the human retina. Altogether, determination of erythema formation and ear weight, local LN reactions (quantification of LN weights and cell counts), and retina changes as well as identification of a no- or lowest-observed-adverse-effect level (NOAEL/LOAEL) are described as key elements supporting later human photosafety assessment. Finally, the implications of an in vitro-in vivo phototoxicity correlation on the preclinical in vivo photosafety testing strategy are discussed.

MATERIALS AND METHODS

UV/visible light absorption spectra. Light absorption spectra within sunlight range (290–700 nm) were recorded on a Cary 300 spectrophotometer (Varian Australia Pty Ltd, Australia) using UV-transparent quartz glass cuvettes (1 cm path length). Substances were dissolved in methanol applying individual solvent-specific baseline correction. For each peak (and for 290 nm if this was the highest observed absorption value) the molar extinction coefficient (ε or MEC) was calculated:

\[ \varepsilon = \frac{A}{(c \times l)} \]

(A, absorbance; c, concentration; l, path length [cm]).

In vitro 3T3 NRU phototoxicity test. The BALB/c mouse fibroblast cell line 3T3.A31 was obtained from the European Collection of Cell Cultures (ECACC, no. 86110401, at passage 82), United Kingdom. Cells were cultivated in Dulbecco’s Modified Eagle Medium (with phenol red) containing 10% fetal calf serum, 1% glutamine, and 1% penicillin/streptomycin. The assay was performed in accordance with OECD Testing Guideline 432. Briefly, 24 h after seeding the mouse fibroblast cells (not exceeding passage 99) into 96-well plates, the medium was removed and the cells were treated with different concentrations of the test compound for 1 h using Hank’s Balanced Salt Solution (HBSS, without phenol red) as medium replacement. Subsequently, these cells were irradiated (+Irr) with simulated sunlight (SOL500 H1, Dr. Hönle, Germany) with a main spectral output from 320 until beyond 700 nm. The integrated H1 filter system attenuated the highly cytotoxic UVB range to a level which was tolerated by the cell culture as suggested by the mentioned guideline. The spectral intensity of the light source has been published in Boudon et al. (2014). In parallel, an identically prepared 96-well plate was kept in the dark (−Irr), serving as control. UV/visible irradiance was measured by a UVA meter (product no. 0037; Dr. Hönle, Germany) with spectral sensitivity in the range from 320 to 400 nm and a measuring range between 0 and 199.9 mW/cm². The yearly calibration using an externally calibrated spectroradiometer covering the full spectral range from 250 to 800 nm was performed by opto.cal GmbH (Switzerland), which is a calibration laboratory accredited by the Swiss Accreditation Service. The applied intensity was 1.67 mW/cm² resulting in a total UVA dose of 5 J/cm² after 50 min of irradiation. After irradiation, the HBSS buffer was replaced by fresh medium. Cell viability was determined 24 h later using neutral red as the vital dye, which was measured at 540 nm after incubation and extraction. The photo irritation factors (PIFs) were calculated according to OECD TG 432 using the following equation: PIF = \( \frac{IC_{50}(-Irr)}{IC_{50}(+Irr)} \).

Mice. Female BALB/c mice, obtained in a specific pathogen-free state from Charles River Laboratories (either L’Arbresle/France or Sulzfeld/Germany for an individual study; choice of breeding facility depended on mouse availability and timing of animal delivery), were used throughout the studies, usually at the age of 8–10 weeks. The photo-LLNA studies were performed in conformity with the Swiss Animal Welfare Law and in accordance with internal standard operating procedures and guidelines for care and use of laboratory animals. Mice had ad libitum access to pelleted standard rodent diet and tap water from the domestic supply and were kept under temperature- and humidity-controlled conditions and an automatic 12 h light/dark cycle with background radio coordinated with light hours.

Treatment of mice. For the establishment of the optimized modified murine systemic photo-LLNA, the following reference compounds (all obtained from Sigma-Aldrich/Fluka, Switzerland) were administered by oral gavage at three dose levels (12 mice per group) once a day for three consecutive days: sparfloxacin (product no. 56968; in 1% (w/v) aqueous solution of carboxymethylcellulose [CMC]); enoxacin (product no. E3764; in water); doxycycline hyclate (product no. D9891; in water); ketoprofen (product no. K1751; in 0.5% CMC); lomefloxacin hydrochloride (product no. L2906; in water; only administered for two consecutive days). For systemic administration of 8-MOP, commercial meladinine tablets (10 mg; Galderma, Switzerland; in water) were used at only two dose levels for three consecutive days.

Drug candidates were administered systemically (by oral gavage or intravenously) at three dose levels in suitable vehicles for three consecutive days. Selection of dose levels was mostly based on expected maximal tolerated exposure (high dose level) upon systemic treatment, pharmacologically relevant exposure (low dose level), and an exposure level in between those two (intermediate dose level).

If necessary, the amount of the administered reference compound or drug candidate was corrected for the content of the active ingredient (e.g., if dosed as hydrochloride).

Exposure of mice to simulated sunlight. During irradiation, mice were kept in specific cages allowing only for lateral movements and ensuring a uniform irradiation of their backs and
ears. Nonirradiated animals were kept in their housing cages under standard room light. Six mice per dose level were exposed to simulated sunlight (Psoriasis 900 H1 lamp; Dr. Hönle, Germany) with a main spectral output from 320 until beyond 590 nm. The spectral intensity of the light source has been published in Boudon et al. (2014). Irradiation was normalized to a dose of 10 J/cm² UVA. The integrated H1 filter system attenuated the highly cytotoxic UVB range to a level that was tolerated by the animals. This adjustment is recommended for testing oral drugs because in such cases photosafety assessment is mainly focusing on UVA and visible light, as only these wavelengths are penetrating sufficiently into skin (ICH S10, 2013). With the sunlight simulator used, spectral output between 450 and 490 nm and beyond 590 nm was under-represented compared with sunlight (Boudon et al., 2014). However, none of the administered compounds had its absorption peak in these ranges. UVA irradiance was measured with a UV radiometer (X1-1, detector XD-9501; Gigahertz-Optik GmbH, Germany). The yearly calibration of this GLP-compliant equipment with an externally calibrated spectroradiometer covering the full spectral range from 250 to 800 nm was performed by opto.cal GmbH (Switzerland). Dose groups were exposed to simulated sunlight separately from each other. Selection of the time point of exposure to simulated sunlight was mostly based on pharmacokinetic properties of the compounds (i.e., expected tmax). Corresponding control groups treated with vehicle, not exposed to simulated sunlight, were included. For the reference compounds, exposure to simulated sunlight started not later than 1.5 h after treatment (exception: 2 h for doxycycline).

Erythema scoring. During the dosing period, formation of ear skin erythema was monitored at least twice daily using a defined scoring system (0 = no erythema; 1 = slight erythema; 2 = moderate erythema; 3 = strong erythema).

Determination of ear biopsy weights and auricular LN weights and cell counts. Approximately 24 h after the last treatment, mice were sacrificed by exposure to carbon dioxide. If not indicated differently in the results part, from both ears circular pieces from the apical area of each ear with a diameter of 8 mm (= 0.5 cm²) were excised using a disposable punch and weighed as pairs on an analytical balance. For assessment of auricular LN weights and cell counts, the superficial parotid LNs that can be found as single LNs at the jugular bifurcation and that are referred to as “auricular LNs” (cf. Van den Broeck et al., 2006; NIH, 1999) here, were excised bilaterally, weighed on an analytical balance, and kept in 1 ml ice-cold 0.5% BSA/PBS per pair. LN cell suspensions were prepared by mechanical disruption of the LNs using a stainless steel mesh. From the resulting suspensions, cell counts were determined in a conductometer (CASY TTC, Schärfe System, Germany).

Histopathology of retina. In murine photo-LLNA studies for sparfloxacin and several drug candidates, one eye from each animal was taken and fixed in Davidson’s solution. Tissue was embedded in Paraplast, sectioned, stained with hematoxylin and eosin, and examined microscopically. For the human retina, only exposure to visible light is relevant because wavelengths below 400 nm do not sufficiently penetrate human cornea, lens, and vitreous body (Dillon et al., 2000; ICH S10, 2013; Lei and Yao, 2006; Sliney, 2002). Therefore, histopathological examination of the retina was not done for all compounds.

Statistical analysis. For statistical calculations, either SigmaStat or SAS was used. A One-Way Analysis of Variance (ANOVA) was used as statistical method. A normality test was performed to assure that the data were normally distributed (significance level = 0.01). The equal variance test was used to check the assumption that the sample was drawn from populations with the same variance (significance level = 0.01). In case of significant results of the One-Way ANOVA (P < 0.05), multiple comparisons were performed with the Student-Newman-Keuls test. If the normality test and/or the equal variance test gave P values < 0.01, a suitable transformation (log, square root) was applied; if the normality test and/or equal variance test still gave P values < 0.01, the nonparametric Kruskal-Wallis test was used, and in case of a significant result of the Kruskal-Wallis test (P < 0.05), multiple comparisons were performed with the Student-Newman-Keuls test for the ranks of the original observations. For the Student-Newman-Keuls test, the confidence level for the difference of the means was set to 95% (α = 0.05). Groups of mice treated with compound were statistically compared with the group of mice treated with vehicle and not exposed to simulated sunlight. Furthermore, groups of mice treated with compound and exposed to simulated sunlight were compared with corresponding groups not exposed to simulated sunlight.

RESULTS

Clinically Phototoxic Reference Compounds in the Modified Murine Oral (Gavage) photo-LLNA

The UV-vis absorption spectra of six clinically relevant phototoxic compounds, i.e., sparfloxacin, enoxacin, lomefloxacin, doxycycline, ketoprofen, and 8-MOP, were recorded and analyzed to identify absorption peaks with associated MECs (Table 1). The in vitro phototoxic potential of these compounds was identified by determination of PIF values using the well-established 3T3 NRU test. All six reference compounds were phototoxic in vitro with PIF values > 25, and they showed a phototoxic potential in the herein described optimized murine oral (gavage) photo-LLNA (Table 1). For completeness, the results with the previously reported reference compound vemurafenib (Boudon et al., 2014) are listed as well.

Signs of ear skin irritation (erythema and/or increased ear biopsy weights) and auricular LN response (increased LN weight and cell count) mostly occurred concomitantly. As an ex-
exception, ketoprofen did not induce skin irritation or auricular LN response up to 300 mg/kg/day. However, during a dose-finding phase, a limited number of mice (n = 2) was also treated with 400 and 500 mg/kg/day ketoprofen. These dose levels were identified to be toxic, but irradiation-dependent erythema formation was observed at 500 mg/kg/day. Hence, the phototoxic properties of ketoprofen were confirmed in BALB/c mice. For all six tested reference compounds, in vivo phototoxicity was dose-dependent.

As shown as an example in Figure 1, sparfloxacin induced weak signs of irradiation-dependent ear skin irritation (increase of ear biopsy weights) and a LN response (increase of auricular LN cell counts) in the modified murine photo-LLNA at 25 mg/kg/day, establishing the LOAEL with regard to phototoxicity. At 100 and 150 mg/kg/day sparfloxacin, all quantitatively determined parameters (ear biopsy weight, auricular LN weight, and cell count) were dose-dependently and statistically significantly increased depending on additional exposure to simulated sunlight. Due to the robust response, 100 mg/kg/day sparfloxacin was chosen as standard positive control item in further studies using the modified murine photo-LLNA for systemically applied drug candidates. The mean values and standard deviations of sparfloxacin/irradiation-induced ear biopsy weight as well as auricular LN weight and cell count changes derived from 21 studies, in which sparfloxacin was used as the positive control item, are described in Table 2. On average, ear biopsy weights increased by a factor of 1.58, auricular LN weights by a factor of 1.77, and auricular LN cell counts by a factor of 2.18, depending on additional exposure to simulated sunlight. Sparfloxacin, which absorbs visible light relevant for the human retina (Boudon et al., 2014), also induced irradiation-dependent pathological alterations in the retina at the dose of 100 mg/kg/day. As shown in Figure 2 (which also contains information for a drug candidate, cf. 3.2), sparfloxacin induced irradiation-dependent minimal to moderate atrophy and degeneration in the retina. Reduced thickness, disorganized appearance of the outer nuclear layer, and loss of nuclei form rods and cones were the most prominent features. Although less prominent, changes were also present in the inner nuclear layer, e.g., loss of cytoplasmic detail in the outer limiting membrane.

### Table 1

**Combined UV-vis, In Vitro, and In Vivo Data for Systemically Applied Phototoxic Drugs (Reference Compounds, in Order of Increasing PIF Values)**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Peak (nm)</th>
<th>MEC (L mol⁻¹ cm⁻¹)</th>
<th>IC₅₀ – irr (µg/ml)</th>
<th>IC₅₀ + irr (µg/ml)</th>
<th>PIF</th>
<th>LOAEL/NOAEL (mg/kg body weight)</th>
<th>Skin</th>
<th>Lymph nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enoxacin</td>
<td>345</td>
<td>18300</td>
<td>1000</td>
<td>39</td>
<td>&gt; 26</td>
<td>400/800/1600</td>
<td>ET n.r.</td>
<td>ET ≥ 800</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LW ≥ 800</td>
<td></td>
</tr>
<tr>
<td>Lomefloxacin</td>
<td>290</td>
<td>35900</td>
<td>2352</td>
<td>84</td>
<td>&gt; 28</td>
<td>200/400/800</td>
<td>ET n.r.</td>
<td>EW ≥ 800</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LC ≥ 200</td>
<td></td>
</tr>
<tr>
<td>Vemurafenib</td>
<td>305</td>
<td>22800</td>
<td>1.5</td>
<td>0.052</td>
<td>&gt; 29</td>
<td>100/350/450 800</td>
<td>ET ≥ 350</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sparfloxacin</td>
<td>305</td>
<td>33600</td>
<td>500</td>
<td>6.2</td>
<td>&gt; 82</td>
<td>25/100/400</td>
<td>ET ≥ 100</td>
<td>EW ≥ 25</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LC ≥ 25</td>
<td></td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>290</td>
<td>9800</td>
<td>1000</td>
<td>4.1</td>
<td>&gt; 240</td>
<td>100/200/300 (400/500)</td>
<td>ET = 500</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxycycline</td>
<td>352</td>
<td>17300</td>
<td>1000</td>
<td>2.3</td>
<td>&gt; 440</td>
<td>100/250/400</td>
<td>ET ≥ 250</td>
<td>EW ≥ 400</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>LC ≥ 250</td>
<td></td>
</tr>
<tr>
<td>8-MOP</td>
<td>299</td>
<td>10700</td>
<td>100</td>
<td>0.22</td>
<td>&gt; 457</td>
<td>10/20</td>
<td>ET n.r.</td>
<td>ET = 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LW = 20</td>
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</tr>
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</table>

*Note.* MEC, molar extinction coefficient; PIF, photoinitiation factor; NOAEL, no-observed-adverse-effect level with regard to phototoxicty; LOAEL, lowest-observed-adverse-effect level with regard to phototoxicty; ET, erythema; EW, ear biopsy weight; LW, auricular lymph node weight; LC, auricular lymph node cell count; n.r., no finding with regard to irradiation-dependent effects; r.n., not recorded.

*Numbers in italics* represent measurements at the lower spectrum cut-off at 290 nm (not at a peak). *aThe IC₅₀ values for cytotoxicity in the absence (−irr) and presence (+irr) of irradiation with simulated sunlight are given in this table. Numbers in italics represent the highest tested concentration (not IC₅₀ values), which was limited by solubility or the maximal assay range (1000 µg/ml), thus preventing the determination of exact PIF values (indicated by PIF “larger than”).

*bThree dose levels (exception: 8-MOP, two dose levels) were tested and are given in this table; the NOAEL is underlined; the LOAEL is bolded.

*cEar skin erythema (ET) and weight (EW) changes are described (with dose levels of occurrence in mg/kg); in the cases of enoxacin, lomefloxacin, and 8-MOP erythema formation has not been recorded (n.r.) and ear weight changes are based on one ear (instead of pairs of ears).

*dLymph node weight (LW) and cell count (LC) changes are described (with dose levels of occurrence in mg/kg).

*eData from Boudon et al. (2014).

*fEar weight increase was observed 1–6 h after irradiation, but decreased to baseline already at 24 h (Boudon et al., 2014; this additional investigation was not performed at dose levels above 350 mg/kg (LOAEL)).

gDuring dose finding, a limited number of mice (n = 2) was also treated with 400 and 500 mg/kg/day ketoprofen for 2 days only; these dose levels were toxic; erythema formation was observed at 500 mg/kg confirming the phototoxic properties of ketoprofen in vivo.
inner and outer segments of rods and cones up to almost complete loss of these structures in more pronounced cases. In addition, minimal to slight hypertrophy was present in the retinal pigment epithelium. The observed changes were generally consistent with those reported after toxic retinal injury. Finally, 100 and 400 (but not 25) mg/kg/day sparfloxacin induced moderate erythema formation within five to six hours after the first treatment depending on additional exposure to simulated sunlight (Fig. 3A). Over the following two days, a dose-dependent increase in the severity and persistence of erythema was noted.

A similar phenomenon was also observed with doxycycline and ketoprofen.

**Drug Candidates in the Modified Murine Systemic photo-LLNA**

The UV-vis absorption spectra of 34 systemically applied drug candidates were recorded and analyzed to identify absorption peaks with associated MECs and to assess the need and relevance of retina evaluation due to residual absorption of visible light, which is relevant for the human retina (Table 3). The phototoxic potential of these drug candidates was determined *in vitro* with the 3T3 NRU test and *in vivo* with the optimized modified murine photo-LLNA. Out of the 34 drug candidates, three had a PIF < 2 ("not phototoxic"), three had a PIF between 2 and 5 ("probably phototoxic"), and 28 had a PIF > 5 ("phototoxic"). As shown in Table 3, all 17 drug candidates with a PIF up to at least 33 did not show a phototoxic potential in the *in vivo* assay. Furthermore, the probability for a drug candidate to cause phototoxicity *in vivo* correlated with the magnitude of the phototoxicity identified *in vitro*. Seventy-six percent of all tested drug candidates with a PIF ≥ 36 and 92% of all drug candidates with a PIF ≥ 56 showed a phototoxic potential in the *in vivo* assay. Figure 4A shows the frequencies of compounds identified as phototoxic *in vivo* relative to *in vitro* PIF (categorized). Figure 4B shows the categorized distribution of PIF values (histogram, n = 100), derived from a historical database of an unbiased selection of drug candidates covering approximately three years. The majority of drug candidates (67%) had a PIF below 5 and were not considered phototoxic *in vitro*. Moreover, 83% of all candidates had PIF values below 25, whereas 8% of drug candidates showed PIF values between 25 and 100, and further 9% of drug candidates were highly phototoxic *in vitro* (PIF > 100).

In most cases, signs of ear skin irritation (erythema and/or increased ear biopsy weights) and auricular LN response (increased LN weight and cell count) occurred together. As exceptions, drug candidates #20, #23, and #27 were characterized by a high sensitivity to irradiation-dependent skin reactions, particularly erythema formation. These started to occur at dose levels, at which local LNs were not responding yet. The most severe case of phototoxicity *in vivo* was associated with drug candidate #32, characterized by a PIF of 630. In the modified murine photo-LLNA for this orally applied drug candidate, exposure to simulated sunlight was not done on day 3 because of persisting compound/irradiation-induced skin reactions.

The use of three dose levels in the modified murine photo-LLNA enabled the establishment of NOAELs and/or LOAELs. As an example, the results of the *in vivo* testing of drug candidate #26 are shown in Figure 5 (ear weight and auricular LN responses), Figure 2 (eye histopathology), and Figure 3B (erythema formation). No significant ear, LN, or eye response was observed at 50 mg/kg/day, representing the NOAEL with regard to phototoxicity. Irradiation-dependent ear irritation (erythema formation and increased ear biopsy weights), auricular LN response (increased LN weights and cell counts), and minimal to slight atrophy and degeneration of the retina that was...
FIG. 2. Irradiation-dependent retina changes (retinal atrophy/degeneration) following oral administration of sparfloxacin or drug candidate #26 to female BALB/c mice in the modified murine photo-LLNA. NAD, no abnormality detected.

TABLE 2
Ear Weight and LN Weight and Cell Count Results (Mean ± SD) from 21 photo-LLNA Studies in Which 100 mg/kg Sparfloxacin Was Used as Positive Control

<table>
<thead>
<tr>
<th>Sparfloxacin (100 mg/kg)</th>
<th>Ear Weight (mg)</th>
<th>LN Weight (mg)</th>
<th>LN Cell Count (× 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irradiation (UVA/vis)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>20.6 ± 0.9</td>
<td>4.7 ± 0.9</td>
<td>9.6 ± 2.6</td>
</tr>
<tr>
<td>+</td>
<td>32.5 ± 6.8</td>
<td>8.3 ± 1.7</td>
<td>20.9 ± 6.9</td>
</tr>
</tbody>
</table>

qualitatively similar to changes observed with sparfloxacin and irradiation became apparent at 100 mg/kg/day (LOAEL) and 150 mg/kg/day. As for sparfloxacin, also in the case of drug candidate #26 and other drug candidates showing a phototoxic potential in vivo, irradiation-dependent erythema formation increased over the treatment period dose-dependently.

Figure 6A shows the distribution of in vivo photo-LLNA results as a function of the PIF value of the in vitro 3T3 NRU phototoxicity test results. With the exception of compound #23, the LOAEL for all drug candidates identified as phototoxic in the photo-LLNA was ≤ 125 mg/kg/day. Among the 12 drug candidates with a PIF ≥ 56, the only drug candidate that did not show a phototoxic potential was compound #30, which was tested only up to 50 mg/kg/day. Figure 6B shows the distribution of in vivo photo-LLNA results as a function of the IC50 value under irradiation of the 3T3 NRU test results. The probability to cause phototoxicity in vivo was higher for drug candidates with low IC50 values. Out of all 24 tested drug candidates
In a first murine photo-LLNA study, dose levels of 10, 30, and 100 mg/kg were tested with no ear, lymph node, or retina finding.

Ear skin erythema (ET) and weight (EW) changes are described with dose levels of occurrence in mg/kg.

**Abbreviations:** see Table 1.

**Note:** Numbers in italics represent measurements at the lower spectrum cut-off at 290 nm (not at a peak).

**TABLE 3**

<table>
<thead>
<tr>
<th>Drug candidate</th>
<th>UV/vis absorption&lt;sup&gt;a&lt;/sup&gt;</th>
<th>In vitro T3N RNU phototoxicity test&lt;sup&gt;b&lt;/sup&gt;</th>
<th>LOAEL&lt;sup&gt;c&lt;/sup&gt;/LOAEL&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Skin&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Lymph nodes&lt;sup&gt;f&lt;/sup&gt;</th>
<th>Retina atrophy&lt;sup&gt;g&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>#21</td>
<td><strong>1000</strong></td>
<td></td>
<td>50/100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#25</td>
<td>2000</td>
<td></td>
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**Note:** Numbers in italics represent measurements at the lower spectrum cut-off at 290 nm (not at a peak).

<sup>a</sup>Numbers in italics represent measurements at the lower spectrum cut-off at 290 nm (not at a peak).

<sup>b</sup>The IC50 values for cytotoxicity in the absence (+/-) and presence (+/+) of irradiation with simulated sunlight are given in this table. Numbers in italics represent the highest tested concentration (not IC50 values), which was limited by solubility or the maximal assay range (1000 µg/ml), thus preventing the determination of exact PIF values (indicated by PIF “larger than”).

<sup>c</sup>Drug candidate #34 induced increased ear weights in the absence of UV/vis irradiation with no clear effect of UV/vis absorption.

<sup>d</sup>Numbers in italics represent measurements at the lower spectrum cut-off at 290 nm (not at a peak).

<sup>e</sup>Reversal of auricular lymph node hyperplasia induced by drug candidate #33 at ≥ 100 mg/kg/day.

<sup>f</sup>Drug candidate #34 induced increased ear weights in the absence of UV/vis irradiation with no clear effect of UV/vis irradiation on this parameter.

<sup>g</sup>Drug candidate #34 induced increased ear weights in the absence of UV/vis irradiation with no clear effect of UV/vis irradiation on this parameter.
with an IC50 value < 3 µg/ml, 13 candidates (= 54%), and out of all 8 tested drug candidates with an IC50 value < 0.2 µg/ml, 7 candidates (= 88%) showed a phototoxic potential in the in vivo assay.

**DISCUSSION**

The clinically relevant phototoxic compounds sparfloxacin, enoxacin, lomefloxacin, doxycycline, 8-MOP, and vemurafenib were reliably identified as phototoxic in the herein described optimized modified murine photo-LLNA. These results are in alignment with previous studies including sparfloxacin, enoxacin, lomefloxacin, and 8-MOP in similar in vivo murine photo-LLNA based on the reported 34 drug candidates. (B) Distribution of PIF values (histogram, n = 100), derived from a historical database of an unbiased selection of drug candidates covering approximately three years of testing.
FIG. 5. Irradiation-dependent increase of ear weight and auricular LN weight and cell count following oral (gavage) administration of drug candidate #26 to female BALB/c mice in the modified murine photo-LLNA. *P < 0.05 versus vehicle control; #, P < 0.05 versus corresponding nonirradiated group.

phototoxicity assays (Matsumoto et al., 2010; Neumann et al., 2005; Vohr et al., 2001). However, whereas enoxacin has been described to exclusively induce irradiation-dependent LN responses but no increase in ear thickness (Vohr et al., 2001), it induced a statistically significant increase of ear biopsy weights in addition to LN responses in our study using the optimized modified murine oral (gavage) photo-LLNA at the same dose level. Furthermore, whereas 8-MOP has been described to induce phototoxicity at 10 mg/kg/day (Neumann et al., 2005; Vohr et al., 2001), 10 mg/kg/day represented the NOAEL and 20 mg/kg/day the LOAEL in our study. Differences in the mouse strain and/or irradiation conditions may explain these differences. Additionally, differences in the chosen endpoints (ear biopsy weight vs. ear thickness) may have contributed to the described differences regarding irradiation-dependent ear skin reactions to enoxacin. The in vivo phototoxic potential of systemically applied ketoprofen could only be identified based on erythema formation at the toxic dose level of 500 mg/kg/day. Clinically, ketoprofen is well known as a phototoxic compound upon topical application (Bagheri et al., 2000). However, only anecdotal cases of ketoprofen-induced phototoxicity upon systemic application have been reported (Foti et al., 2011). Therefore, the modified murine photo-LLNA confirmed the relatively weak potential of ketoprofen to induce phototoxicity upon systemic treatment and emphasizes the clinically observed difference of ketoprofen-associated phototoxicity risks following topical versus systemic administration. Promethazine, another clinically phototoxic compound upon topical application (Sidi et al., 1955), did not show a phototoxic potential in the modified murine photo-LLNA up to the maximally tolerated oral dose of 100 mg/kg/day (data not shown). It should be noted that incidence and relevance of phototoxicity seen clinically after oral administration of promethazine remain unclear as well, even though such cases have been reported occasionally (e.g., Epstein and Rowe, 1957; Newill, 1960). Compared with other phenothiazine derivatives, the in vitro phototoxicity potential of promethazine is slightly below chlorpromazine (photo-hemolysis test, Eberlein-König et al., 1997; in vitro 3T3 NRU, in-house data, not shown). However, in mice promethazine showed hardly any phototoxicity reaction after intraperitoneal administration, whereas chlorpromazine was clearly positive (Ljunggren and Möller, 1977), thus confirming an overall low phototoxicity potential after systemic administration.

Sparfloxacin at a dose of 100 mg/kg/day was chosen as standard positive control item in further murine photo-LLNA studies for drug candidates. This reference compound absorbs visible light in addition to UV light so that it also represents a relevant reference compound with regard to retinal phototoxicity. Indeed, sparfloxacin did not only reliably induce irradiation-dependent local ear irritation and an auricular LN response, but also retina atrophy, a strongly adverse phototoxic effect relevant for systemically applied compounds that absorb light above 400 nm. Histopathological evaluation of the retina for systemically applied compounds absorbing light at > 400 nm represents an important endpoint of the optimized modified murine photo-LLNA.

For lomefloxacin (Matsumoto et al., 2010) as well as for sparfloxacin, doxycycline, ketoprofen, and several drug candidates showing a phototoxic potential in the modified
FIG. 6. Distribution of \textit{in vivo} photo-LLNA results as a function of PIF or IC\textsubscript{50} values (\textit{in vitro} 3T3 NRU phototoxicity test). The horizontal level indicates the dose level; the symbol indicates the outcome (phototoxic/nonphototoxic) including any identified NOAEL or LOAEL. Individual dose groups are plotted versus (A) the associated PIF value or versus (B) the associated IC\textsubscript{50} value under irradiation of the respective test compound. Thus, all dose groups of a single animal study are vertically stacked above the associated \textit{in vitro} result. It is apparent that positive \textit{in vivo} results were only obtained at PIF values above 25 and IC\textsubscript{50} values below 5 \(\mu\text{g/ml}\). Moreover, the lowest phototoxic dose level (LOAEL) tends to decrease the higher the associated PIF value is. In most cases, signs of phototoxicity were found around 100 \(\text{mg/kg}\) or even at significantly lower levels.
murine photo-LLNA, irradiation-dependent erythema formation increased over the treatment period dose-dependently. This strongly argues for the general need of multiple treatment days rather than only one for in vivo systemic photocytotoxic testing. The three-day treatment as used here for the modified murine photo-LLNA appears to be appropriate.

In vivo photocytotoxicity is a dose-dependent effect. In addition, photosafety assessment may not only consider the phototoxic potential of a drug candidate but also the relevance for the therapeutic treatment. Therefore, inclusion of multiple dose levels in the in vivo photocytotoxicity test, considering the maximal tolerated and pharmacologically efficacious dose levels upon systemic treatment with the aim to identify NOAELs and/or LOAELs and potential safety margins versus therapeutically relevant drug levels, is important. Because exposure to simulated sunlight is done for a limited period of time, determination of drug exposure at the time of irradiation is considered to be relevant for photosafety assessment. Overall, the results obtained with the clinically relevant reference compounds convincingly demonstrate the general suitability of the selected study design including irradiation conditions and endpoints that are in line with current regulatory recommendations (ICH S10, 2013).

An in vitro-in vivo correlation demonstrated that a drug candidate classified as "phototoxic" in vitro based on the 3T3 NRU test is not necessarily phototoxic in vivo. However, the probability of a drug candidate to cause photocytotoxicity in vivo clearly correlated with the magnitude of the phototoxicity identified in vitro. This has implications on the preclinical in vivo photosafety testing strategy. For example, none of the 15 tested drug candidates, which were characterized by a PIF < 25, showed a phototoxic potential in the modified murine systemic photo-LLNA. Because based on historical data 83% of all drug candidates fell into this category, in vivo efforts early in drug development may primarily focus on the 17% of drug candidates with a PIF > 25, which were associated with a probability of 68% to show a phototoxic potential in vivo. Most strikingly, this probability increased to 90% for drug candidates with a PIF > 100. It should be noted that due to interlaboratory differences regarding the in vitro 3T3 NRU photocytotoxicity test, the mentioned PIF threshold of 25 may not be applicable to other laboratories. Thus, corresponding PIF thresholds need to be identified by laboratories individually.

CONCLUSION

Taken together, the modified murine photo-LLNA, based on quantification of skin irritation (erythema and ear biopsy weight) and auricular LN weight and cell count, is suitable to support preclinical photosafety assessment of systemically applied drug candidates. For drug candidates absorbing visible light, additional histopathological analysis of the retina is informative and can thus be recommended. The observed increase of erythema formation over the treatment period as well as pharmacokinetic considerations support the need for multiple treatment days, and a three-day treatment design as used in our study seemed appropriate. The establishment of NOAELs and LOAELs is supported by the inclusion of three dose levels. This allows for the calculation of multiples (safety margin) between nonphototoxic and pharmacologically relevant drug levels in order to determine therapeutic indices and support human photosafety assessment.

Because the probability for a drug candidate to cause phototoxicity in vivo correlated with the magnitude of the phototoxicity identified in vitro, further in vivo efforts early in drug development may primarily focus on drug candidates with PIF values above a certain threshold. This PIF threshold needs to be defined individually for each laboratory due to potential interlaboratory variability; in our case it is 25. For all other drug candidates identified as phototoxic in vitro, the in vivo photosafety testing may be delayed to a later time point in drug development.

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