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## Luminescent threat: toxicity of light stick attractors used in pelagic fishery

## SUBJECT AREAS:

CHEMICAL  
MODIFICATION

DNA

RISK FACTORS

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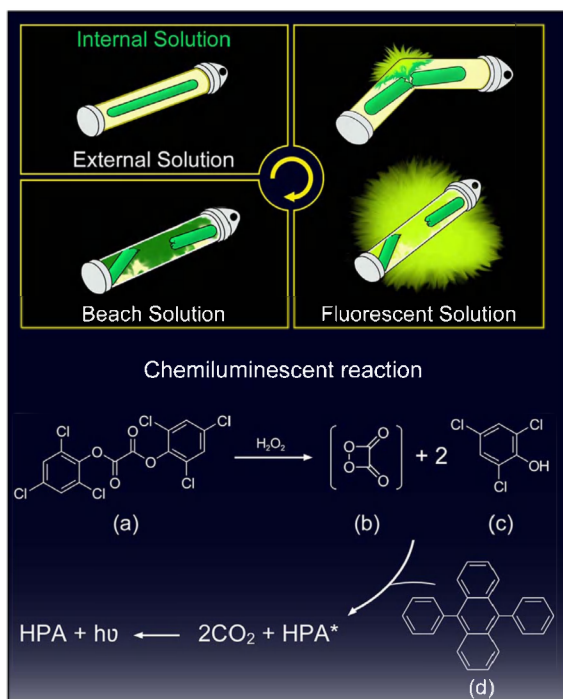
Light sticks (LS) are sources of chemiluminescence commonly used in pelagic fishery, where hundreds are discarded and reach the shores. Residents from fishing villages report an improper use of LS contents on the skin. Given the scarce information regarding LS toxicity, the effects of LS solutions in cell cultures were evaluated herein. Loss of viability, cell cycle changes and DNA fragmentation were observed in HepG2 cell line and skin fibroblasts. A non-cytotoxic LS concentration increased the occurrence of the mutagenic lesion 1,N<sup>6</sup>-ε-Ado in HepG2 DNA by three-fold. Additionally, *in vitro* incubations of spent LS contents with DNA generated dGuo-LS adducts, whose structure elucidation revealed the presence of a reactive chlorinated product. In conclusion, the LS contents were found to be highly cyto- and genotoxic. Our data indicate an urgent need for LS waste management guidelines and for adequate information regarding toxic outcomes that may arise from human exposure.

Light sticks (LS) are plastic devices that emit oxalate- and polyaromatic-based chemiluminescence<sup>1</sup>, predominantly used as fish attractants in pelagic fishery<sup>2</sup>. Hundreds of LS are replaced in each fishing multi hook, and most are discarded in the ocean, where they ultimately pollute coastal shores<sup>3</sup>.

Thousands of LS are frequently found in the overseas floating garbage that reaches the Brazilian coastline<sup>4</sup>. Members of a non-governmental organization (NGO) named Global Garbage collected approximately 7,000 LS in a monitoring campaign performed 90 km off the northern coast of Bahia State. This group is concerned with alerting the population and fishing companies of the risks that can result from the improper use and disposal of LS<sup>5</sup>.

Residents from fishing villages located on Brazilian beaches report the use of LS contents on the skin in the belief that they can serve as sunblock, suntan oil, massage oil, repellent, and medicine for muscle pains, skin marks, and vitiligo<sup>3</sup>. This belief is partly due to the scarce information on LS, although local efforts have been made to reverse this situation<sup>3</sup>. The improper use of LS contents by individuals in dance clubs and parties, where individuals unknowingly spread the luminous oil over their skin, is also known. Hoffman et al.<sup>6</sup> reported LS content exposure through ingestion, application on the eyelids, and dermal contact.

Unused LS contain two separated solutions in a transparent flexible tube. One of the solutions is inside a glass capillary tube that is surrounded by the outer solution in a polyethylene tube. In general, one solution contains an oxalate ester and a polycyclic aromatic hydrocarbon (PAH) dissolved in dibutyl phthalate, and the other solution contains hydrogen peroxide and a salicylate dissolved in dimethyl phthalate. The chemiluminescent reaction occurs immediately after the plastic tube is bent and the glass ampoule is broken, resulting in the mixing of the two solutions<sup>1</sup>. The chemiluminescent reaction is initiated by salicylate-catalyzed nucleophilic attack of hydrogen peroxide on the oxalate ester, generally a (bis)2,4-dinitrophenyl- or (bis)2,4,6-trichlorophenyl derivative, yielding an energy-rich intermediate (1,2-dioxetanodione) that cleaves and excites the fluorophore<sup>7</sup>. According to Rauhut<sup>8</sup>, a viscous solvent, such as di-*n*-butyl phthalate, is required to allow for electron transfer from PAH to the CO<sub>2</sub> dimer, yielding the hypothetical complex [C<sub>2</sub>O<sub>4</sub>]<sup>-</sup>-PAH<sup>+</sup> that decays to CO<sub>2</sub> and electronically excited PAH, which emits fluorescent light. These reactions were collectively coined as the peroxyoxalate chemiluminescent system<sup>8,9</sup> (Figure 1).



**Figure 1** | The solutions used in the present study and the chemiluminescent reaction are illustrated: (A) bis(2,4,6-trichlorophenyl)oxalate (TCPO), (B) 1,2-dioxetanedione, (C) 2,4,6-trichlorophenol, and (D) 9,10-diphenylanthracene (DPA). Figure 1 was created by the author T.F.O., using Adobe® Fireworks® CS6 software.

Upon exposure to LS contents, local irritation may be experienced<sup>6</sup>. The devices that are found on Brazilian beaches lack any information about their utility or potential hazards, confounding local inhabitants who apply the solutions to the skin<sup>3</sup>. Additionally, the composition of the LS solutions collected from beaches is expected to be different from that of the original reaction mixtures as these tubes are exposed to high temperatures and solar irradiation for a long period. When applied to rat skin for four consecutive days, these solutions led to the appearance of erythema, edema, and vesicles, followed by epidermis proliferation, stratum corneum hyperkeratosis, and the presence of inflammatory infiltrates<sup>3</sup>.

Data concerning the toxicity of LS solutions are scarce, and there is no information about possible pathways involved in the toxic outcomes. Considering that humans are inadvertently exposed to these solutions through oral and dermal routes, we evaluated their toxicity in a human HepG2 cell line and a normal human dermal fibroblast (NHDF) culture. The *in vitro* reactivity of LS solutions towards dGuo and isolated DNA was also examined to gain insight into possible mechanisms of toxicity.

## Results

**Chemical Characterization of the Light Stick Solutions.** The LS solutions were characterized by HPLC/PDA-ESI-MS/MS to identify the PAH, oxalate ester, and alkyl phthalate present in the LS formulation. The main components were identified by HPLC-ESI-MS/MS as follows: (i) “internal” solution – di-*n*-butyl phthalate ( $[M+H]^+$   $m/z$  279; fragments:  $m/z$  149 as base peak,  $m/z$  121,  $m/z$  93, and  $m/z$  59), bis(2,4,6-trichlorophenyl)oxalate (TCPO) ( $[M+H]^+$   $m/z$  223/225/227/229 corresponding, respectively, to the presence of  $3Cl^{35}/2Cl^{35}+1Cl^{37}/1Cl^{35}+2Cl^{37}/3Cl^{37}$  in the molecule, which is an ESI source fragment of TCPO [nominal mass 446 Da]; fragments:  $m/z$  167 as base peak,  $m/z$  195, and  $m/z$  182), and 9,10-diphenylanthracene (DPA) ( $[M+H]^+$   $m/z$  330; fragments:  $m/z$  252 as base peak and  $m/z$  313); (ii) external solution – dimethyl

phthalate ( $[M+H]^+$   $m/z$  195; fragments:  $m/z$  163 as base peak,  $m/z$  133,  $m/z$  135, and  $m/z$  105). All mass spectra were consistent with the NIST Standard Reference Database<sup>10</sup>. Although not analyzed here, hydrogen peroxide is expected to be the oxidant present in the external solution, along with a general base catalyst that may be sodium or tetrabutylammonium salicylate<sup>11</sup>.

Analysis of the “beach” LS solution revealed the presence of additional compounds that were absent from the freshly “fluorescent” solution (Figure 2). As these compounds may be the result of secondary reactions after a long period of sunlight exposure, we analyzed the “fluorescent” solution exposed to sunlight for periods of four, six, and ten days. As shown in Figure 2, new compounds emerge in the sunlight-exposed solutions, and the DPA was consumed.

**Cytotoxicity.** Data regarding cytotoxicity in HepG2 cells and NHDF are presented on Tables 1 and 2. Except for the “external” solution, the lowest concentration tested with each LS solution (0.0125%) was found to significantly induce HepG2 cell death, which increased in a concentration-dependent manner. However, cell membrane damage leading to LDH release occurred only with the highest concentrations tested (0.05% and 0.12%). The collapse of the HepG2 mitochondrial respiratory chain activity (XTT assay) was observed after exposure to the lowest concentrations of all tested solutions, with the exception of the “external” sample. Conversely, a significant increase in mitochondrial respiratory chain activity was observed in HepG2 cells incubated with the lowest concentrations of the “external” solution, which was followed by the concentration-dependent loss of cell viability as measured by the XTT assay.

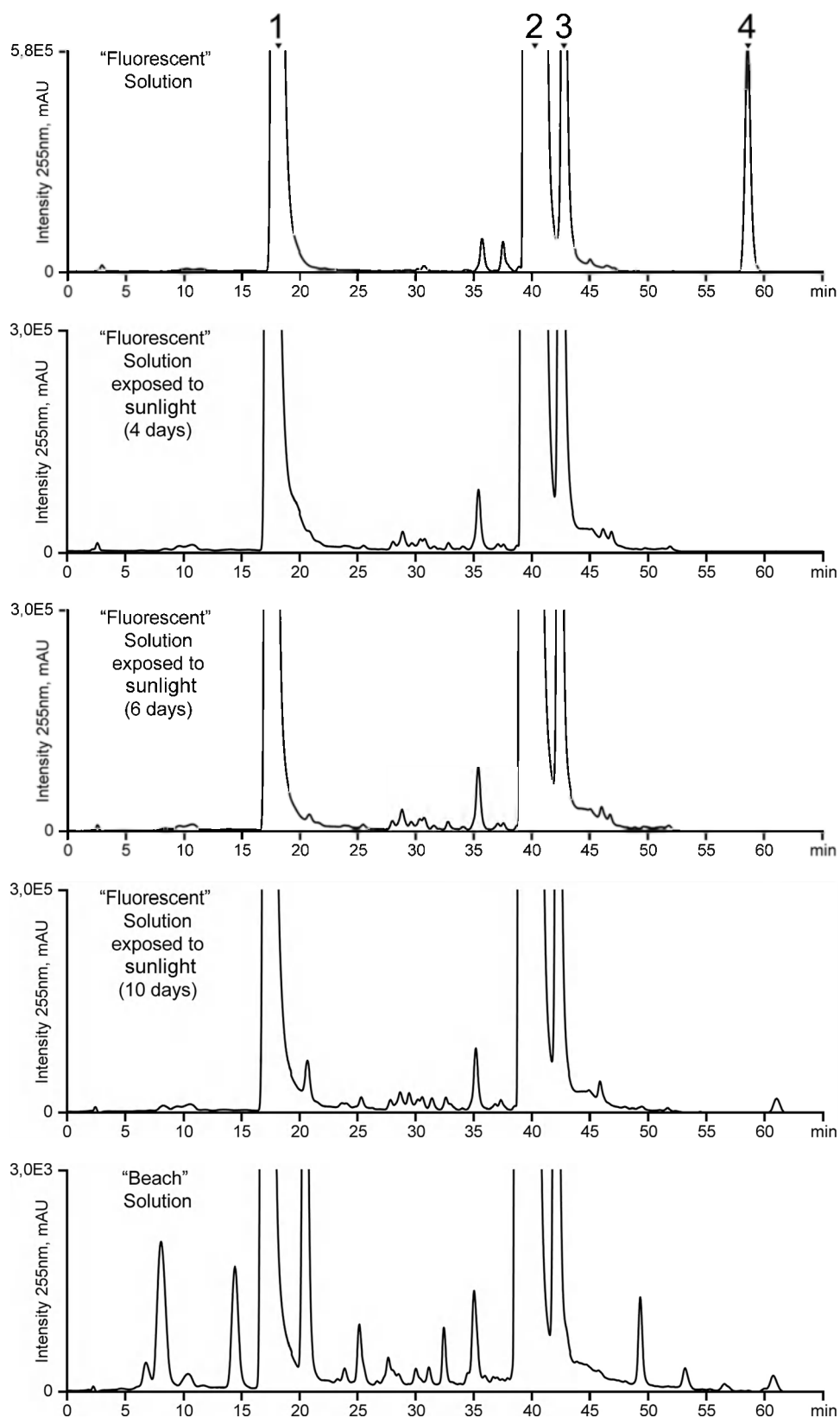
NHDF were very sensitive to all of the LS solutions tested, as measured by the CVD assay. The XTT assay also revealed the loss of cell viability in a concentration-dependent manner for all solutions. However, taking into account the IC<sub>50</sub> value in the XTT assay, the “external” solution was less toxic to NHDF than the other solutions tested, reiterating the trend observed with the HepG2 cells.

**Cell Cycle.** Data regarding the cell cycle changes in HepG2 cells and NHDF are presented on Tables 3 and 4. All solutions induced changes in the HepG2 cell cycle. A decrease in the fraction of the G0/G1 cell population and an increase in the S phase population were observed as the LS concentration increased. The “internal” solution also led to a significant increase in fragmented DNA.

The fibroblasts also exhibited changes in the different cell cycle phases upon incubation with the LS solutions. Predominantly, the highest concentration tested lessened the number of cells in G0/G1 phase and increased the number in G2/M (“external”) or S (“beach” and “fluorescent”) phase. Lower concentrations led to distinct effects by the “external” and “fluorescent” solutions. Exposure to the “internal” and “fluorescent” solutions led to augmented DNA fragmentation. Given the severe cell damage induced by the “internal” solution, cell cycle data for the cells incubated with 0.05% of this solution could not be obtained.

**DNA Damage in HepG2 Cells.** A non-cytotoxic LS concentration (0.003%), as assessed by the XTT and CVD assays, was chosen for these studies. Both the “beach” and “fluorescent” solutions induced the formation of the mutagenic lesion 1,N<sup>6</sup>-*ed*Ado in cell DNA (levels of 1,N<sup>6</sup>-*ed*Ado/10<sup>9</sup> deoxynucleosides, N = 4, mean ± SD: control, 1.11 ± 0.42; “internal”, 2.53 ± 2.24; external, 0.61 ± 0.14; “beach”, 3.69 ± 0.37; “fluorescent”, 3.38 ± 0.89). The statistical significance between groups was assessed using the Kruskal-Wallis test ( $p = 0.0061$ ).

**Reactivity with dGuo and DNA *in vitro*.** LS solutions were examined to verify direct reactions with biomolecules. *In vitro* incubations of dGuo with each LS solution revealed the presence of a reactive electrophile in the “beach” solution that reacts with dGuo, yielding the pair of diastereoisomeric adducts named 9-(4-



**Figure 2** | Chromatograms obtained by HPLC/PDA ( $\lambda = 255$  nm) of “beach” and “fluorescent” solutions following 4 to 10 days of sunlight exposure. The main constituents of the LS solutions were the following: (1) dimethyl phthalate, (2) di-*n*-butyl phthalate, (3) bis(2,4,6-trichlorophenyl)oxalate (TCPO), and (4) 9,10-diphenylanthracene (DPA).

hydroxy-5-hydroxymethyl-oxolan-2-yl)-2-((1,2-dichloro-3-hydroxybutyl) amino)-1,9-dihydro-purin-6-one (Figure 3). The same adducts were not detected when dGuo was incubated with the “internal,”

“external,” or “fluorescent” LS solutions. Adduct formation occurred rapidly within the first 2 h of incubation and then more slowly for up to 24 h of incubation (Figure 3). Aliquots of 12 out of



Table 1 | HepG2 cell line survival after 16 hours of exposure

Groups	Concentration	Crystal Violet Assay			XTT Assay			LDH Assay	
	% in medium	Mean (%) ± S.D.	p value	IC <sub>50</sub>	Mean (%) ± S.D.	p value	IC <sub>50</sub>	Mean (%) ± S.D.	p value
Control	-	100.0 ± 21.3	-	-	100.00 ± 16.7	-	-	8.6 ± 3.4	-
Internal Solution	0.0125	35.6 ± 6.3	p = 0.0065	<0.0125%	60.2 ± 13.7	p = 0.0042	0.0338%	11.4 ± 3.2	p = 0.0085
	0.0250	38.2 ± 7.9			61.3 ± 7.9			12.9 ± 2.8	
	0.0500	31.8 ± 8.3			54.7 ± 2.7			22.9 ± 9.8	
	0.1200	15.6 ± 7.7			20.9 ± 7.2			37.5 ± 6.0	
External Solution	0.0125	87.2 ± 2.4	p = 0.0019	0.0636%	120.8 ± 5.3	p = 0.0034	>0.1200%	6.7 ± 1.4	p = 0.0063
	0.0250	77.7 ± 7.8			121.6 ± 10.2			5.2 ± 1.8	
	0.0500	54.9 ± 12.9			112.3 ± 4.1			10.9 ± 1.1	
	0.1200	32.2 ± 5.9			65.9 ± 6.4			13.6 ± 1.3	
Beach Solution	0.0125	52.9 ± 12.6	p = 0.0014	0.0119%	66.8 ± 18.8	p = 0.0029	0.0177%	8.7 ± 1.4	p = 0.0048
	0.0250	29.4 ± 6.5			33.6 ± 11.5			10.2 ± 1.3	
	0.0500	18.0 ± 4.7			11.8 ± 3.6			14.8 ± 1.3	
	0.1200	10.8 ± 5.1			2.4 ± 0.2			31.6 ± 6.8	
Fluorescent Solution	0.0125	38.6 ± 12.5	p = 0.0044	<0.0125%	94.5 ± 22.6	p = 0.0049	nc	9.1 ± 0.7	p = 0.0116
	0.0250	26.8 ± 12.0			52.3 ± 11.7			8.8 ± 3.7	
	0.0500	20.2 ± 8.3			0.0 ± nc			19.5 ± 7.4	
	0.1200	7.1 ± 5.5			0.0 ± nc			24.1 ± 3.9	

nc, not calculated.

20 randomly selected “beach” LS (60%) yielded the same pair of adducts at distinct concentrations upon incubation with dGuo (data not shown).

Mass spectra of the purified pair of adducts were traced for structure elucidation to identify the source of the reactive electrophile in the “beach” LS. The full adduct mass spectrum is shown in Figure 4, where the protonated molecular ions  $[M+H]^+$  with  $m/z$  408/410/412 and the main fragment ions  $[M-dR+H]^+$  with  $m/z$  292/294/296 were found. The observed  $m/z$  408/410/412 and  $m/z$  292/294/296 isotopic distributions are 100/70/15 (%) and 100/65/10 (%), respectively, in accordance with the theoretical values 100/63.96/10.23 (%) for the presence of two chlorine atoms in the molecule. Further structural characterization was obtained through the collision-induced dissociation of the  $m/z$  408, 410, 292, 294, 274, and 246 ions, as depicted in Figure 4. The adduct and fragment structures are illustrated in Figure 5.

In vitro incubation of DNA with the “beach” LS solution was performed to verify whether the nucleic acid can also be a target of the same reactive electrophile. The adduct dGuo-LS was detected by HPLC-ESI-MS/MS (MRM) after enzymatic hydrolysis of the damaged DNA (Figure 6).

## Discussion

As stated in LS patents, the main ingredients for peroxyoxalate chemiluminescence comprise an oxalate ester (10–1,500 mM), a fluorescer (PAHs, 1–10 mM), a peroxide (anhydrous hydrogen peroxide, 200–15,000 mM), and a catalyst (salicylate derivative, 0.1–1 mM). The reactants are separated in a two-component system consisting of (1) oxalate + fluorescer and (2) hydrogen peroxide + catalyst. Suitable viscous solvents for the two LS compartments are mainly di-*n*-butyl- and dimethyl phthalate<sup>11</sup>. LS solutions toxicity to human cell cultures was expected due to their high concentrations of aromatic and chlorinated reactants and products. In fact, this work presents evidence of high toxicity, as LS solutions diluted to 0.0125% (v/v) in culture media induced both HepG2 and NHDF death. Previous studies on the toxicity of “beach” LS solutions using distinct experimental designs and organisms produced similar results, such as EC<sub>50</sub> values of 0.0285% for sea urchin embryos<sup>12</sup> and 0.0063% for *Artemia*<sup>13</sup>.

The “fluorescent” and “beach” solutions were found to be more toxic to HepG2 cells and NHDF than the “internal” and “external” solutions (XTT assay), even using the original LS formulation in the

Table 2 | Normal human dermal fibroblast survival after 16 hours of exposure

Groups	Concentration	Crystal Violet Assay			XTT Assay		
	% in medium	Mean (%) ± S.D.	p value	IC <sub>50</sub>	Mean (%) ± S.D.	p value	IC <sub>50</sub>
Control	-	100 ± 17.6	-	-	100.0 ± 9.8	-	-
Internal Solution	0.0125	30.4 ± 6.9	p = 0.0045	<0.0125%	87.8 ± 15.4	p = 0.0063	0.0306%
	0.0250	25.4 ± 13.0			29.4 ± 3.8		
	0.0500	15.5 ± 7.8			23.6 ± 14.0		
	0.1200	3.2 ± 1.9			32.5 ± 13.7		
External Solution	0.0125	25.5 ± 2.2	p = 0.0094	<0.0125%	79.6 ± 10.4	p = 0.0016	0.0468%
	0.0250	27.8 ± 15.9			51.9 ± 6.3		
	0.0500	9.5 ± 7.7			53.9 ± 6.3		
	0.1200	10.5 (n=2) ± nc			31.2 ± 6.7		
Beach Solution	0.0125	25.7 ± 6.6	p = 0.0085	<0.0125%	53.4 ± 2.1	p = 0.0021	0.0122%
	0.0250	21.1 ± 4.9			36.9 ± 2.3		
	0.0500	18.3 ± 7.2			28.5 ± 6.2		
	0.1200	7.9 ± 2.3			24.2 ± 3.6		
Fluorescent Solution	0.0125	30.8 ± 28.6	p = 0.0383	<0.0125%	42.3 ± 0.7	p = 0.0053	<0.0125%
	0.0250	9.3 ± 6.4			38.1 ± 4.8		
	0.0500	11.5 ± 6.1			34.6 ± 15.9		
	0.1200	9.9 ± 12.9			6.3 ± 2.6		

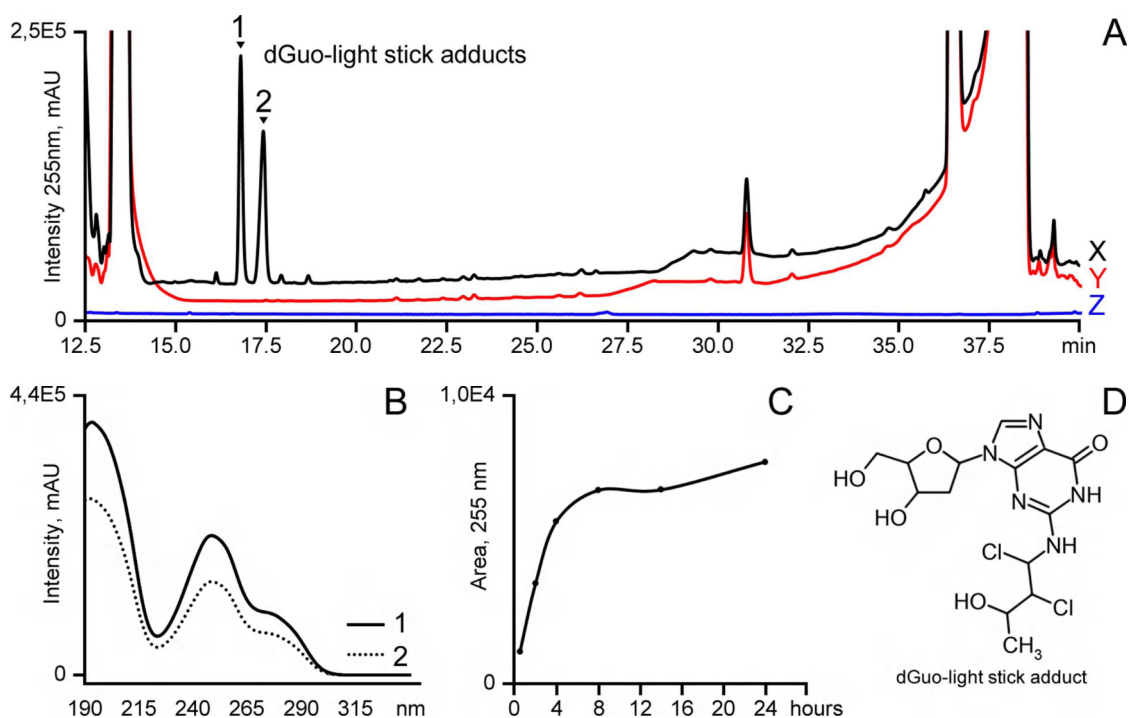


Table 3 | HepG2 cell cycle and DNA fragmentation

Groups	Concentration		Cell Cycle		SubG1		
	% in medium		G0/G1	S	G2/M	% of cells with fragmented DNA	p value
	Mean (%) ± S.D.	p value	Mean (%) ± S.D.	p value	Mean (%) ± S.D.	p value	
Control	68.5 ± 1.8	-	24.9 ± 1.3	-	6.5 ± 2.6	-	3.2 ± 0.5
Internal Solution	75.9 ± 4.6	-	17.9 ± 3.2	-	6.1 ± 2.0	-	5.0 ± 0.5
Solution	60.3 ± 3.7	p = 0.0216	27.0 ± 3.4	p = 0.0378	12.6 ± 0.8	p = 0.0534	6.0 ± 0.8
External	55.9 ± 2.9	-	33.2 ± 4.7	-	11.1 ± 2.4	-	7.7 ± 1.0
Solution	70.5 ± 1.2	p = 0.0216	22.9 ± 0.1	p = 0.0188	6.6 ± 1.3	p = 0.0534	3.4 ± 0.3
Solution	74.5 ± 1.6	-	16.8 ± 1.2	-	8.6 ± 1.0	-	3.5 ± 0.2
Beach	61.0 ± 2.0	-	27.9 ± 1.5	-	11.0 ± 0.5	-	3.0 ± 0.5
	73.9 ± 0.9	-	18.3 ± 1.7	-	7.7 ± 2.6	-	3.2 ± 0.4
	53.8 ± 3.4	p = 0.0216	35.5 ± 3.1	p = 0.0237	10.7 ± 1.3	p = 0.2181	2.6 ± 0.2
	59.1 ± 6.2	-	33.0 ± 5.1	-	7.8 ± 2.1	-	4.8 ± 1.6
Fluorescent Solution	64.2 ± 2.7	p = 0.0330	25.7 ± 1.5	p = 0.0378	10.0 ± 2.2	p = 0.2228	4.0 ± 0.4
Solution	58.8 ± 1.1	-	34.1 ± 1.3	-	7.0 ± 1.5	-	3.6 ± 0.7
	58.5 ± 4.6	-	33.6 ± 3.3	-	7.8 ± 1.3	-	5.5 ± 1.0

Table 4 | Normal human dermal fibroblast cell cycle and DNA fragmentation

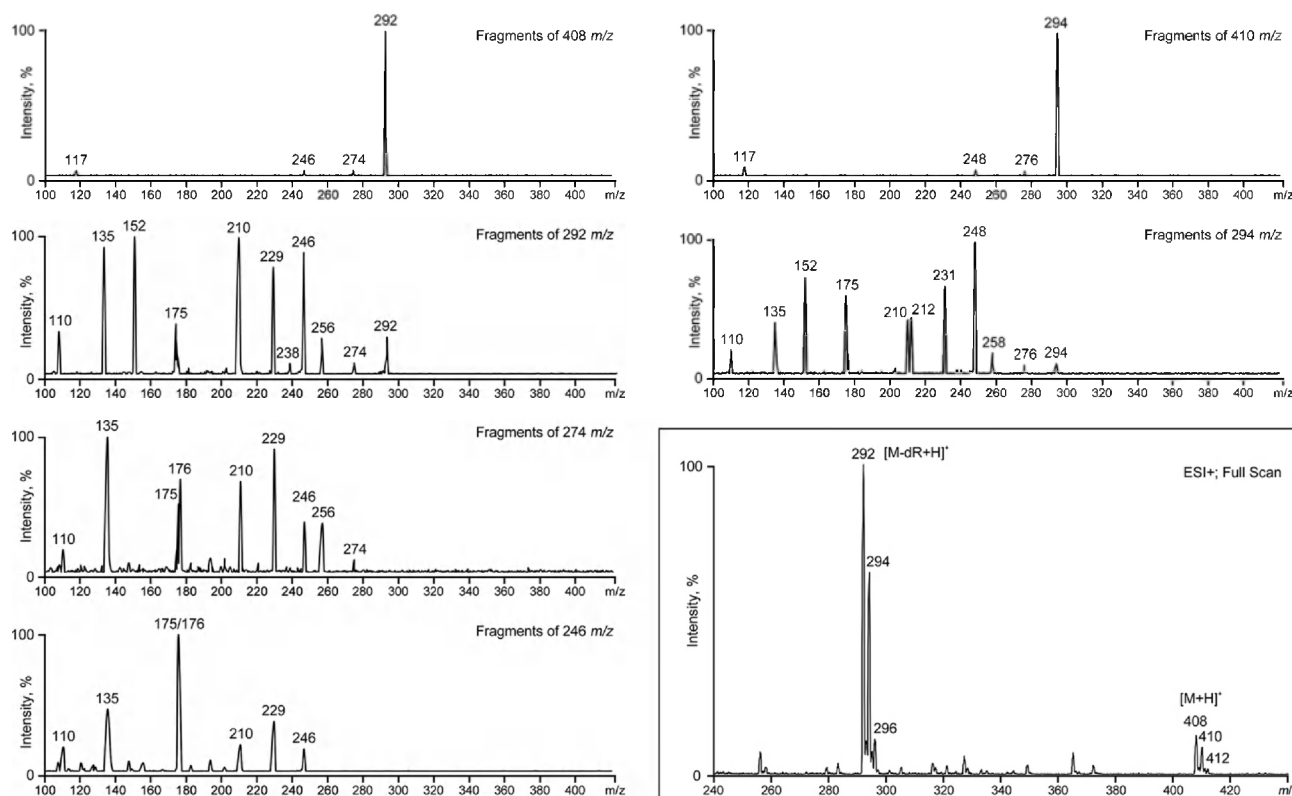
Groups	Concentration		Cell Cycle		SubG1		
	% in medium		G0/G1	S	G2/M	% of cells with fragmented DNA	p value
	Mean (%) ± S.D.	p value	Mean (%) ± S.D.	p value	Mean (%) ± S.D.	p value	
Control	56.8 ± 1.2	-	23.6 ± 1.2	-	19.6 ± 0.4	-	1.9 ± 0.4
Internal Solution	62.6 ± 3.4	-	26.8 ± 2.7	-	10.6 ± 0.7	-	5.0 ± 1.5
Solution	56.3 ± 5.3	p = 0.1184	26.3 ± 3.2	p = 0.2881	17.3 ± 2.2	p = 0.0273	10.5 ± 0.8
External Solution	57.8 ± 0.4	-	31.5 ± 1.9	-	13.5 ± 2.7	-	4.0 ± 1.0
Solution	59.0 ± 5.4	p = 0.0752	33.1 ± 4.1	p = 0.0329	14.4 ± 1.7	p = 0.0249	4.3 ± 1.5
Beach	46.2 ± 2.3	-	21.8 ± 1.8	-	30.0 ± 1.3	-	4.2 ± 0.1
	54.9 ± 4.3	-	31.7 ± 0.1	-	10.4 ± 0.5	-	2.3 ± 0.4
	52.4 ± 3.8	p = 0.0216	28.4 ± 1.0	p = 0.0434	12.5 ± 6.3	p = 0.0627	2.2 ± 1.2
Fluorescent Solution	48.1 ± 2.0	-	32.7 ± 4.0	-	21.0 ± 3.0	-	7.9 ± 7.6
Solution	66.3 ± 4.2	p = 0.0752	24.8 ± 3.4	p = 0.0249	8.8 ± 1.0	p = 0.1129	2.0 ± 0.2
	61.9 ± 3.0	-	16.4 ± 1.0	-	21.5 ± 2.1	-	2.9 ± 0.4
	44.9 ± 5.3	-	34.3 ± 1.6	-	20.7 ± 3.8	-	7.6 ± 5.4



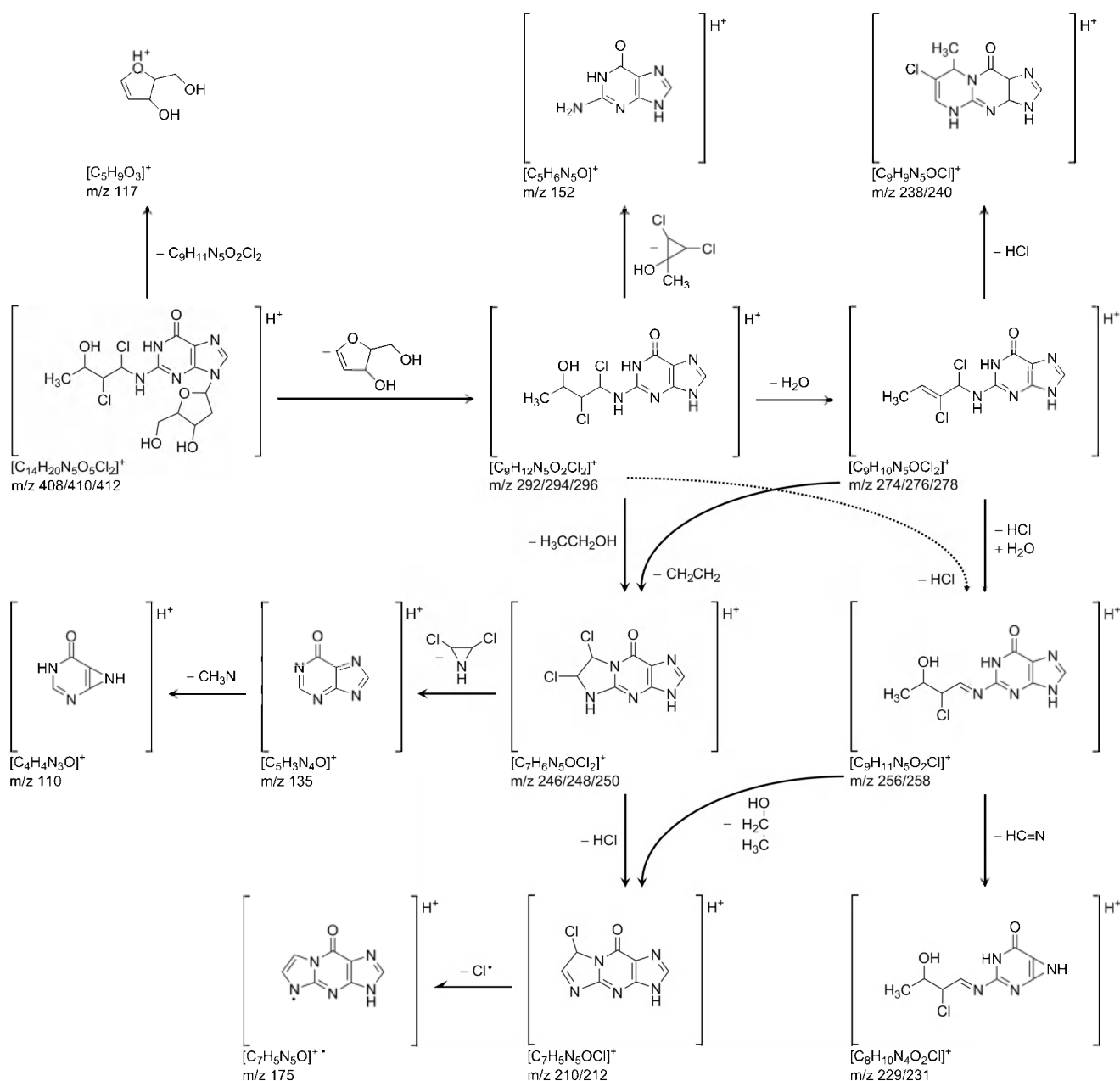
**Figure 3** | (A) *In vitro* adduct formation from the reaction of dGuo with “beach” solution (X, complete solution; Y, “beach” solution; Z, dGuo solution). Chromatograms were obtained by HPLC/PDA; (B) Absorbance spectra of the pair of diastereoisomeric adducts obtained by HPLC/PDA; (C) Adduct formation as a function of time (0.5–24 h); (D) dGuo-LS adduct structure based on Figure 5.

4:1 (“external” : “internal”) solutions. On the beach, the reaction products, catalyst, and solvent exposed to sunlight for long periods of time may greatly contribute to the observed increase in LS toxicity. Because

humans are mainly exposed to the “fluorescent” and “beach” LS contents, care must be taken when attempting to extrapolate toxicity data from the initial components to the spent and sun-exposed mixtures.



**Figure 4** | Mass spectra (full scan and MS<sub>2</sub>) of the dGuo-LS adduct obtained by the collision-induced dissociation of  $m/z$  408, 410, 292, 294, 274, and 246 ions. A collision energy of 10 eV was used.

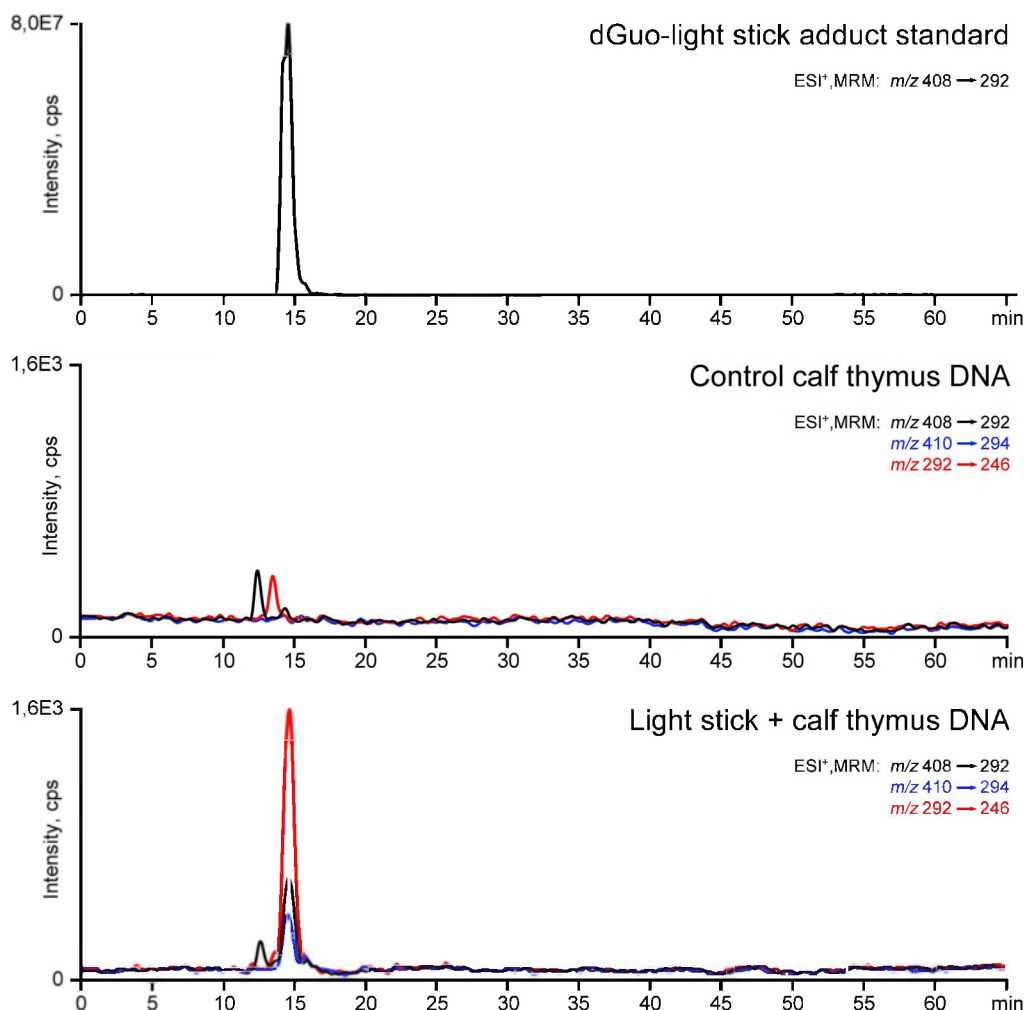


**Figure 5** | Suggested structure and fragmentation pathway for the dGuo-LS adduct.

When external and internal LS solutions are mixed, TCPO reacts with hydrogen peroxide, yielding 2,4,6-trichlorophenol and the highly energized 1,2-dioxetanodione intermediate, whose DPA-activated decomposition to  $\text{CO}_2$  places the fluoroscer in the fluorescent state. The chlorinated product 2,4,6-trichlorophenol is listed as reasonably anticipated to be a human carcinogen by the US National Toxicology Program<sup>14</sup>, as a probable human carcinogen (group B2) by the US Environmental Protection Agency<sup>15</sup>, and as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer<sup>16</sup>. Leukemia, lymphoma, leukocytosis, monocytosis, and bone marrow hyperplasia were induced in Fischer 344 rats, and hepatocellular adenoma and carcinoma were provoked in B6C3F1 mice after a two-year dietary exposure to high concentrations of 2,4,6-trichlorophenol (5000 and 10000 ppm)<sup>17,18</sup>. The genotoxicity of 2,4,6-trichlorophenol has also been evaluated in various *in vitro* and *in vivo* systems. Mutagenic responses were verified in different *Salmonella* Typhimurium strains (5 or 25  $\mu\text{g}/\text{mL}$ , in the presence of S9) and in the *tk* locus of mouse lymphoma L5178Y cells

(80  $\mu\text{g}/\text{mL}$ , without S9)<sup>19,20</sup>. Additionally, chromosomal aberrations, aneuploidy, and micronucleus induction were detected in Chinese hamster lung V79 cells (30 or 400  $\mu\text{g}/\text{mL}$ , without S9)<sup>21,22</sup>. Furthermore, increased micronuclei frequencies in peripheral erythrocytes were observed following the exposure of Japanese medaka (*Oryzias latipes*) to 2,4,6-trichlorophenol (0.01 to 1  $\mu\text{g}/\text{mL}$ ) for 28 days<sup>23</sup>.

Based on the LS patented formulations of oxalate ester concentrations, the cells used herein were exposed to 0.5–800  $\mu\text{g}/\text{mL}$  TCPO when the “internal” solution was tested, considering the lowest and highest reported concentrations. The concentration of TCPO in the “fluorescent” solution was 5 times diluted due to the high ratio of “external” : “internal” solutions, and its oxidative cleavage to 2,4,6-trichlorophenol must be considered. Human exposure to LS solutions usually occurs without any additional dilution. As chlorophenols are promptly absorbed through oral, dermal, and inhalation routes, both local and systemic effects are expected<sup>16</sup>. After absorption, major concentrations of chlorophenols have been reported to be



**Figure 6** | Chromatograms obtained by HPLC-ESI-MS/MS of calf thymus DNA incubated *in vitro* with spent LS solution. The analyses were conducted with multiple reaction monitoring (MRM) using the following fragmentations:  $m/z$  408 >  $m/z$  292,  $m/z$  292 > 246,  $m/z$  410 >  $m/z$  294.

found in the kidney and liver, with excretion occurring mainly in the urine as glucuronide and sulfate conjugates, with half-lives of 10–20 days<sup>16,24</sup>. Additionally, when 2,4,6-trichlorophenol was incubated *in vitro* with rat liver S9 mix, the metabolite 2,6-dichloro-1,4-semiquinone radical was detected. Furthermore, after competitive dismutation and one-electron transfer to molecular oxygen, the semiquinone radical produces the corresponding benzoquinone and reactive oxygen species during the redox cycling process<sup>25</sup>.

The “fluorescent” and “beach” solutions tested herein in a non-cytotoxic concentration (0.003%) led to 1,*N*<sup>6</sup>- $\epsilon$ dAdo formation in HepG2 cell DNA. As the 1,*N*<sup>6</sup>- $\epsilon$ dAdo levels did not increase upon incubation with 0.003% of the “external” or “internal” solutions, the products generated by the chemiluminescent reaction may indeed contribute to oxidative stress. The detected exocyclic DNA adduct is mutagenic and formed by reaction of  $\alpha,\beta$ -unsaturated aldehydes from lipid peroxidation with the adenine in DNA. Currently, 1,*N*<sup>6</sup>- $\epsilon$ dAdo is credited as an important biomarker of oxidative stress<sup>26</sup>. This finding, even after more than 30,000-fold LS dilution, is a clear indicator of the potential cytotoxicity and genotoxicity of these solutions.

Cellular alterations attributed to oxidative stress and reactive electrophiles may be involved in the cell cycle changes observed herein. Generally, a decrease in the number of HepG2 cells in G0/G1 phase and increase in the number of cells in S phase were found in all of the incubations with all of the solutions. These findings may pose an additional threat to the exposed cells, as induction of the replication

of damaged cells favors mutagenesis and cell transformation. The same effect was observed for the “beach,” “fluorescent,” and lowest “external” solution concentrations administered to the fibroblasts. However, the highest “external” solution concentration induced G2/M cell cycle arrest in NHDF cells.

Among chlorophenols, pentachlorophenol has been noted as a liver tumor promoter in mice<sup>27</sup> and as an apoptosis inhibitor in HepG2 cells<sup>28</sup>. Proliferative signaling is also known to be activated under low ROS concentrations, whereas high ROS concentrations lead to cell cycle arrest and death<sup>29</sup>. The chlorinated products present in the “fluorescent” and “beach” solutions, as well as the distinct intracellular ROS concentrations after cell exposure to the different LS solutions, may account for the observed cell cycle changes. Dilution of the compounds present in the “fluorescent” and “beach” solutions compared with the initial LS solution may also be responsible for the diverse effects in NHDF cells.

Phthalates present as solvents of all the solutions tested are additional contributors to cell toxicity<sup>30</sup>. Dilutions of the LS solutions (0.0125%–0.12%) led to phthalate concentrations in the culture medium in the 130–1430  $\mu\text{g}/\text{mL}$  range. Concentrations of di-*n*-butyl phthalate above 100  $\mu\text{g}/\text{mL}$  are reportedly cytotoxic to MVLN cells derived from the MCF-7 breast cancer cell line<sup>30</sup>. Growth inhibition, G1 cell cycle arrest, increased levels of the apoptotic gene *Bid*, and decreased levels of the antiapoptotic gene *Bcl2* were reported in mouse ovarian antral follicles exposed to 1000  $\mu\text{g}/\text{mL}$  of di-*n*-butyl phthalate for 24 or 168 h<sup>31</sup>. Objections to human phthalate exposure





due to the potential health risks have increased<sup>30</sup>. Accordingly, endocrine disruption, impairment of normal human development, induction of respiratory and allergic diseases in children, and liver and testes tumors in rats have been related to phthalate exposure<sup>30,32</sup>. Considering the reported adverse health outcomes, the US Consumer Product Safety Improvement Act (CPSIA) banned the use of butylbenzyl phthalate, di-2-ethylhexyl phthalate, and di-*n*-butyl phthalate in children's toys and child care articles<sup>32</sup>. It is important to note that LS are available at toy, sporting goods, camping, emergency, and party goods stores.

Another relevant finding of our study is that a reactive electrophile is present in LS collected on the beaches. When different "beach" LS solutions were incubated with dGuo, a pair of diastereoisomeric adducts was formed promptly after incubation. Adduct characterization through ESI-MS/MS revealed a four-carbon side chain holding two chlorine atoms and one hydroxyl group covalently bound to dGuo (Figure 5). The same pair of adducts was also detected in DNA incubated with an aliquot of a "beach" LS solution (Figure 6). These data clearly show that several "beach" LS solutions contain genotoxic materials. Additionally, these products may also attack nucleophilic moieties in proteins, RNA, and lipids. Taking into account the LS components, TCPO is considered the main source of the chlorinated electrophile. The photodegradation of 2,4,6-trichlorophenol produces several oxidized intermediates, including aromatic ring cleavage products<sup>33,34</sup>. Solar light and high H<sub>2</sub>O<sub>2</sub> concentrations in LS may be involved in the degradation process through the generation of hydroxyl radicals. Although these degradation products were not directly identified in the "beach" LS solutions, we verified that several additional products were present in these spent solutions (Figure 2). The direct reaction of the electrophile with biomolecules, in addition to generating ROS, may increase the risk of cell transformation in subjects who are inadvertently exposed to spent LS.

In conclusion, we observed herein the high toxicity of LS solutions in two cell types. Even when the "beach" or "fluorescent" LS were diluted 30,000 times, albeit not driving cell cytotoxicity, the levels of the putative mutagenic lesion 1, N<sup>6</sup>-edAdo were increased in HepG2 cell DNA, indicating the occurrence of oxidative stress. To the best of our knowledge, this report is the first to show the presence of reactive electrophiles in LS "beach" solutions prone to promptly reacting with electron-rich groups of biopolymers such as the amino groups of proteins and nucleic acids. These findings demand the assessment of the mutagenic activity of these solutions. Additional studies are necessary to identify the reactive electrophiles and conditions that favor their formation, although the dGuo-LS adduct identified herein indicates that the main electrophile is chlorinated. Therefore, it can be derived from the chlorophenyl leaving group of the chemiluminescent oxalate ester when reacting with hydrogen peroxide.

Altogether, these findings reinforce the conclusion that fresh or spent LS solutions are hazardous and that the health risks associated with exposure have not yet been properly evaluated. An urgent need exists to adequately manage the discarded LS and to foster awareness about the health risks that may result from exposure.

## Methods

**Chemicals.** Chromatography grade 2-propanol, acetonitrile, and methanol were obtained from Merck (Germany). The cytotoxicity assay kits were purchased from Xenometrix (Switzerland). The DNA extraction solutions were obtained from QIAGEN (Valencia, CA). The culture media, FCS, and trypsin/EDTA were acquired from Vitrocell – Embriolife (Brazil). All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO).

**DNA Adduct Standards.** The isotopic standard [<sup>15</sup>N<sub>5</sub>]1, N<sup>6</sup>-edAdo was obtained as described by Loureiro *et al.*<sup>35</sup>. Its identity was confirmed by its spectral properties: UV, λ<sub>max</sub> 275 nm, ε 10300 M<sup>-1</sup>cm<sup>-1</sup>, pH 7.0; positive ESI-MS: *m/z* 281 [M + H]<sup>+</sup>, *m/z* 165 [M - 2'-deoxyribose + H]<sup>+</sup>.

**Light Stick Solutions.** Spent LS were collected on Brazilian beaches by the NGO Global Garbage and kindly donated to our group. These solutions were named "beach". New LS (Firefly™, 6" Light Stick, 15 × 165 mm, Y-Green, Bandi CO. LTD,

Korea) were carefully opened, and the "external" and "internal" solutions were separated. Another LS sample of the same brand was folded to start the chemiluminescent reaction. After a few minutes, the "fluorescing" solution was collected. All solutions were stored in polypropylene tubes and kept at -20°C until use.

**HPLC-UV-ESI-MS/MS Systems.** **System 1** – A Quattro II mass spectrometer (Micromass, U.K.) coupled to an HPLC system (Shimadzu, Japan). Mass spectra in the positive mode were obtained in the 100–800 *m/z* range, with cone voltages of 15–50 V. Collision-induced dissociation was obtained with 1 × 10<sup>-6</sup> mBar argon pressure in the collision cell and a collision energy of 10 eV. Simultaneously, compounds were detected at 255 nm. The data were processed using MassLynx software (Micromass). The following chromatographic condition was used for the chemical characterization of the LS solutions: a 150 × 2.0 mm i.d., 3.0 μm, Luna C18(2) column (Phenomenex, Torrance, CA) was eluted with a gradient of 0.1% formic acid in water (A) and acetonitrile (B) at a 120 μL/min flow rate and 25°C as follows: from 0 to 60 min, 5–100% of B.

**System 2** – A linear Quadrupole Ion Trap mass spectrometer, Model 4000 QTRAP (Applied Biosystems/MDS Sciex Instruments, Foster City) coupled to an Agilent 1200 series HPLC system (Wilmington, DE, USA) was used for 1, N<sup>6</sup>-edAdo quantitation. The analyses were performed with multiple reaction monitoring (MRM; *m/z* 276 → *m/z* 160 and *m/z* 281 → *m/z* 165) for the detection of 1, N<sup>6</sup>-edAdo and [<sup>15</sup>N<sub>5</sub>]1, N<sup>6</sup>-edAdo, respectively. The ESI-MS parameters were set in the positive ion mode as follows: CUR, 20 psi; CAD, medium; IS, 4500 V; temperature, 450°C; GS1, 55 psi; GS2, 40 psi; and EP, 10 V. Calibration curves were constructed for the interval of 0.5 to 80 fmol of 1, N<sup>6</sup>-edAdo, with fixed amounts of [<sup>15</sup>N<sub>5</sub>]1, N<sup>6</sup>-edAdo (200 fmol). The data were processed using Analyst software 1.6 (Applied Biosystems/MDS Sciex). The chromatographic condition involved a 150 × 2.0 mm i.d., 3.0 μm, Luna C18(2) column (Phenomenex, Torrance, CA) eluted with a gradient of 5 mM ammonium acetate, pH 6.6 (A) and acetonitrile (B) at a flow rate of 130 μL/min and 25°C as follows: from 0 to 10 min, 0% B; 10 to 39 min, 0–20% B; 39 to 41 min, 20–75% B; 41 to 46 min, 75% B; 46 to 47 min, 75–0% B; and 47 to 60 min, 0% B. The first 35 min of eluent was directed to waste, and the 35–42 min fraction was diverted to the ESI source.

**Chemical Characterization of Light Stick Solutions.** Aliquots of the solutions were diluted 30 times in acetonitrile, and 10 μL of the diluted solutions were subsequently injected into the HPLC-UV-ESI-MS/MS system 1.

**HepG2 and Normal Human Dermal Fibroblasts Cultures.** The human hepatocellular carcinoma cell line HepG2 was kindly provided by Professor Mari C. Sogayar (University of São Paulo, Brazil), who purchased the cells from ATCC® (HB-8065™). Primary normal human dermal fibroblasts (NHDF) were kindly provided by Professor Silvyia Stuchi Maria-Engler (University of São Paulo, Brazil), according to CEP/FCF/169/2009.

The HepG2 culture was maintained in DMEM (15% FCS, 10 U/mL penicillin, and 0.1 mg/mL streptomycin). The cells were incubated at 37°C in atmosphere containing 5% CO<sub>2</sub>. The NHDF culture was used until the 15th passage. The cells were grown at 37°C and 5% CO<sub>2</sub> in DMEM containing 4 mM L-glutamine, 10% FCS, 25 mg/mL ampicillin, and 100 mg/mL streptomycin.

**Cytotoxicity Screening.** Mitochondrial respiratory chain activity (XTT assay), cell survival (using crystal violet dye - CVD), and plasma membrane integrity (lactate dehydrogenase assay - LDH) were evaluated using In Cytotox kits from Xenometrix. The cells were exposed to 0.0125%, 0.025%, 0.05%, or 0.12% of each LS solution in complete culture medium.

Cells were plated at a density of 5 × 10<sup>4</sup> cells/well in 96-well plates. After 24 h, the culture medium was replaced with medium containing 0.0125–0.12% of each LS solution. Cell exposure lasted for 16 h. Cytotoxicity tests were performed according to the manufacturer's instructions (Xenometrix). Absorbance was measured with an ELISA reader (Power Wave X340, Bio-Tek Instruments). Optical density readings were normalized to the control cells.

**XTT Assay** – The cells were carefully washed twice with PBS, and 200 μL of fresh culture medium (FCS, 5%) and 50 μL of XTT reagent were added to each well. The cells were incubated for 1 h at 37°C. The absorbance values were read at 540/690 nm.

**CVD Assay** – The cells were washed twice with PBS to remove non-adherent cells, and 100 μL CVD solution was added to each well for cell staining (10 min). The wells were then washed with water (200 μL, 4x), and the dye was dissolved with a solubilization solution (100 μL/well). The absorbance values were read at 540/690 nm.

**LDH Assay** – The culture media (20 μL) was incubated with a solution containing NADH and pyruvate (5:1, 240 μL). The absorbance measurement was immediately started at 340 nm in kinetic mode at 37°C, with readings every 20 sec over 25 min. The results are expressed as the percentage of the maximum consumption rate of NADH (NADH/min), which was obtained using a positive control (Triton X-100, 1%).

**Cell Cycle Analyses.** Cells were plated at a density of 5 × 10<sup>5</sup> cells/well in 24-well culture plates. After 24 h, the culture medium was replaced by new medium containing 0.0125% to 0.12% of each LS solution. After 16 h, the cells were suspended in 1,000 μL of DMEM (phenol red-free) supplemented with 10% FCS. Cell suspensions were centrifuged (600 g, 10 min, 4°C) and suspended in 300 μL of cell lysis buffer (PBS, 2% FCS, 0.05% Triton X-100, 0.1% sodium citrate) containing 20 μg/mL propidium iodide (PI) and 100 μL of RNase A solution (15 mg/mL). After



30 min at room temperature, the fluorescence intensity of the PI bound to DNA was monitored in 10,000 events per sample at  $\lambda_{exc.} = 488$  nm and  $\lambda_{emi.} = 695$  nm in a FACS CANTO II (BD Biosciences).

**DNA Extraction.** HepG2 cells were plated in 150 × 20 mm dishes and grown to a density of  $1 \times 10^7$  cells. The culture medium was replaced by new medium containing 0.003% of each LS solution. Cell exposure occurred for 16 h. Cell DNA was isolated as described in the Puregene Gentra® protocol (QIAGEN). The DNA concentration was determined by UV absorbance at 260 nm.

**DNA Sample Preparation for 1,N<sup>6</sup>- $\epsilon$ -dAdo Analysis.** DNA aliquots (150  $\mu$ g) were transferred to 186.5  $\mu$ L of water containing 7.5  $\mu$ L of 200 mM Tris/MgCl<sub>2</sub> buffer (pH 7.4), 1.4  $\mu$ L of [<sup>15</sup>N<sub>5</sub>]1,N<sup>6</sup>- $\epsilon$ -dAdo internal standard solution (250 fmol/ $\mu$ L), and 6  $\mu$ L (15 units) of DNase I. The samples were incubated at 37°C for 1 h. Then, 6  $\mu$ L (0.006 units) of phosphodiesterase I and 7.5  $\mu$ L (15 units) of alkaline phosphatase were added, followed by further incubation at 37°C for 1 h. Aliquots of 10  $\mu$ L were analyzed by HPLC/PDA for deoxynucleoside quantification. The residual sample volume was submitted to solid phase extraction using SPE-C18 cartridges (30 mg/mL, 33  $\mu$ m, 1 mL; Strata-X, Phenomenex, Torrance, CA). The cartridges were loaded as follows: methanol, water, DNA hydrolysate, water, 10% methanol, 15% methanol, and 100% methanol. The last fraction was then collected. The samples were vacuum-dried and resuspended in 83.1  $\mu$ L of water prior to HPLC-ESI-MS/MS analysis (System 2). The molar fraction of 1,N<sup>6</sup>- $\epsilon$ -dAdo/10<sup>9</sup> deoxynucleosides in each DNA sample was determined.

**Normal Deoxynucleoside Quantitation in DNA Samples.** A Shimadzu (Kyoto, Japan) HPLC/PDA system was used as follows: a 250 mm × 4.6 mm i.d., 5  $\mu$ m, Luna C18(2) column (Phenomenex, Torrance, CA), eluted with a gradient of 0.1% formic acid and CH<sub>3</sub>OH (from 0 to 25 min, 0 to 18% CH<sub>3</sub>OH; from 25 to 27 min, 18 to 0% CH<sub>3</sub>OH; from 27 to 37 min, 0% CH<sub>3</sub>OH) at a 1 mL/min flow rate and 30°C. The PDA detector was set at 260 nm. Calibration curves were constructed at intervals of 1–8 nmol for normal 2'-deoxynucleosides.

**Reaction of dGuo with Light Stick Solutions.** dGuo (1 mg) in 800  $\mu$ L of sodium phosphate buffer (50 mM, pH 7.4) was added to 200  $\mu$ L of each LS solution and incubated at 37°C under agitation. Control incubations were performed in the absence of dGuo or the LS solutions. Aliquots of 100  $\mu$ L of each solution were collected after 0.5, 2, 4, 8, 14, and 24 h and centrifuged (600 g, 5 min), and the aqueous phase was injected into the HPLC-UV-ESI-MS/MS System 1.

**Reaction of DNA with Light Stick Solutions.** Calf thymus DNA (1 mg) in 500  $\mu$ L of sodium phosphate buffer (50 mM, pH 7.4) was added to 100  $\mu$ L of each LS solution and incubated at 37°C for 21 h under agitation. Control incubations were performed in the absence of DNA or LS solutions. The samples were then centrifuged, and the aqueous phase was extracted with 500  $\mu$ L of chloroform. The DNA concentration in the aqueous phase was determined by UV absorbance at 260 nm. DNA aliquots were enzymatically hydrolyzed as described above, without the addition of an internal standard, and submitted to HPLC-ESI-MS/MS (System 1) analysis. The analyses were conducted with multiple reaction monitoring (MRM) using the following fragmentations of the characterized dGuo-LS adduct:  $m/z$  408 [M+H]<sup>+</sup> →  $m/z$  292 [M - 2'-deoxyribose + H]<sup>+</sup>,  $m/z$  410 [M+H]<sup>+</sup> →  $m/z$  294 [M - 2'-deoxyribose + H]<sup>+</sup>, and  $m/z$  292 →  $m/z$  246 [M - 2'-deoxyribose - H<sub>3</sub>CCH<sub>2</sub>OH + H]<sup>+</sup>.

**Statistical analyses.** The data are presented as the mean ± standard deviation (S.D.). The significance of the differences ( $p < 0.05$ ) between groups was assessed using the Kruskal-Wallis test, using the software GraphPad Prism®.

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### Author contributions

E.J.H.B. and A.P.M.L. conceived and designed the project. F.P.B. collected spent LS on Brazilian beaches. T.F.O., A.L.M.S., R.A.M. and R.B. performed the experiments and analyzed the data. I.P.A.C. analyzed the spectroscopic data. A.P.M.L., T.F.O., A.A.F.O. and E.J.H.B. wrote and edited the manuscript. M.H.G.M. and P.D.M. co-wrote the manuscript. A.P.M.L. supervised the project. T.F.O. and A.L.M.S. contributed equally to the work.

### Additional information

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