Characterization of Vemurafenib Phototoxicity in a Mouse Model

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Vemurafenib is a first-in-class, small molecule B-Raf kinase inhibitor for the treatment of patients with unresectable or metastatic melanoma carrying the BRAFV600E mutation, commercially available since 2011. A general phototoxic potential was identified early during development; however, based on results of an animal study in hairless rats, it was concluded that there would exist no relevant risk for humans. Surprisingly, signs of clinical photosensitivity were reported in many patients during clinical development. Therefore, it became a fundamental question to understand this discrepancy. An established mouse model (oral UV-Local Lymph Node Assay, UV-LLNA) for the assessment of *in vivo* photosafety was used to investigate the impact of formulations, dose levels, duration of treatment, and timing of irradiation. Moreover, a basic pharmacokinetic profile was established within the same mouse strain. We were able to demonstrate dose- and time-dependent phototoxicity of vemurafenib using commercially available tablets (stabilized amorphous material). The lowest phototoxic dose was 350 mg/kg administered for 3 consecutive days followed by exposure to UV-visible irradiation at a UVA-normalized dose of 10 J/cm². In comparison, pure vemurafenib, which easily forms crystalline variants and is known to have poor bioavailability, was tested at 350 mg/kg, and no signs of phototoxicity could be seen. The most apparent difference between the early study in hairless rats and this study in mice was the spectral range of the irradiation light source (350–400 nm vs 320–700 nm). Because vemurafenib does not absorb sufficiently light above 350 nm, this difference can easily explain the negative earlier study result in hairless rats.

Key Words: **phototoxicity; photosensitivity; photosafety; LLNA; BRAF; vemurafenib.**

Vemurafenib is a first-in-class, small molecule B-Raf kinase inhibitor for the treatment of patients with unresectable or metastatic melanoma carrying the BRAFV600E mutation. It was approved in 2011 in the United States (FDA review, 2011), in the European Union (CHMP review, 2011), and in Switzerland.

Although a general phototoxic potential was identified early for this compound (based on UV-vis spectra and *in vitro* 3T3 Neutral Red Uptake (NRU) phototoxicity data), the initial conclusion was made that no relevant human risk would exist. Apparently, this was driven by the results of an animal study, suggesting that no increased sensitivity to light could be induced (CHMP review, 2011; FDA review, 2011). Surprisingly, signs of clinical photosensitivity were reported in 42% of patients included in the phase I trial extension cohort. Similarly, during phase II and phase III, 52% and 30% of vemurafenib-treated patients were affected, respectively (Chapman *et al.*, 2011; Flaherty *et al.*, 2010; Lacouture *et al.*, 2013). The findings included severe (grade 2 and 3) sunburn-like reactions, which occurred even after exposure to sun light through window glass (eg, while driving a car), and thus had a significant impact on the quality of life. Later, Dummer *et al.* (2012) confirmed that the minimal erythema dose (MED) in patients receiving vemurafenib was markedly reduced in the UVA range, whereas the UVB-dependent MED remained unchanged compared with untreated subjects. Therefore, we felt encouraged to understand the reasons behind this situation that a clinically relevant strong phototoxicity had gone undetected preclinically. In particular, it was of great interest to learn how such potentially "false negative" animal studies can be avoided in the future.

In this study, we utilized an established mouse model for the assessment of *in vivo* photosafety. This model is based on a modified (cell count-based) UV-LLNA in albino Balb/c mice as described by Ulrich *et al.* (2001). However, with oral administration, the main endpoint of this model is acute phototoxicity rather than (photo)-contact allergy. A similar approach was also described by Vohr *et al.* (2000). Typically, mice were treated for 3 days including daily exposure to simulated sunlight. The light source used provided a reasonable coverage of the UVA and visible light range, whereas highly cytotoxic UVB irradiation was attenuated in order to not limit the overall irradiation (which was normalized to a UVA dose of 10 J/cm² UVA). Any skin reactions (mainly erythema seen at the ears) were recorded during these days. Subsequently, edema (by measuring ear weight) and markers of local inflammation (weight and cell count of the ear-draining auricular lymph nodes) were

assessed. Historically, a treatment period of 3 days has been used to allow for sufficient activation of the local lymph nodes. However, in the context of photosafety evaluation of systemically administered drugs, the repeated-dose protocol does also ensure sufficient distribution of compounds to skin.

During clinical development of vemurafenib, it became apparent that reaching sufficient systemic exposure in patients was a key challenge because this drug substance is practically insoluble in an aqueous environment. Finally, solubility was improved by using a stabilized amorphous variant of vemurafenib. This solid dispersion contains amorphous vemurafenib and hypromellose acetate succinate in which the drug substance is uniformly dispersed within the polymeric substrate. Currently, the approved daily dose of vemurafenib is 1920 mg (which equals twice daily 4 tablets containing 240 mg each) (Bollag *et al.*, 2010; Shah *et al.*, 2013). Because reaching sufficient bioavailability is also a key challenge in animal studies with vemurafenib, we performed our experiments with both crystalline and stabilized amorphous material in order to test different conditions. In addition, a pharmacokinetic profile was established within the same mouse strain in order to support comparisons with published exposure data from both nonclinical safety studies in animals and human clinical trials.

MATERIALS AND METHODS

Test Compounds and Positive and Negative Control Items

For most animal studies, amorphous vemurafenib was used, which was commercially available as ZELBORAF tablets (240 mg/tablet; Roche, Basel, Switzerland). Prior to immediate dosing, the tablets were finely grinded with a pestle and a mortar. An appropriate amount of aqueous carboxymethylcellulose (CMC) 0.5% was added to form a fine suspension (final dosage volume of 20 ml/kg), which was sonicated without heating for 5 min and shaken/vortexed. The vehicle, CMC 0.5%, was used as control.

In addition, in-house synthesized vemurafenib was used, mainly for experiments requiring solutions (eg, recording of UV-vis spectra, *in vitro* 3T3 phototoxicity test). This material was assumed to be composed of crystalline forms, which are obtained from chemical synthesis if no special precautions are taken. Identity was confirmed based on 1H-NMR, LC-MS, and UPLC. The obtained data are in agreement with the known structure of vemurafenib. Purity was assessed using UPLC demonstrating 98% content with ethyl acetate as major remaining impurity.

Sparfloxacin and 8-methoxypsoralene (8-MOP), used as positive control reference compounds, were obtained from Sigma-Aldrich (Buchs, Switzerland) with at least 98% purity and available certificates of analysis.

UV-Visible Light Absorption Spectra

Light absorption spectra in the UV-visible range were recorded on a Cary 300 spectrophotometer (Varian Australia Pty Ltd, Mulgrave, 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, the molar extinction coefficient (ε or MEC) was calculated: $\varepsilon = A/(c \cdot l)$, A: absorbance, c: concentration of the solution in methanol, l: path length (cuvette).

In Vitro 3T3 Neutral Red Uptake 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 (OECD, 2004). Briefly, 24 h after seeding the mouse fibroblast cells 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 Buffered Salt Solution (HBSS) without phenol red as medium replacement. Subsequently, these cells were irradiated (+Irr) with simulated sun light (SOL 500 H1, Dr. Hönle GmbH, Gräfelfing, 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 that was tolerated by the cell culture as suggested by the mentioned guideline. (Fig. 1) The polystyrene lids were on the 96-well plates during light exposure. Calibration was performed through the lids as well. In parallel, an identically prepared 96-well plate was kept in the dark (−Irr), serving as control. UVA irradiance was measured by a UVA meter (Dr. Hönle AG) with spectral sensitivity in the range from 320 to 400 nm and a measuring range between 0 and 199.9 mW/cm². The applied intensity was 1.67 mW/cm², resulting in a total UVA dose of 5 J/cm² after 50 min of irradiation. The related UVB exposure (calculated from the spectral irradiance of the light source) was around 15 mJ/cm². 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 after incubation and extraction at 540 nm. The Photo-Irritation-Factor (PIF) was calculated according to OECD TG 432 using the following equation: $\text{PIF} = IC_{50}(-\text{Irr})/IC_{50}(+\text{Irr})$.

Animal Experiments

Animal Husbandry

Female BALB/C mice aged of about 8 weeks at the start of the experiment, purchased from Charles River (L'Arbresle, France), were acclimatized for around 1 week. The experiments were performed in conformity with the Swiss Animal Welfare Law and in accordance with internal SOPs and guidelines for care and use of laboratory animals. Animals had *ad libitum* access to pelleted standard rodent diet and tap water from the domestic supply and were kept in an air-conditioned animal room under periodic bacteriological control, at 22°C ± 2°C with monitored 30%–80% humidity, a 12-h light/dark cycle, and background radio coordinated with light hours.

Irradiation Conditions for Animal Experiments

During irradiation, mice were kept in specific cages allowing only for lateral movements and ensuring a uniform irradiation of their back and ears. Nonirradiated animals were kept in their housing cages under standard room light. For irradiation, a sun light simulator (Psorisan 900 H1, Dr. Hönle GmbH) was used showing a main spectral output from 320 until beyond 590 nm. The integrated H1 filter system attenuated the highly cytotoxic UVB range to a level that was tolerated by the animals. UVA irradiance was measured by a UV-radiometer (Dr. Hönle GmbH) with a spectral sensitivity in a range from 320 to 400 nm and a measuring range between 0 and 199.9 mW/cm². Typically, the applied intensity was 4.8 mW/cm² at a distance of 50 cm. Irradiation was normalized to a dose of 10 J/cm² UVA, which was typically achieved after 35 min of light exposure. The related UVB exposure (calculated from the spectral irradiance of the light source) was around 30 mJ/cm². External calibration of the equipment was performed by Opto.cal GmbH (Movelier, Switzerland), which is a calibration laboratory accredited by the Swiss Accreditation Service.

Treatment Protocols and Endpoints

Oral UV-LLNA in BALB/c mice. Studies A, B, and C: On 3 consecutive days, 6 mice/group were treated orally with the test compound (see Table 1 and Fig. 2 for details) dissolved in 0.5% aqueous CMC or with vehicle. Two hours after each treatment, mice from the groups "with UV" were irradiated.

Study D: On 3 consecutive days, 6 mice/group were treated orally with 350 or 450 mg/kg of the amorphous form of vemurafenib. Mice from the 350 mg/kg "with UV" group were irradiated 2 h after each treatment, whereas mice from group 450 mg/kg "with UV" were irradiated 2 h after last treatment only on day 3.

Measurement of LLNA endpoints was done as described before (Ulrich *et al.*, 2001): 24 h after the last irradiation, mice were sacrificed using carbon dioxide asphyxiation. Circular biopsies (0.5 cm²) from the apical area of each
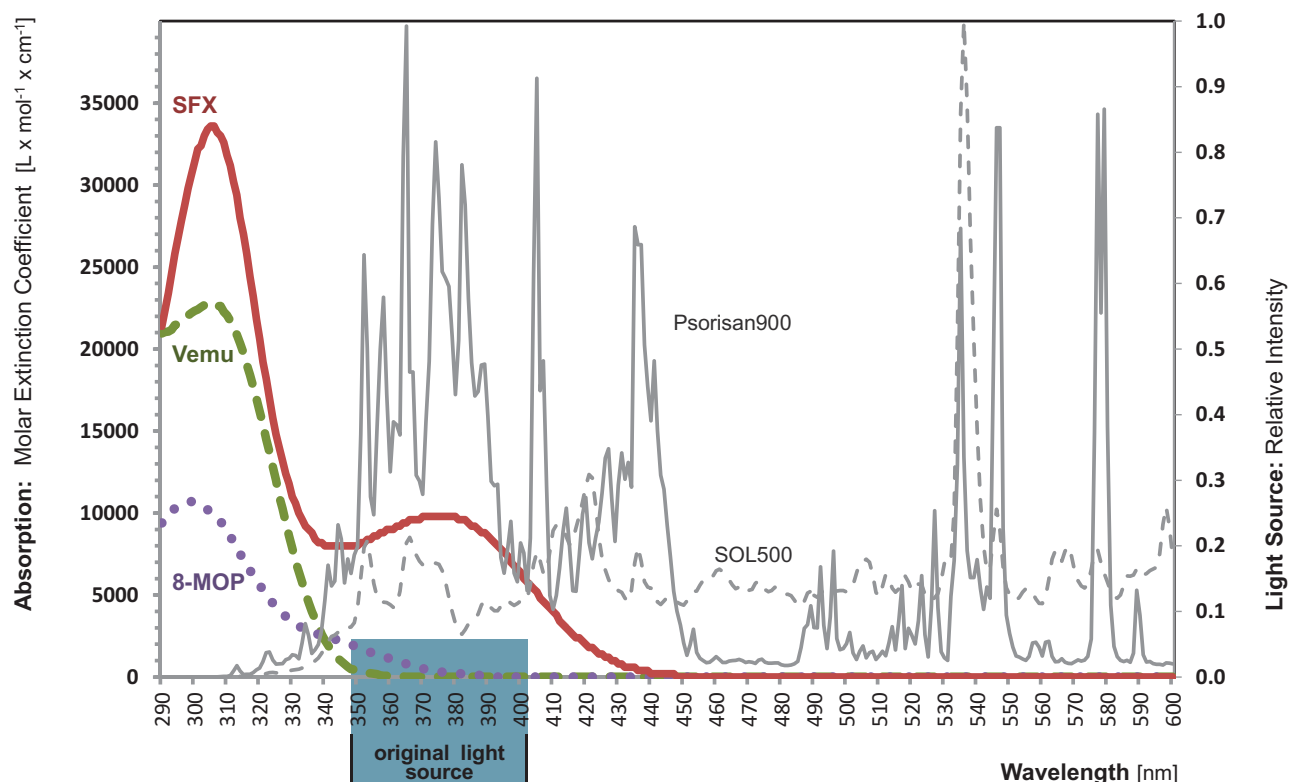
FIG. 1. Normalized UV-visible light absorption spectra of Vemu (dashed), SFX (solid), and 8-MOP (dotted) recorded at concentrations of 50 or 100µM in methanol. For comparison, the spectral intensity of the light sources used in this study are shown as overlap (SOL500, dashed gray; Psorisan900, solid grey). Abbreviations: 8-MOP, 8-methoxypsoralene; SFX, sparfloxacin; Vemu, vemurafenib.

ear were excised using a disposable punch and weighed as pairs on an analytical scale. Lymph node weights were obtained from lymph node pairs taken from individual animals and weighed using analytical scales. For the determination of individual lymph node cell counts, single-cell suspensions from the lymph node pairs from individual animals were prepared by mechanical tissue disaggregation through a sterile stainless steel gauze in 1 ml PBS (Ca^{2+}/Mg^{2+} free) containing 0.5% bovine serum albumin. Individual cell counts were determined in a cell counter (CASYTTC cell counter, Schärfe System, Reutlingen, Germany) gating on a particle diameter above 4.88 µm.

Time profile of erythema and edema formation after irradiation. Study E: On 3 consecutive days, 6 mice/group were treated orally with 350 mg/kg of the amorphous form of vemurafenib or with vehicle. Mice were irradiated 2 h after each treatment and sacrificed at 1, 2, 3, 4, and 6 h after light exposure on day 3. Circular biopsies (0.5 cm²) from the apical area of each ear were excised using a disposable punch and weighed as pairs on analytical scales.

Pharmacokinetic profile of vemurafenib in BALB/c mice. On 3 consecutive days, 6 mice/group were treated orally with a suspension of the amorphous form of vemurafenib. Blood samples from 3 animals per time point per group were collected from the vena saphena 1, 2, 4, and 7 h postdosing; blood specimens from 6 mice per time point per group were collected from terminal heart puncture at 24 h after treatment on day 1 for animals from group 1, on day 2 for animals from group 2, and on day 3 for animals from group 3. Plasma was prepared from blood specimens and stored on ice water until all plasma specimens were prepared. Specimens from all animals were analyzed. Twenty-four hours after the administration on day 1, 2, and 3, mice were sacrificed using carbon dioxide asphyxiation. Circular biopsies (= 0.5 cm²) from the apical area of each ear were excised using a disposable punch and weighed as pairs on an analytical scale. They were snap-frozen in liquid nitrogen and stored in a deep freezer at −70°C or below. Determination of vemurafenib in mouse plasma was performed

by protein precipitation followed by LC-MS/MS using electrospray ionization in positive mode. The ear samples were homogenized with 9 volume equivalents of acetonitrile/water and then processed and analyzed like the plasma samples.

Statistical Analysis

For all statistical calculations, SigmaPlot for Windows (version 11.0) was used. A one-way ANOVA was used as the statistical method (Glantz, 1992). A normality test was performed to assure that the specimens were drawn from a normal population (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, multiple comparisons were performed with the Student-Newman-Keuls test. If the normality test and/or the equal variance test gave p values < .01, a suitable transformation (log, square root) was applied; if the normality test and/or equal variance test still gave p values < .01, the nonparametric Kruskal-Wallis test was used, and multiple comparisons for the ranks of the original observations were performed. The confidence interval for the difference of the means was set to 95% (α= .05).

RESULTS

UV-Visible Light Absorption Spectra

UV-visible spectra of vemurafenib and the reference compounds sparfloxacin and 8-methoxy psoralene were recorded from 290 to 700 nm, which is the spectral region relevant for photosafety assessment (sun light) (Table 2). Vemurafenib shows absorption mainly in the UVB (peak at 305 nm) and the short UVA region. Importantly, above

TABLE 1
Skin Irritation and Lymph Node (LN) Activation Induced by Vemurafenib (Vemu) Using Crystalline (crystal) and Amorphous Material (amorph)

Study		Ear Weights		LN Weights		LN Cell Count		Erythema After UV Exposure (day 1/2/3)
		Mean (mg)	SD	Mean (mg)	SD	Mean ($\times 10^6$)	SD	
A	Sparfloxacin 100 mg/kg	19.26	0.58	4.44	0.52	8.11	1.66	—
	Sparfloxacin 100 mg/kg UV	30.72***	1.63	9.45***	1.11	20.60***	2.37	+/+/+
B	Vehicle CMC 0.5%	24.55	1.54	5.99	0.97	9.96	2.24	—
	Vemu crystal 20 mg/kg	20.03	0.62	5.99	0.99	11.78	1.66	—
	Vemu crystal 20 mg/kg UV	20.87	0.86	5.18	0.91	9.77	2.86	–/–/–
	Vemu crystal 100 mg/kg	20.62	0.57	5.48	0.79	10.19	1.50	—
	Vemu crystal 100 mg/kg UV	20.69	0.68	6.51	0.79	11.99	1.01	–/–/–
	Vemu crystal 350 mg/kg	20.98	0.38	5.90	1.12	11.86	2.75	—
	Vemu crystal 350 mg/kg UV	21.25	0.6	6.11	0.69	11.95	1.84	–/–/–
C	Vehicle CMC 0.5%	20.33	0.6	4.23	0.41	5.85	0.42	—
	Vemu amorph 100 mg/kg	21.41	0.89	4.61	0.41	6.18	0.65	—
	Vemu amorph 100 mg/kg UV	21.73	0.49	4.85	0.27	7.48	1.13	–/–/–
	Vemu amorph 450 mg/kg	20.66	0.46	4.58	0.33	7.68	0.87	—
	Vemu amorph 450 mg/kg UV	22.02	0.36	4.63	0.77	7.56	1.40	–/–/+
	Vemu amorph 800 mg/kg	20.65	1.04	4.50	0.77	7.53	1.98	—
	Vemu amorph 800 mg/kg UV	22.13	0.95	4.97	1.14	9.28	1.23	–/–/+
D	Vemu amorph 350 mg/kg	21.12	0.40	5.07	0.33	5.72	0.59	—
	Vemu amorph 350 mg/kg UV	21.11	0.46	5.69	0.94	6.58	1.08	–/–/+
	Vemu amorph 450 mg/kg UV (UV on day 3 only)	20.70	0.39	5.35	1.12	6.81	1.50	–/–/+
E	Vehicle CMC 0.5%	21.57	0.84	—	—	—	—	—
	Vemu amorph 350 mg/kg 6 h	21.43	0.94	—	—	—	—	—
	Vemu amorph 350 mg/kg UV 1 h	23.55**	0.71	—	—	—	—	–/–/+
	Vemu amorph 350 mg/kg UV 2 h	23.48**	0.77	—	—	—	—	–/–/+
	Vemu amorph 350 mg/kg UV 3 h	24.00***	0.84	—	—	—	—	–/–/+
	Vemu amorph 350 mg/kg UV 4 h	23.82**	0.83	—	—	—	—	–/–/+
	Vemu amorph 350 mg/kg UV 6 h	24.31***	1.25	—	—	—	—	–/–/+

Note. Six female Balb/c mice per group were treated orally on 3 consecutive days by gavage. Mean ear weights were obtained 1 day after the last exposure using the weights of circular pieces ($0.5\,cm^2$) punched from the apical area of 1 ear. Mean LN weights were derived from pairs of auricular LN from an individual animal, and mean LN cell count values represent the corresponding total cellularity of the LN. **$0.1\% < p < 1\%$, ***$p < 0.1\%$, versus corresponding dose control. Data of the positive control, sparfloxacin, are displayed to illustrate the expected responses for each endpoint.

350 nm, no relevant absorption is observed. In comparison, 8-MOP shows a similar absorption profile (peak at 299 nm), but absorption extends into the long UVA region up to 380 nm. Sparfloxacin shows an additional peak (375 nm) in the long UVA, and absorption extends into the visible region up to 440 nm. For comparison, an overlay of these absorption spectra with the spectral irradiance of the light sources (*in vitro*: SOL500/H1 filter, *in vivo*: Psorisan900/H1 filter) is shown up to 600 nm in Figure 1. It should be noted that the obvious absorption of all 3 compounds in the UVB range is a common phenomenon among the majority of low molecular weight drug substances. However, for oral drugs, photosafety assessment is mainly focusing on UVA and visible light as these wavelengths are penetrating sufficiently into skin (ICH S10, 2013).

In Vitro Phototoxicity Test Results

The *in vitro* 3T3 NRU phototoxicity test measures cytotoxicity profiles in the presence or absence of simulated sun light using neutral red as vital dye. This assay is based on the calculation of the PIF, which represents the ratio of the IC_{50} values obtained with (+Irr) or without (−Irr) irradiation. According to the respective OECD Testing Guideline 432, compounds showing a PIF value above 5 (which equals a 5-fold shift of the IC_{50} value toward lower concentrations) are considered to be phototoxic. Vemurafenib (Table 2) was insoluble in cell culture buffer at concentrations higher than 1.5 µg/ml, without showing significant cytotoxicity up to this concentration. However, in the presence of simulated sun light, a defined cytotoxicity profile was obtained (IC_{50} value of 0.052 µg/ml). The resulting PIF value was 29 (using the solubility limit of 1.5 µg/ml because no IC_{50} was obtained in the absence of irradiation), indicating that vemurafenib was clearly phototoxic *in vitro* to cultured cells. In addition, *in vitro* phototoxicity results for sparfloxacin and 8-MOP are shown. PIF values for both compounds are limited by solubility as well. However, achieved concentrations were significantly higher, which resulted in much higher PIF values. Under these conditions, the extremely low IC_{50} value of vemurafenib (0.052 µg/ml) illustrates the potent inherent photoreactivity while the
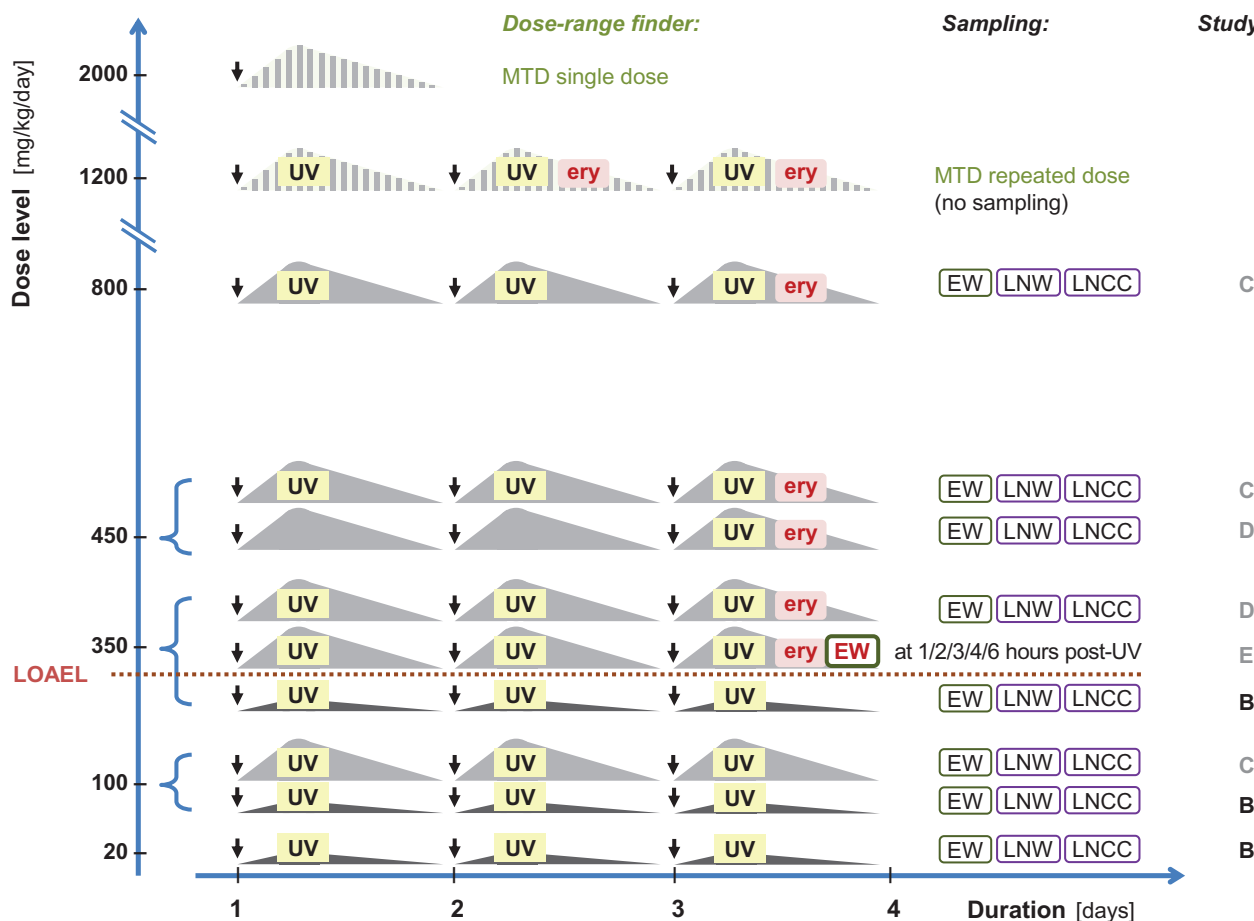
FIG. 2. Schematic overview of *in vivo* phototoxicity studies performed with 2 different variants of vemurafenib (crystalline [black] and amorphous [grey]). The MTD was assessed in dose-finding experiments using individual animals (dashed grey). The shape of the grey and black figures intends to reflect the temporal profile of vemurafenib's concentration in blood. For the main studies (solid grey or black), 2 groups of 6 female BALB/c mice were allocated to each dose, one with and one without subsequent exposure to simulated sun light ("UV"). During the treatment phase, typically daily administration (arrow) during 3 consecutive days, ery (if observed) was monitored during the first 6 h following daily irradiation. At necropsy, mean EW (bold boxed if significantly increased) were calculated using the weights of circular pieces (0.5 cm²) punched from the apical area of each ear. Mean LNW were derived from pairs of auricular lymph nodes, and mean LNCC values represent the corresponding total cellularity of the lymph node. The horizontal dashed line defines the LOAEL. Abbreviation: EW, ear weights; ery, erythema; LNCC, lymph node cell count; LNW, lymph node weights; LOAEL, lowest-observed-adverse-effect level; MTD, maximum tolerated dose.

solubility-limited PIF value of 29 is likely an underestimate of the true phototoxic potential.

Oral UV-LLNA

Data obtained with the positive control sparfloxacin are displayed in Table 1 (study A) to illustrate the expected responses for each endpoint. After oral administration of sparfloxacin (100 mg/kg), redness of the ears (erythema) was observed after each irradiation. At the time of necropsy, increased ear weight (edema) and a proliferation response in the ear-draining auricular lymph nodes were seen.

For vemurafenib (Fig. 2), the maximum tolerated dose (MTD) was established at 800 mg/kg in a dose-finding experiment using individual animals. Clinical symptoms were monitored during the dose-finding experiment, which started at a dose of 2000 mg/kg body weight/day using amorphous material (finely

grinded tablets). Following the first administration, reduced activity, piloerection, and hunched posture were observed during the initial 5 h. In concordance with Mackay *et al.* (1992), 1200 mg/kg was used as the next lower dose. Similar symptoms, although less pronounced, were observed allowing for additional irradiation after treatment. During the second and third day of treatment, erythema at the ear skin was observed. The next lower dose, 800 mg/kg, was well tolerated by mice for 3 days (apart from the irradiation-dependent erythema on day 3) and, therefore, was regarded as the MTD for amorphous vemurafenib to be considered in subsequent studies.

The oral UV-LLNA with vemurafenib was performed with doses of 20, 100, and 350 mg/kg of crystalline material and with doses of 100, 350, 450, and 800 mg/kg of amorphous material (Table 1, respectively, studies B, C, and D). After oral administration of crystalline vemurafenib, no clinical signs

TABLE 2
Summary of Spectrophotometric and *In Vitro* Phototoxicity Data for Vemurafenib and for the Reference Compounds Sparfloxacin and 8-Methoxypsoralene

Compound	MEC l/(mol·cm)	Precipitation (µg/ml)	IC_{50} (−Irr) (µg/ml)	IC_{50} (+Irr) (µg/ml)	PIF
Vemurafenib	22 800 (305 nm)	> 1.50	—	0.052	> 29
Sparfloxacin	33 600 (305 nm)	> 500	—	6.16	> 82
8-Methoxy psoralene	10 700 (299 nm)	> 100	—	0.22	> 457

and no redness of the ears were observed, and the ear-draining lymph nodes showed no proliferation response. After oral administration of amorphous vemurafenib, signs of phototoxicity (erythema) appeared on day 3 directly after UV exposure at doses of 350, 450, and 800 mg/kg, but not at 100 mg/kg; this effect disappeared within the next 15 h. In addition, a less pronounced erythema was already apparent after irradiation on day 3 at the dose level of 800 mg/kg. However, at time of necropsy, the mice did not show a UV-dependent increase in ear weight at any dose. The ear-draining lymph nodes showed no proliferation response at any dose. Based on the evident erythema reaction, 350 mg/kg was considered to be the lowest observable adverse effect level (LOAEL), whereas 100 mg/kg may be the no observable adverse effect level (NOAEL).

An alternative design of the UV-LLNA was used at a dose level of 450 mg/kg with the amorphous form in order to address the relevance of daily versus single irradiation (Study D). Mice were administrated 3 consecutive days with vemurafenib but exposed to simulated sun light only on day 3. Signs of phototoxicity appeared on day 3 directly after UV exposure although the ear reddening was reduced compared with the results obtained with the standard study design. As before, no ear weight increase and no proliferation response of the ear-draining lymph nodes were seen at the time of necropsy. This result suggests that for vemurafenib, repeated administration is needed in this mouse model in order to induce increased light sensitivity of the skin. Furthermore, induction of lymph node reactions driven by acute ear skin inflammation (as seen, for instance, with sparfloxacin) starts on day 3 and would only become visible after an extension of the treatment protocol up to 5 days.

Time Profile of Erythema and Edema Formation After Irradiation

The time profile obtained on the last of 3 consecutive days with daily treatment (Study E, 350 mg/kg amorphous vemurafenib followed by irradiation) is shown in Figure 3. Ear weights were significantly increased at 1, 2, 3, 4, and 6 h postirradiation compared with the control group, illustrating that a pronounced edema is formed at the same time at which the erythema can be observed. However, 24 h later (the standard sampling time point of the UV-LLNA), the ear weight increase after vemurafenib had already decreased to predose levels.

Pharmacokinetic Profile

Concentrations of vemurafenib found in mouse plasma over 3 days were found to be similar (Fig. 4), ranging from 54 to 193 µg/ml (C_{min}, C_{max}) on day 3. The time to reach the highest plasma concentration was between 2 and 4 h after the administration irrespective of the day. The apparent half-life time was 10–13 h, showing a slight decrease during treatment days. The amount of vemurafenib found in ear skin tended to decrease after repetitive administration and was about 2-fold lower compared with plasma. Distinct accumulation was neither found in plasma nor in ear skin.

DISCUSSION AND CONCLUSION

From a chemical point of view, vemurafenib carries all features of a clinically relevant phototoxic substance: (1) It does absorb sun light within UVB and UVA up to 350 nm; (2) it shows *in vitro* a significant phototoxic reaction in cell culture (proving photochemical reactivity), and (3) it contains a fundamental structural element (diaryl ketone or benzophenone chromophore) also seen in known clinically phototoxic drugs such as ketoprofen or amiodarone. However, it is important to remember that without sufficient distribution to sunlight-exposed tissues, such compounds do not lead to clinically relevant phototoxic reactions. Therefore, it is essential to confirm these *in vitro* findings in established animal models of phototoxicity as long as meaningful human phototoxicity data cannot be generated easily.

In our established in-house *in vivo* phototoxicity model (oral UV-LLNA in mice), we were able to demonstrate dose- and time-dependent phototoxicity of vemurafenib using commercially available tablets (stabilized amorphous material). The lowest phototoxic dose was 350 mg/kg given for 3 consecutive days followed by exposure to UV-visible irradiation at a UVA-normalized dose of 10 J/cm² (related blood plasma levels of vemurafenib on day 3, C_{max}: 193 µg/ml, C_{min}: 54 µg/ml). In comparison, pure vemurafenib, which forms easily crystalline variants and is known to have poor bioavailability, was tested at 350 mg/kg. Indeed, no signs of phototoxicity could be seen, which emphasizes the importance of adequate formulations and confirmed systemic exposure (either measured in blood or tissue or indirectly by clinical signs).
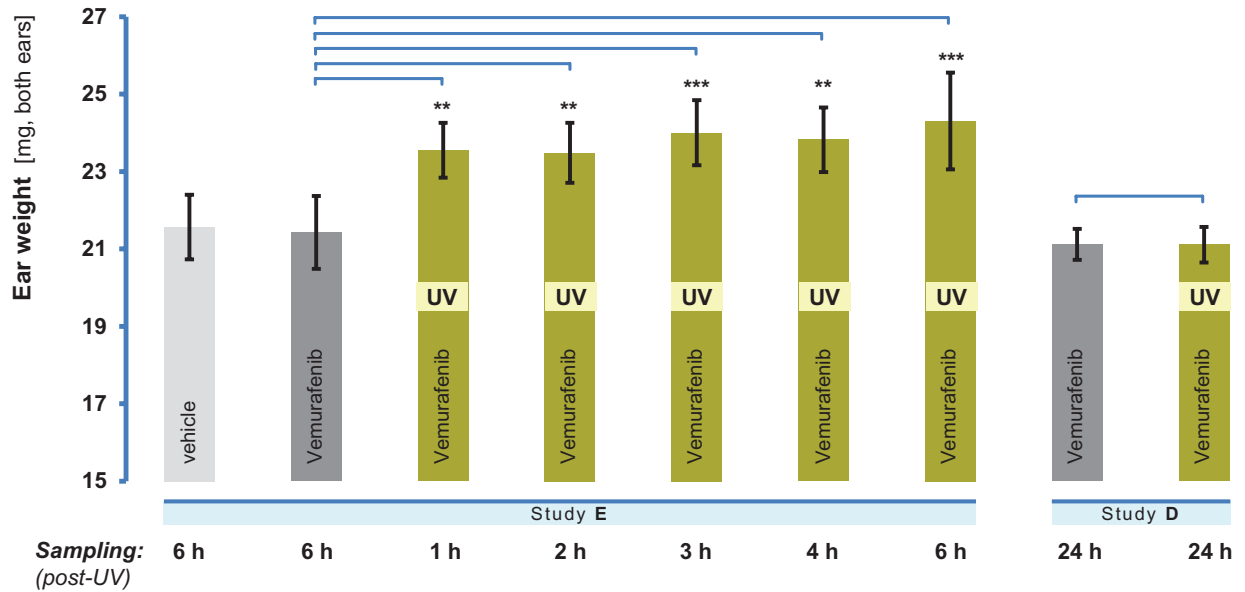
FIG. 3. Time-dependent edema reaction in mouse ears. A significant, time-dependent increase (up to 13%) of the ear weights (punch-out biopsies) within 6h post-UV exposure is seen. Ear weight at 24h from Study D. *Vemurafenib-treated groups (350mg/kg), comparison of UV exposed versus non-UV exposed. Student's t test (unpaired), significance levels: $**p < .01$, $***p < .001$.
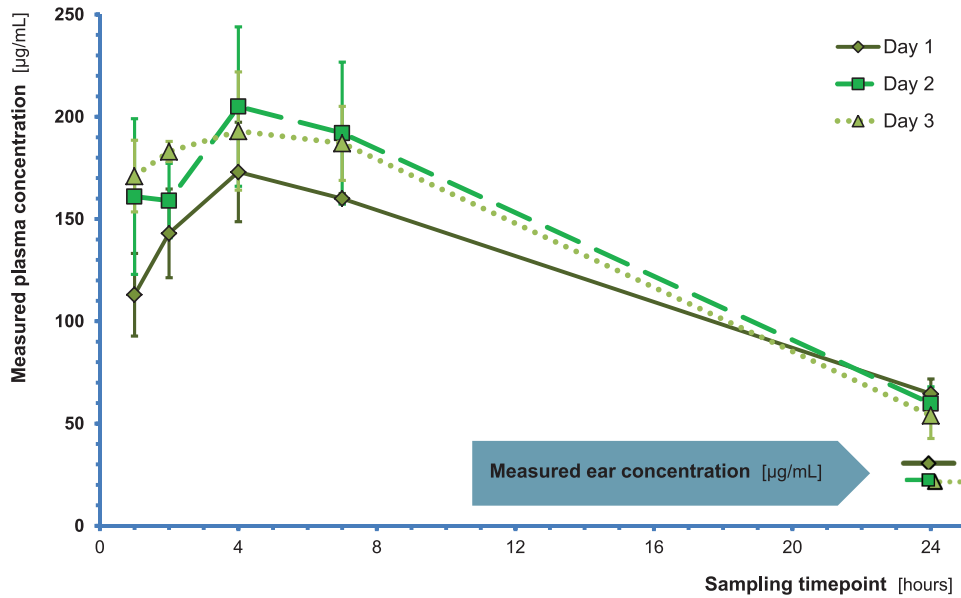

FIG. 4. Mean concentrations of vemurafenib versus time in mouse plasma. Six mice per group were treated orally with a suspension of amorphous vemurafenib on up to 3 consecutive days. Blood samples from 3 animals per time point per group were collected from the vena saphena at 1, 2, 4, and 7h postdose. In addition, from all animals of a group (1 per day), terminal samples at 24h were taken from heart puncture. At the same time, point ear samples were taken (punch-out biopsies).

Interestingly, initial studies performed as part of the nonclinical development of vemurafenib could not confirm any phototoxicity *in vivo* (for details, see CHMP review, 2011, FDA review, 2011). At that time, hairless female rats (Ico:OFA-hr/hr) were treated daily for 7 days at dose levels of 30, 150, and 450mg/kg using stabilized amorphous material comparable to that used later in commercial tablets. Although not reported for the hairless rat, sufficient systemic exposure can be assumed based on toxikokinetic data available from general toxicity studies (26-week toxicity study, female Crl:CD(SD) rats, daily dose of 450mg/kg:

C_{max} at day 1: 70 µg/ml, C_{max} at day 91: 115 µg/ml, converted from reported molar concentrations: 143.1 and 234.7µM, respectively). Irradiation of the treated hairless rats was performed on the last day of treatment starting 90 min after last administration of vemurafenib with UVA doses ranging from 5 to 35 J/cm². The light source had apparently the characteristics of fluorescent tubes with a reported maximal spectral output range from 350 to 400 nm and a peak at 370 nm (Fig. 1). In comparison to the light absorption spectrum of vemurafenib, it is becoming evident that there is no spectral overlap between this original light source used during development of vemurafenib and the test compound. This fact alone may fully explain the negative results of this earlier study in hairless rats because duration of treatment and exposure to both vemurafenib and the formal UVA dose (limited to 350–400 nm) was clearly exceeding the conditions we have used in our studies in mice reported above.

In patients, efficacious dose levels have been reported to show average C_{max} values around 60 µg/ml (CHMP review, 960 mg b.i.d., day 15), which is comparable to the exposure reached in mice at 350 mg/kg and in rats at 450 mg/kg. However, human PK profiles differ significantly from those seen in preclinical animal species. Particularly half-life in blood plasma appears to be several-fold longer in men (57 h), which suggests that a steady state is reached only after many days of treatment. Nevertheless, it should be noted that phototoxicity *in vivo* (both animal and human) is driven by the presence of photoreactive molecules in light-exposed tissues. Therefore, comparison of achieved peak concentrations (C_{max})—even at different T_{max}—remains the most appropriate exposure assessment (see also ICH S10, 2013), whereas AUC-based evaluations are of limited value.

So far, human phototoxicity data have only been reported from patient populations (see earlier). Two groups (Dummer *et al.*, 2012; Gelot *et al.*, 2013) have reported follow-on investigations for individual patients. In summary, clinical representation of vemurafenib-induced phototoxicity was described as a quickly occurring erythema accompanied by an edema and in some cases a burning sensation during light exposure and was apparently UVA dependent. As described by Ferguson (2002), this clinical presentation is typical for direct photochemical mechanisms of phototoxicity. Gerlot *et al.* speculated that the observed UVA dependency could be explained by increased systemic porphyrin levels. However, the authors did not discuss the known intrinsic photoreactivity of vemurafenib, which is—as confirmed by our own results—indeed UVA driven (UV-vis spectrum, *in vitro* and *in vivo* phototoxicity tests) and can easily explain the clinical reactions. The quick onset of edema formation in mice (Fig. 3) resembles the acute clinical reactions in men. Although vemurafenib does not show typical signs of accumulation or retention in skin (neither in animals nor in men), phototoxicity may be linked to steady-state conditions. In our *in vivo* studies, an irradiation-induced skin reaction at the lowest effective dose level (350 mg/kg) became apparent only after 3 consecutive days of dosing. However, at higher dose levels, these skin reactions

started already on day 2. Currently, there is no data providing further insight. Assuming that vemurafenib molecules represent the photoreactive species (rather than endogenous molecules as discussed above), there may be slower but critical redistribution processes (within skin, within cells), which are ultimately driving susceptibility of skin to UVA light.

In conclusion, our investigations on the kinase inhibitor vemurafenib confirm a nonclinical safety profile, which is consistent with the clinical signs of phototoxicity seen in many treated patients. Furthermore, these results highlight once again the impact of carefully designed *in vivo* phototoxicity studies. It is apparent that duration of treatment and timing of irradiation are key parameters to ensure an appropriate sensitivity. These elements of the study design should be supported by relevant pharmacokinetic data. The common perception is that if a compound presents an identical pharmacokinetic profile over several days, a single-treatment/single-irradiation design is appropriate as it would not be affected by an accumulation of the compound into the skin. However, this case clearly shows that even for compounds without apparent overproportional distribution to skin, a single-treatment/single-irradiation design can be inappropriate. Finally, it is evident that appropriate irradiation conditions are crucial. The more general use of "solar simulator" light sources covering at least the full range of UVA and visible light should be considered state of the art.

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