Vandetanib-induced phototoxicity in human keratinocytes NCTC-2544

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Abstract
The phototoxicity of the new anticancer drug vandetanib was evaluated using human keratinocyte cell line, NCTC-2544. This study was started since many clinical cases of vandetanib photosensitizing reactions were recently reported in literature. Vandetanib induces a clear drop in human keratinocytes viability after cell irradiation in concentration and UV-A dose dependent mode. Since vandetanib can photolyze with the formation of two main photoproducts after UV-A exposure, the contribution of these new species was also evaluated. These two photoproducts did not have a main role in the phototoxicity of their parent drug. In our opinion, the main hypothesis for the vandetanib phototoxic potential is the formation of a reactive species, such as an aryl radical, which can react promptly with different targets inside the cells. In fact, a massive DNA photodamage was detected both in the in vitro DNA photocleavage experiments, and in cells. Moreover, vandetanib was able to photoinduce lipid peroxidation and protein oxidations. Vandetanib photoinduced cell death by apoptosis with the involvement of mitochondria and lysosomes.

Keywords:
Vandetanib
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1. Introduction

Vandetanib is a novel, orally available, anticancer agent which was approved for the treatment of symptomatic or progressive medullary thyroid cancer (MTC) in patients with unresectable, locally advanced, or metastatic disease (Thornton et al., 2012). MTCs are rare endocrine malignancies that arise from the parafollicular C cells of the thyroid. They account for less than 5% of all thyroid cancers. A germline rearranged during transfection (RET) mutation is present in most familial forms of MTC (Schlumberger et al., 2012) while somatic RET activation is found in 40–50% of sporadic MTCs (Elisei et al., 2008).

Vandetanib is a small-molecule inhibitor of vascular endothelial growth factor receptor-2 (VEGFR-2) and epidermal growth factor receptor (EGFR)-dependent signaling as well as RET, which is the most important growth driver in this type of neuroendocrine thyroid cancer (Campbell et al., 2013).

The most commonly observed side effects during its use are mainly mild or moderate; however, 12% of patients receiving vandetanib discontinue treatment due to toxicity and 35% require dose reduction because of an adverse event (Schlumberger et al., 2012). An adverse effect that has received a great deal of attention is heart-rate corrected QT (QTc) interval prolongation. In fact, the FDA issued a boxed warning for QTc prolongation, torsades de pointes, and sudden death and implemented a Risk Evaluation and Mitigation Strategy (REMS) to limit the prescription of vandetanib to trained and certified doctors and pharmacists (Campbell et al., 2013).

Moreover, severe skin reactions (including Stevens-Johnson syndrome) have been reported during vandetanib therapy. Among these skin side effects, photosensitivity problems have also been observed and they include eczematous dermatitis, skin hyperpigmentation, acniform lesions, delayed wound healing, xerosis, erythematous edematous scaly lesions and pruritus (Kong et al., 2009; Fava et al., 2010; Chang et al., 2009). For this reason, AstraZeneca advises to use sun photoprotections for 4 months after vandetanib discontinuation due to its long half-life.

In a recent study, our research group (Dall’Acqua et al., 2013) showed that vandetanib underwent photodegradation after UV-A light exposure. In particular, the formation of two main photoproducts was demonstrated after UV-A irradiation of the drug in aqueous media and their chemical structures were determined by different analytical techniques (Fig. 1). These two new species derived from the loss of the vandetanib bromine atom: dehalogenation is quite usual in the photodegradation profile of other drugs (Musa and Eriksson, 2009; Fasani et al., 1998).
Thus, the aim of this work was to evaluate the light-induced toxicity of vandetanib and its main photoproducts in cultured NCTC-2544 human keratinocytes and to study the mechanisms of drug phototoxicity.

To the best of our knowledge, besides the clinical cases, this is the first report in which the phototoxic effects of vandetanib are demonstrated at cellular level.

2. Materials and methods

2.1. Chemicals and cellular media

Vandetanib, N-(4-bromo-2-fluorophenyl)-6-methoxy-7-((1-methylpiperidin-4 yl)methoxy)quinazolin-4-amine, was provided by Selleck Chemicals. All chemicals and cellular media were obtained from Sigma Aldrich, if not elsewhere indicated.

2.2. Irradiation procedure

HPW 125 Philips lamps, mainly emitting at 365 nm, were used as UV-A source. The UV-A dose was determined by a radiometer type VLX-3 W, Vilber Lourmat, with a sensor CX-365, to be each time of 0.25 J/cm$^2$ min$^{-1}$.

2.3. Isolation of photoproducts

Aqueous solutions of vandetanib were irradiated as described in Dall'Acqua et al. (2013) with 100 J/cm$^2$ to maximize the photolysis process. Then, photoproducts were isolated and characterized as described in Dall'Acqua et al. (2013).

2.4. Cell cultures

Cultures of human immortalized keratinocytes (NCTC-2544), human epidermoid carcinoma cells (A-431) and of Balb/c mouse (3T3) fibroblasts were grown in Dulbecco's modified Eagle medium; cultures of human T-cell leukemia (Jurkat) and of human chronic myeloid leukemia (K-562) were grown in RPMI-1640 medium. All media were supplemented with 115 U/mL of penicillin G, 115 µg/mL streptomycin and 10% fetal bovine serum (Invitrogen).

2.5. Cytotoxicity and phototoxicity tests

About 100 µL of complete medium containing 5 x 10$^3$ adhesion cells or 1 x 10$^4$ leukemic cells were seeded in individual wells of 96-well tissue culture microtiter plate and plates were incubated in an atmosphere of 5% CO$_2$ at 37°C for 24 h. Cytotoxic experiments were performed as previously described (Diana et al., 2011). For phototoxicity, after the medium was removed, 100 µL of vandetanib, dissolved in DMSO, were added to each well after dilution with Hank's balanced salt solution (HBSS; pH = 7.2). The plate was then incubated for 30 min. After irradiation, the solution was replaced by the medium and the plates were placed in the incubator for 72 h. Cell viability was assayed as described previously (Viola et al., 2007).

2.6. Cell irradiation for cytofluorimetric experiments and immunoblotting

NCTC-2544 cells (300,000 cells/mL) were seeded in 6 cm-dishes and incubated for 24 h prior irradiation. After medium removal, 3 mL of the drug solution in HBSS was added to each dish, incubated at 37°C for 30 min and then irradiated (3.75 J/cm$^2$). After irradiation, the drug solution was replaced by fresh medium and the dishes were incubated for different times prior to cytofluorimetric or immunoblotting experiments.

2.7. Cell cycle analysis

For flow cytometric analysis of DNA content, NCTC-2544 cells in exponential growth were irradiated as described above. After 24 h, the cells were trypsinized and together with floating cells, centrifuged, and fixed with ice-cold ethanol (70%). Subsequently, the keratinocytes were treated with RNase A and then stained with propidium iodide (PI) (Awtar, 1975). Samples were analyzed on a BD FACS Calibur flow cytometer (Becton Dickinson, New York, USA). Results of cell-cycle analysis were examined using WinMDI 2.9 (Windows Multiple document Interface for Flow Cytometry).

2.8. Externalization of phosphatidylserine

Surface exposure of phosphatidylserine (PS) by apoptotic cells was measured by flow cytometry by adding Annexin V conjugated with fluorescein isothiocyanate (FITC) to cells according to the manufacturer’s instructions (Annexin V-FITC apoptosis detection

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Fig. 1. Chemical structure of vandetanib and of its main photoproducts FP2 and FP3.
2.9. Mitochondrial dysfunction

After 24 h from irradiation, cells were trypsinized, counted by centrifugation and re-suspended in 1 μM 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine (JC-1, Invitrogen) solution in HBSS (Salvioli et al., 1997) or in 100 nM 10-N-nonyl acridine orange (NAO, Invitrogen) solution in DMEM medium (Kaewsuya et al., 2007). The cytofluorimetric analysis (FACS Calibur flow cytometer) was performed collecting green (FL1) and orange (FL2) fluorescence for JC-1 staining and only the green one for NAO staining in at least 10,000 events for each sample.

2.10. Lysosomal dysfunction

After 24 h from the irradiation in the presence of vandetanib, cells were assessed for lysosomal stability using the acridine orange (AO) uptake method (Zhao et al., 2000): cells were stained with AO at 5 mg/mL at 37 °C for 15 min, washed, and then analyzed by flow cytometry using excitation at 488 nm and detecting the red emission at FL3.

2.11. Lipid peroxidation: thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation was measured using a thiobarbituric acid assay as described previously (Viola et al., 2007). A standard curve of 1,1,3,3 tetraethoxypropane was used to quantify the amount of malonaldehyde produced. Data are expressed in terms of nanomoles of TBARS normalized to the total protein content, measured in each cell extract.

2.12. Protein oxidation

Solutions of bovine serum albumin (BSA) (0.5 mg/ml) in phosphate buffer 10 mM were irradiated in the presence of 20 μM vandetanib for various times in a quartz cuvette. At each time, the tryptophan (Trp) content was followed by monitoring the characteristic Trp fluorescence as described by Balasubramanian and Zigler (1990). In further experiments, the degree of protein oxidation was monitored spectrophotometrically by the method of Levine et al. (1990), by derivatization with 2,4-dinitrophenylhydrazine (DNPH).

2.13. DNA strand breaks

Irradiation was performed on samples containing 100 ng of supercoiled pBR322 DNA (Fermentas) dissolved in phosphate buffer (10 mM) at pH 7.2. After different irradiation doses (3.75 and 7.5 J/cm²), samples were loaded on 1% agarose gel. The electrophoretic run was carried out in TAE buffer (0.04 M Tris–acetate, 1 mM EDTA) at 75 V for 2 h. After staining in ethidium bromide solution, the DNA bands were detected under UV radiation with a UV illuminator. Pictures were taken with a digital photocamera Kodak DC256, and the quantification of the bands was achieved by the image analyzer software Quantity One (Bio RAD). The fraction of each DNA form was calculated as described by Ciulla et al. (1989).

2.14. Western blot analysis

NCTC-2544 cells were irradiated in the presence of vandetanib and, after different times, were trypsinized, centrifuged, and washed two times with ice cold phosphate-buffered saline (PBS). The pellet was resuspended in lysis buffer (Diana et al., 2011). After the cells were lysed on ice for 20 min, lysates were centrifuged at 10,000 g at 4 °C for 20 min. The protein concentration in the supernatant was determined. Equal amounts of protein (20 μg) were resolved using 8–16% gradient polyacrylamide precast gels (Thermo Scientific) and transferred on a nitrocellulose Hybond-p membrane (GE Healthcare). Membranes were blocked with 5% skim milk powder in Tween-PBS for at least 2 h. Membranes were incubated with primary antibodies against caspase-3, phospho-histone γH2AX and β-actin (all rabbit, 1:1000, Cell Signaling), for 2 h or overnight. Membranes were next incubated with peroxidase-labeled goat antirabbit IgG (1:3000, Cell Signaling) for 60 min. All membranes were visualized using ECL Advance (GE Healthcare) and exposed to Hyperfilm MP (GE Healthcare).

2.15. Statistic analysis

Unless indicated otherwise, results are presented as mean ± SEM. The differences between different treatments were analyzed by One-Way ANOVA using the Dunnett test; p values lower than 0.05 were considered significant (* p < 0.05).

3. Results

3.1. Cellular phototoxicity

Since vandetanib causes photosensitizing reactions in some patients in vivo, cellular phototoxicity was evaluated irradiating two different cell lines in vitro. For this test, human immortalized keratinocytes (NCTC-2544) and the classical cells for in vitro phototoxic studies, 3T3, were chosen. Vandetanib was dissolved in HBSS at different concentrations and was added to these cell lines for 30 min prior irradiation (0, 2.5, 3.75 and 6.25 J/cm²). As can be clearly observed in Table 1, vandetanib was phototoxic in both cell lines and the GI₀₀ – the concentration of drug to cause 50% reduction in proliferation of cells- was UV-A dose dependent. Fig. 2 shows the reduction in viability in both NCTC-2544 and 3T3 cells at different concentrations and different UV-A doses. As can be observed, a concentration-dependent reduction in cell viability was also induced by vandetanib.

We recently demonstrated that vandetanib undergoes UV-A photodegradation and that two main photoproducts are produced in aqueous medium. The photoproduct FP2 derives from the loss of bromine atom and from the subsequent solvent nucleophilic substitution (i.e. an addition of a hydroxyl group), while the photoproduct FP1 develops from the mere loss of the bromine (Dall’Acqua et al., 2013) (Fig. 1). Thus, the contribution of the formation of these new species in solution was also assessed. In particular, some cytotoxic tests were performed incubating different human cell lines for 72 h with vandetanib or their main photoproducts.

| Table 1 |
|------------------|---------|---------|---------|---------|
| UV-A DOSES       | 0 J/cm² | 2.5 J/cm² | 3.75 J/cm² | 6.25 J/cm² |
| NCTC-2544a       |       |         |         |        |
| GI₀₀ (μM) in vandetanib irradiated cells after 72 h from irradiation. |
| 3T3b             | 41.8 ± 2.2 | 7.2 ± 0.8 | 3.1 ± 1.2 | 0.4 ± 0.1 |

a NCTC-2544 = human immortalized keratinocytes; 3T3 = murine immortalized fibroblasts.

b Values represent mean ± S.E.M for at least 3 independent experiments.
etanib was not photodegraded. Described in Dall’Acqua et al. (2013) with 20 J/cm² dose. Then these solutions were evaporated and the irradiated mixtures were resuspended to reach the desired concentration. Cell viability was measured by MTT test after 72 h from irradiation. Data represent mean ± S.E.M for at least four independent experiments.

Effectively, our intention was also to assess if photoproducts could be effective antiproliferative compounds and thus we also tested their cytotoxicity in tumor cell lines. Moreover, these cell lines were also let grow in the presence of an UV-A irradiated vandetanib solution to check the possibility of a loss in activity of the drug. Results were presented in Table 2. The irradiated mixture was less cytotoxic than the parent compound and in fact both photoproducts often were at least 10 times less active than vandetanib, even in tumor cell lines. Moreover, the phototoxicity tests were also carried out irradiating NCTC-2544 cells in the presence of FP2 and FP3 and they did not induce any viability reduction in comparison to control. Thus, the formation of new species in solution seems to be marginal for the phototoxic mechanism of vandetanib.

### 3.2. Cellular death photoinduced by vandetanib

The mode of vandetanib photoinduced cellular death was evaluated with different tests using NCTC-2544 cells since keratinocytes are likely the most involved cells in phototoxic reactions. NCTC-2544 cells were irradiated (3.75 J/cm²) in the presence of different vandetanib concentrations.

First of all, the classical cytofluorimetric test using Annexin V-FITC (A) and propidium iodide (PI) was used to assess the loss of plasmatic membrane asymmetry (Vermes et al., 1995). Dual staining for Annexin V and with PI allows the discrimination between live cells (A−/PI−), early apoptotic cells (A+/PI−), late apoptotic cells (A+/PI+) and necrotic cells (A−/PI+). After 24 h from irradiation (Fig. 3A), while most of control cells were located at the left and bottom part of the histogram (A−/PI−), cells irradiated in the presence of 20 μM vandetanib were mainly found in the right part of it (A+/PI− and A+/PI+). As depicted in Fig. 3B, vandetanib photoinduced an Annexin V positive cells accumulation in comparison with the control, in a concentration-dependent manner. This loss of membrane asymmetry is indicative of apoptosis.

Cell cycle analysis can also be a useful instrument to study apoptosis as a new peak with low DNA content (subG1 peak) appears in the presence of apoptotic cells. In fact, during apoptosis an ordered DNA degradation occurs. The different DNA content during each cycle phase can be used to distinguish the different phases after PI DNA staining (Salvador et al., 2013). Table 3 showed the percentages of each cell cycle phase after 24 h from irradiation. A concentration-dependent increase in subG1 percentage was observed when cell irradiation was carried out in the presence of the drug. Moreover, especially at low doses, a clear increase in G2-M phase together with a G1 phase percentage decrease were evident.

The activation of caspase 3 is considered as a hallmark of apoptotic process (Oliver and Vallette, 2005). The levels of cleaved active subunits of executioner caspase-3 were evaluated by immunoblotting cell lysates after 3, 6, 15 and 24 h from vandetanib irradiation (Fig. 3C). Very little amount of cleaved caspase 3 was present after 3 h from irradiation in the presence of the highest vandetanib concentration (20 μM), while the apoptotic process was clearly activated after 6 h from irradiation with the same vandetanib concentration. After 15 h from irradiation, the increase of the cleaved form of caspase 3 was evident with 10 μM vandetanib.

### 3.3. Mitochondrial involvement in apoptosis

Mitochondrion, the organelle that helps to provide cells with energy to live, also serves as a platform to actively initiate cell death: in fact during apoptosis, the signals that regulate the fate of the cell are integrated at functioning mitochondria. Upon induction of apoptosis, the integrity of the mitochondrial outer membrane is breached, resulting in the release of cytochrome c and other intermembrane space proteins into the cytoplasm which triggers the activation of multiple pathways that result in the demise of the cell (Kale et al., 2012).

The mitochondrial involvement in apoptosis was evaluated by two different flow cytometric tests, which analyzed the mitochondrial potential (ΔΨm) and the production of reactive oxygen species. The lipophilic cation JC-1 was used to monitor the changes...
in $\Delta \Psi_M$ induced by the tested compound in combination with UV-A irradiation. JC-1 has the unique property of changing its fluorescence properties with the loss of mitochondrial potential. In fact, it forms orange fluorescent aggregates under high mitochondrial $\Delta \Psi_M$, while after loss of potential, JC-1 is present in monomeric form and fluoresces in green (Salvioli et al., 1997). Another consequence of mitochondrial dysfunction is the production of reactive oxygen species which oxidize the mitochondrial phospholipid cardiolipin (CL). CL oxidation was monitored by staining irradiated cells with N-nonyl acridine orange (NAO) as described in Section 2.

As can be clearly observed in Fig. 4, vandetanib was able to induce mitochondrial dysfunction in a concentration-dependent mode after 24 h from irradiation.

### 3.4. Lysosomal involvement in apoptosis

Lysosomal alterations can be involved in cell death caused by many photosensitizers such as fluoroquinolones (Ouedraogo et al., 1999), psoralen derivatives (Barraja et al., 2010) and statins (Viola et al., 2010).

To investigate the integrity of lysosomes after irradiation in the presence of vandetanib, flow cytometric analysis was performed with the fluorescent dye acridine orange (AO). AO is a lysosomotropic base and a metachromatic fluorochrome with red fluorescence when highly concentrated in intact lysosomes and with green fluorescence at low concentrations, as in damaged lysosomes. The percentage of cells with intact lysosomes can be evaluated by assaying red fluorescence after AO staining of cells exposed to the photosensitizer. A significant extent of lysosomal

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**Table 3**

| Effect of vandetanib on the cell cycle of NCTC-2544 after 24 h from UV-A irradiation (3.75 J/cm²). |
|---|---|---|---|---|
| $\mu$M | % G1$^a$ | % S | % G2-M | % subG1$^b$ |
| NIC | 70.2 | 12.0 | 17.8 | 6.7 |
| IC | 69.0 | 11.6 | 19.4 | 5.9 |
| Vandetanib 2.5 | 22.3 | 9.9 | 67.8 | 8.8 |
| Vandetanib 5 | 11.8 | 28.0 | 60.2 | 16.2 |
| Vandetanib 10 | 44.6 | 27.5 | 27.9 | 58.1 |
| Vandetanib 20 | 46.9 | 29.2 | 23.9 | 79.2 |

$^a$ Percentages of each phase of the cell cycle excluding the subG1 peak.

$^b$ Percentage of the cell population with hypodiploid DNA content peak (apoptotic cells).

$^c$ NIC = not irradiated control; IC = irradiated control.
damage was photoinduced by vandetanib 24 h postirradiation (Fig. 5).

3.5. Lipid peroxidation and protein photodamage

As vandetanib is a lipophilic drug and its mechanism of action supposes the binding to plasmatic membrane receptor, the first considered targets for its phototoxicity were the main membrane components, lipids and proteins.

The thiobarbituric acid reactive substance (TBARS) assay was used in order to determine whether lipid peroxidation occurred upon irradiation of NCTC-2544 cells incubated in the presence of vandetanib. In fact, this assay detects the formation of a secondary product of lipid peroxidation such as malondialdehyde, which is able to react promptly with thiobarbituric acid. The TBARS test was performed 24 h after irradiation. Fig. 6A presented the results as a function of vandetanib doses. TBARS were significantly produced when the cells were exposed to the compound and UV-A.

The photosensitization capacity of vandetanib towards the other components of cellular membranes, such as proteins, was estimated using bovine serum albumin (BSA) as a model. Solutions of BSA containing 20 μM vandetanib were irradiated with different UV-A doses.

The amount of the aromatic aminoacid tryptophan (Trp) was directly analyzed by monitoring its characteristic fluorescence. As depicted in Fig. 6B, a slow decrease of the emission fluorescence was observed for vandetanib. No effects were observed when BSA was irradiated in the same conditions without the drug.

The degree of oxidative modifications was measured by monitoring the carbonyl content, an index of photodamage of proteins. The results were reported in Fig. 6C and demonstrated that vandetanib significantly increased the carbonyl content of BSA after irradiation.

3.6. DNA photodamage

DNA photodamage can be responsible for the mechanism of the phototoxicity of many drugs such as phenothiazines (Kochevar et al., 1998), non steroidal anti-inflammatories and fibrates (Marguery et al., 1998). To investigate a possible DNA photodamage induced by vandetanib some photocleavage experiments were carried out: buffered aqueous solutions of the drug at different concentrations were irradiated (3.75 and 7.5 J/cm²) in the presence of supercoiled pBR322 plasmid DNA and analyzed by agarose gel electrophoresis. When kept in the dark, vandetanib did not promote any DNA damages (data not shown), but after UV-A irradiation, an increase of the nicked form of DNA with a parallel decrease of the supercoiled form was observed. The level of DNA photocleavage was very high and dependent on vandetanib concentration but also on UV-A dose (Fig. 7A). When the irradiation dose was 3.75 J/cm², the formation of the open circular form (single strand breaks) was evident even at the lowest employed concentration (0.5 μM) and using 5 μM vandetanib, no more supercoiled form was detected but there was the presence of linear form (double strand breaks), indicative of a massive DNA photodamage. When the UV-A dose was increased to 7.5 J/cm², the formation of the linear form occurred even at 1 μM.

A clear DNA photodamage was induced by vandetanib in solution, however this damage had to be proved in cells too.

Phosphorylation of the Ser-139 residue of the histone variant H2AX, forming γH2AX, is an early cellular response to the induction of DNA double-strand breaks and can be used as a marker of this kind of damage (Mah et al., 2010). Thus, some immunoblotting experiments for the phosphorylated form of H2AX were performed after 3, 6, 15 and 24 h from vandetanib irradiation in NCTC-2544...
After 3 h from irradiation, an increase in phosphorylated form of H2AX was detected with both vandetanib concentrations with respect to control. With the highest vandetanib concentration (20 μM), the level of this phosphorylated protein was constant with time and decreased after 24 h; after irradiation in the presence of 10 μM, this band became more intense with time. Altogether, these data suggested that vandetanib phototoxicity was mediated by DNA photodamage.

4. Discussion

Many drugs with different chemical structure and therapeutic use are responsible for photosensitizing reactions when patients are exposed to sunlight (Stein and Scheinfield, 2007). The potential photosensitizers normally have the ability to absorb radiation in the ultraviolet (λ > 290 nm since UV-C light is not relevant because it is effectively absorbed by the ozone layer) and visible ranges (Ferguson, 2002). Vandetanib demonstrated to induce skin adverse reactions after sunlight exposure, thus the aim of our work was to study the mechanism of its phototoxicity. Vandetanib has a quinazoline moiety that lets it absorb in the UV-A range. Thus, we focused our attention on UV-A light since it does not absorb visible light. Moreover, we recently reported that this drug easily underwent UV-A photodegradation with the formation of two main photoproducts, FP2 and FP3 (Dall’Acqua et al., 2013).

We used human immortalized keratinocytes for our in vitro studies as skin is always exposed to sunlight and it is one of the main organs affected by phototoxic reactions together with eyes (Deleo, 2004 and Roberts, 2001). Vandetanib was phototoxic in concentration and UV-A dose dependent way in this cell line model. The phototoxicity of a drug can derive from the formation of photoproducts after sun exposure, thus we analyzed a possible role of the main compounds of vandetanib UV-A photolysis in its mechanism. Both photoproducts were less cytotoxic than the parent compound and were not phototoxic, thus we hypothesized that they accounted only marginally for vandetanib phototoxicity.

Moreover, drug photodegradation can also cause loss of efficacy and for these reasons is an event to avoid. UV-A vandetanib irradiated mixture showed a reduced antiproliferative activity in many human cancer lines thus this fact is relevant for the process of formulation to avoid the loss of the drug activity.

The mode of cellular death photoinduced by vandetanib is clearly very important for phototoxic reactions: in fact, while
apoptosis is a well organized process. During necrosis, the rapid swelling of cells leads to the leakage of cellular components in the extracellular matrix and to the consequent onset of inflammatory process.

We found out with different experiments (Annexin V/PI test, cell cycle analysis and caspase 3 activation) that the main cellular mode of death photoinduced by vandetanib was apoptosis. Mitochondria and lysosomes were involved in cell death photoinduction.

We performed some tests to evaluate which biomolecules can be affected by the photosensitizing reaction of vandetanib. Since vandetanib presents its main physiological anticancer targets in plasmatic membranes, we started to evaluate photoreactions towards their main components, i.e. lipids and proteins. While vandetanib caused a clear increase in lipid peroxidation in irradiated keratinocytes, some experiments with the model protein BSA let us think that this latter biomolecule was less affected by vandetanib photoreactions.

Our results indicated that one of the mechanism of phototoxicity of vandetanib involved DNA. In fact, we assessed that vandetanib was able to photoinduce massive photocleavage in plasmidic DNA in vitro: the formation of frank strand breaks in DNA can be explained by the formation of an aryl radical during the UV-A induced breakage of the C–Br bond of vandetanib. We proposed the photochemical reaction scheme of vandetanib in Dall’Acqua et al. (2013), in which we hypothesized that after UV absorption, the typical C–Br bond homolysis can occur, leading to an aryl radical. It is also well known that aryl radicals are able to cause DNA frank strand breaks (Wender and Jeon, 1999). It is important to note that the two photoproducts that lack the bromine atom were not phototoxic at all and were not able to photoinduce DNA strand breaks (data not shown).

Moreover, we demonstrated that DNA damage also occurred in cells thanks to the activation of histone H2AX, which is normally recruited for DNA double strand breaks. The presence of a DNA damage can be also supported by the fact that vandetanib photoinduced a cell cycle arrest in G2-M phase.

In summary, we have established for the first time that vandetanib was endowed with a clear phototoxic potential in vitro in a human keratinocyte cell line. Its phototoxicity could be mediated by the formation of a very reactive species such as aryl radical. Moreover, we identified DNA as one of the major targets of the vandetanib action, which ultimately led to apoptosis as the principal mode of cell death. Further researches in finding effective aryl radical scavengers could be useful to reduce the phototoxic potential of this new drug, especially for the fact that its therapeutic applications could be amplified since some clinical trials are carried out for other cancer kinds (see for example, Broniscer et al., 2013 or Leboulleux et al., 2012).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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