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Buthionine sulfoximine diverts the melanogenesis pathway toward the production of more soluble and degradable pigments

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ABSTRACT

Buthionine sulfoximine (BSO) is a specific inhibitor of γ-glutamylcysteine synthetase, thus blocking the synthesis of glutathione (GSH). It is known that this makes that BSO affects melanin synthesis because of the role of thiols in melanogenesis. However, BSO may also react with the intermediate oxidation products of melanogenesis, a possibility that has not been investigated from the initial steps of the pathway. We created in vitro conditions simulating eumelanogenesis (oxidation of L-DOPA in the absence of GSH) and pheomelanogenesis (oxidation of L-DOPA in the presence of GSH) under presence or absence of BSO. BSO made that eumelanogenesis results in pigments more soluble and less resistant to degradation by hydrogen peroxide than pigments obtained without BSO. A similar but less marked effect was observed for pheomelanogenesis only at subsaturating concentrations of GSH. These results suggest that BSO diverts the melanogenesis pathway toward the production of more soluble and degradable pigments.

Buthionine sulfoximine (BSO) is a specific inhibitor of γ-glutamylcysteine synthetase, the enzyme that catalyzes the rate-limiting step in the synthesis of glutathione (GSH), where two of its three constitutive amino acids (glutamate and cysteine) are bonded.[1] BSO thus decreases intracellular GSH levels with no side, toxic effects.[2–4] The important antioxidant activity of GSH makes that BSO inhibits the growth of different tumour cell lines and increases their sensitivity to antineoplastic drugs.[5] The inhibitory effect of BSO is particularly high against melanoma-derived cell lines,[6] as melanoma may be dependent on the role of GSH and its linked enzymes in melanin synthesis.[7] However, the effect of BSO on the synthesis of different types of melanin has received little attention. This may have important consequences for understanding possible side effects of BSO use.

The first step in the melanogenesis pathway, catalyzed by the enzyme tyrosinase, is the oxidation of the amino acid L-tyrosine to L-dopaquinone (see Fig. 1) which undergoes an intramolecular cyclization of the amino group to give L-dopachrome via L-cyclodopa, which in turn suffers a redox exchange with L-dopaquinone that produces L-dopachrome and 3,4-dihydroxy-L-phenylalanine (DOPA).[8] In this process, the recruiting of L-DOPA to be re-oxidized to L-dopaquinone is again catalyzed by tyrosinase, so that half of the DOPA oxidized to L-dopaquinone is reduced back to DOPA. L-Dopaquinone is the common precursor of the two connected biosynthetic routes in melanogenesis that lead to the formation of either eumelanin or pheomelanin.[9] In the absence (or below certain concentration) of sulfhydryl groups from thiol compounds such as cysteine or GSH, dopachrome evolves to two dihydroxyindoles, DHICA (5,6-dihydroxyindole-2-carboxylic acid) or DHI (5,6-dihydroxyindole) by tautomerization or decarboxylation, respectively. The resulting DHI/DHICA ratios depend on the level of dopachrome tautomerase activity and/or the presence of some metal ions.[10] DHICA and DHI are further oxidized and polymerized to form eumelanin (Fig. 1). In the presence (or above certain concentration) of sulfhydryl-containing compounds, these conjugate with L-dopaquinone to generate mainly 5-*S*-cysteinyldopa (in the presence of cysteine) or 5-*S*-glutathionyldopa (in the presence of GSH). These and other thiol–DOPA conjugates are further oxidized and polymerized to form pheomelanin[8,9,11] (Fig. 1). Given this biochemical process, the reduction of GSH levels exerted by BSO should decrease pheomelanin production and increase eumelanin production.

However, the effect of BSO on the synthesis of melanins may not be only mediated by the inhibition of γ-glutamylcysteine synthetase and cysteine production, because the S=NH group of BSO

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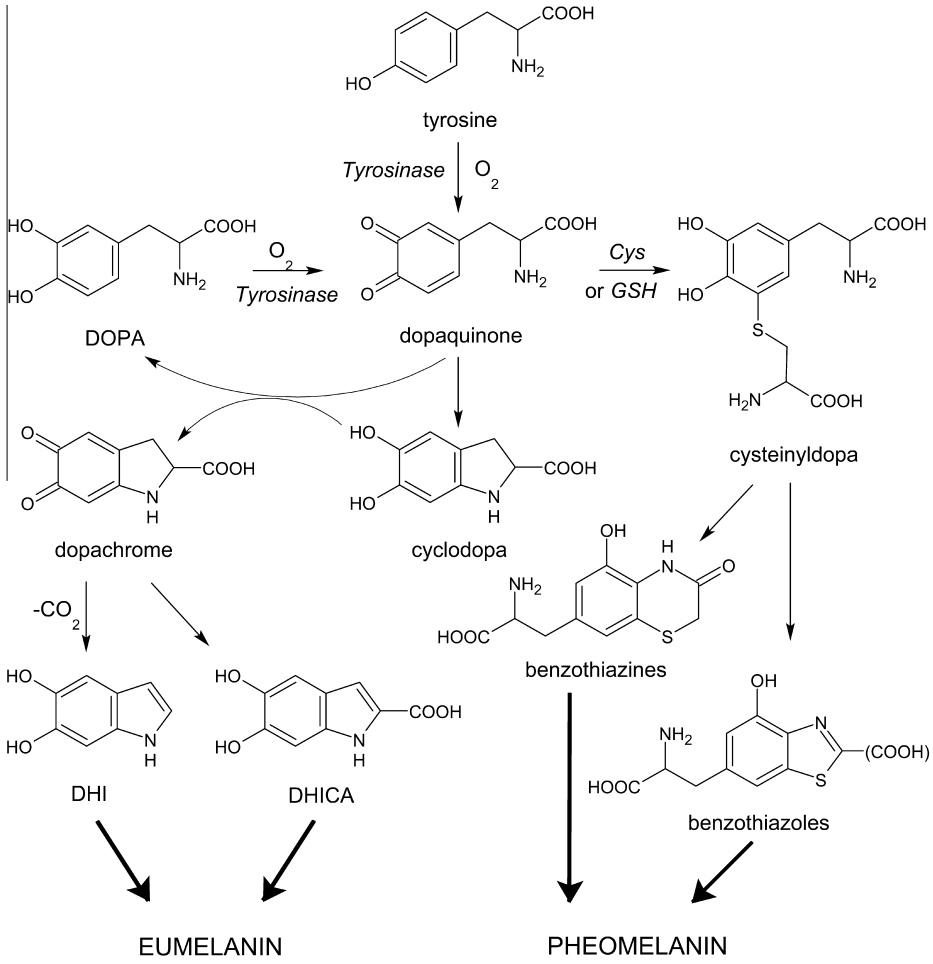

Figure 1. Schematic representation of the melanogenesis pathway. Tyrosinase catalyzes the oxidation of L-tyrosine to L-dopaquinone, which is the common precursor of the two connected biosynthetic routes in melanogenesis that lead to the formation of either eumelanin or pheomelanin depending on the absence or availability of thiol-containing agents. Adapted from Ito et al. (2011).

may also behave as the sulfhydryl groups (S–H) of cysteine and GSH, and thus BSO may react directly with some of the intermediate oxidation products of the melanogenesis pathway and that would affect the production of pigments. To investigate the latter possibility, we conducted an in vitro experiment to go deeper into the chemical reactivity properties of BSO. Benathan and co-workers[12,13] have previously reported increases in the pheomelanin precursor 5-*S*-cysteinyldopa and decreases in total pigmentation in in vivo cells exposed to BSO. However, the possibility that the S=NH group of BSO reacts with melanogenesis intermediates has never been explored. The expectation should be that BSO reacts with dopaquinone to form BSO–DOPA conjugates and thus diverts the melanogenesis route, decreasing the synthesis of both pheomelanin and eumelanin similarly to other agents containing sulfhydryl groups.[14]

To investigate the possibility that BSO reacts with intermediates of the melanogenesis pathway, we reproduced under in vitro conditions the initial steps of the melanogenesis pathway oxidizing L-DOPA with tyrosinase in the presence or absence of GSH, and in the presence or absence of BSO. The oxidation of L-DOPA without GSH should thus be similar to eumelanogenesis, while the oxidation of L-DOPA in the presence of GSH should simulate pheomelanogenesis.[11] We used GSH instead of cysteine as a sulfhydryl compound because the former is more abundant in intracellular media than free cysteine, so pheomelanogenesis in the presence of GSH may resemble more closely the in vivo situation.[9] In any case, 5-*S*-glutathionyldopa, which is the major thiol-conjugated species formed when L-dopaquinone reacts with GSH, releases 5-*S*-cysteinyldopa,[15] the main intermediate of the monomeric subunits for pheomelanin, after the action of a dipeptidase and the pathway then progresses as when only cysteine is present.[9]

To explore the influence of BSO on melanogenesis, five solutions were prepared in cryogenic vials all containing 10 mM L-DOPA[11,16] and 90 mM BSO (DL-buthionine-(*S*,*R*)-sulfoximine) in 1 ml of saline phosphate buffer (4 mM $KHCO_3$, 2 mM $CaCl_2$ $2H_2O$, 20 mM $NaHCO_3$, 138 mM NaCl and 2 mM KCl), pH 7.4. One of these solutions did not contain GSH, other two solutions contained GSH at concentrations (0.65 and 3.2 mM) that were lower than the concentration of the substrate L-DOPA, and finally other two solutions contained GSH at concentrations (16.3 and 81.3 mM) that were higher than the concentration of the substrate L-DOPA but lower than the one of BSO. 36.1 μg mushroom tyrosinase (1715 units/mg) diluted in 20 μl of the saline phosphate buffer were added to all tubes. Other five solutions were prepared as explained before, except that these did not contain BSO, thus serving as appropriate controls for standard eu- or pheomelanogenesis. All solutions were made in duplicates. All products were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The chosen concentration of L-DOPA uses to be employed in studies of melanin synthesis in vitro[17] and is around the natural concentrations found in at least some cells.[18] The chosen concentration of BSO was intermediate between the lowest (4.5 mM) and medium (184.4 mM) doses used in a previous in vivo experiment with a bird species (the great tit *Parus major*), which did not detect significant mortality or effects on body condition.[4] The chosen concentrations of GSH were also around the concentration of total GSH found in the erythrocytes of control birds (3.9 mM) in the same study.[4] This makes that the results of this in vitro experiment should be useful to make biologically significant predictions for the influence of BSO on melanogenesis.

To characterize the products of the oxidation of L-DOPA under different conditions, after the addition of tyrosinase the solutions were kept in a shaker for 48 h and then centrifuged at 3000*g* and 10 °C during 10 min. The absorbance of the resulting supernatant was measured in the range 325–800 nm (2 nm intervals) with a Thermo Scientific Genesys 10S Vis spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA). As precipitates were formed in some tubes, we also measured their resistance to chemical degradation. For this, the precipitates were redissolved after previous drying all tubes in a heater at 65 °C for 1.5 h. Then, 250 μl of 10 M NaOH was added to 500 μl of supernatant in the tubes where precipitate was not formed and 750 μl of 3.3 M NaOH was added to the tubes with precipitate to keep NaOH concentration 3.3 M in all tubes. The precipitates were then exposed to alkaline degradation in a dry block heater at 90 °C during 24 h until complete re-solution. Then, tubes were centrifuged at 3000*g* and 20 °C during 5 min, and the absorbance at 400 nm was measured as an estimate of total melanin present.[19] As H_2O_2 degrades melanin polymers and produces the bleaching of the pigments,[20] 100 μl of 35% H_2O_2 was then added to all tubes, which were then vortexed two times during 15 s and kept for 1.5 h. The absorbance at 400 nm was then measured again in all tubes. By comparing the absorbance at 400 nm of the solutions before and after the addition of H_2O_2, we could therefore determine the resistance to chemical degradation of the products obtained from the oxidation of L-DOPA in the presence of both GSH and /or BSO at different concentrations as stated above.

The shape of the curves with and without BSO was compared by analyzing the mean values of absorbance and the mean values of the slopes of all tangential lines in the curves ($(y_a - y_b/x_a - x_b)$; being y absorbance, x wavelength and a and b two adjacent points in the curve). Mean values of absorbance and slopes were compared by one-way ANOVA considering all values of absorbance ($N = 238$) and slopes ($N = 237$) in the curves. Mean values in the absorbance decrease produced by H_2O_2 bleaching were not compared by ANOVA because these means were calculated with only two points (i.e., absorbance at 400 nm of duplicate solutions), so only the mean values are shown.

The absorbance spectra of the solutions resulted from the oxidation of L-DOPA under different conditions are shown in Figure 2. The simulation of eumelanogenesis (i.e., oxidation of L-DOPA without GSH) showed that, when L-DOPA was oxidized in the absence of BSO, the result was a dark solution with a typical absorbance spectrum of eumelanin with absorbance progressively decreasing with increasing wavelength in the range 300–800 nm.[21] Insoluble black precipitate was formed. When L-DOPA was oxidized in the presence of BSO, the result was a very dark solution with different spectra in which absorbance decreased less steeply and thus absorbance values were greater (Fig. 2). As a consequence, the mean absorbance value and the mean slope value of the curve were higher and lower, respectively, than those of the solution resulted from the oxidation of L-DOPA in the absence of BSO (mean ± SE: absorbance: no BSO = 1.21 ± 0.2, BSO = 1.67 ± 0.02, $F_{1,474} = 180.27$, $P < 0.0001$; slope: no BSO = $2.76 \times 10^{-3} \pm 2.40 \times 10^{-4}$, BSO = $1.96 \times 10^{-3} \pm 2.40 \times 10^{-4}$, $F_{1,472} = 5.49$, $P = 0.019$). Precipitate was not observed in the solution with BSO. These results suggest that BSO diverts the eumelanogenesis pathway towards the formation of more soluble products which remain dissolved in the solution. Thus, the solution in the presence of BSO is therefore darker (i.e., higher absorbance and lower slope values) and does not form precipitate (i.e., insoluble eumelanin) than in the absence of BSO, where the extensive polymerization of intermediates gives place to large eumelanin polymers that precipitate leaving a relatively light solution.

H_2O_2 bleached the oxidation products as reflected by a decrease in absorbance at 400 nm in all solutions, but this decrease was higher in the solutions with BSO (mean absorbance at 400 nm: 0.54 ± 0.11) than in those without BSO (0.39 ± 0.28). This suggests that the products formed in the presence of BSO may be more easily metabolized and excreted than insoluble eumelanins formed in the absence of BSO. Thus, BSO under these in vitro conditions inhibited the production of standard eumelanin and probably induced the production of other unknown pigments. These pigments may result from the addition of the S=NH group of BSO to L-dopaquinone to form BSO–DOPA conjugates, thus decreasing the formation of L-dopachrome (Fig. 1) and impairing, at least partially, the eumelanogenesis pathway.

The simulation of pheomelanogenesis (i.e., oxidation of L-DOPA in the presence of GSH) showed that, at subsaturating concentrations of GSH (0.65 and 3.2 mM), the oxidation of L-DOPA resulted in spectra that were not very different in shape from those generated when L-DOPA was oxidized alone (Fig. 2). Indeed, absorbance values were even lower in the solutions with BSO than in those without BSO (0.65 mM GSH: no BSO = 1.86 ± 0.2, BSO = 1.67 ± 0.02, $F_{1,474} = 69.87$, $P < 0.0001$; 3.2 mM GSH: no BSO = 1.81 ± 0.2, BSO = 1.45 ± 0.02, $F_{1,474} = 124.70$, $P < 0.0001$), and the slope values of the curves were also higher in the solutions with BSO (0.65 mM GSH: no BSO = $9.40 \times 10^{-4} \pm 2.27 \times 10^{-4}$, BSO = $2.27 \times 10^{-3} \pm 2.27 \times 10^{-4}$, $F_{1,472} = 17.16$, $P < 0.0001$; 3.2 mM GSH: no BSO = $1.64 \times 10^{-3} \pm 2.22 \times 10^{-4}$, BSO = $2.83 \times 10^{-3} \pm 2.22 \times 10^{-4}$, $F_{1,472} = 14.61$, $P < 0.001$). At the lowest concentration of GSH (0.65 mM), dark insoluble precipitate, which may be insoluble pheomelanin or eumelanin escaping trapping of dopaquinone by GSH, was formed in the solution without BSO, but not when BSO was present. Under higher GSH concentration (3.2 mM), precipitate was not observed regardless BSO presence.

The oxidation products formed in the presence of BSO were always degraded by H_2O_2 easier than when BSO was not present, as reflected by a greater decrease in absorbance of the oxidation products with BSO plus 3.2 mM GSH (mean absorbance at 400 nm: no BSO: 0.10 ± 0.33; BSO: 0.44 ± 0.08). Thus, BSO also inhibits the production of pheomelanin and instead induces the production of other related pigments which may arise from BSO DOPA conjugates avoiding the formation of cysteinyldopas and thus impairing the pheomelanogenesis pathway (Fig. 1). However, this effect is less marked than in eumelanogenesis.

Lastly, oxidation of L-DOPA under saturating concentrations of GSH (16.3 and 81.3 mM) resulted in transparent solutions and thus a lack of absorbance, regardless the presence of BSO (Fig. 2). This lack of absorbance is expected from the fact that GSH bonds to the copper active site of tyrosinase, thus inhibiting the enzyme.[8,22] GSH does not react with L-DOPA, but with L-dopaquinone, so it is necessary that tyrosinase first oxidizes L-DOPA to L-dopaquinone. Under high concentrations of GSH, tyrosinase is almost completely inhibited by GSH. This greatly prevents the formation of 5-*S*-glutationyldopa and the further generation of pheomelanins. In agreement with that, the solutions were thus transparent because L-DOPA, GSH and BSO (when present) were mostly unaltered without the initial tyrosinase action on L-DOPA. This suggests that the effect of BSO on melanogenesis will depend on the levels of thiol-agents such as L-cysteine and GSH.

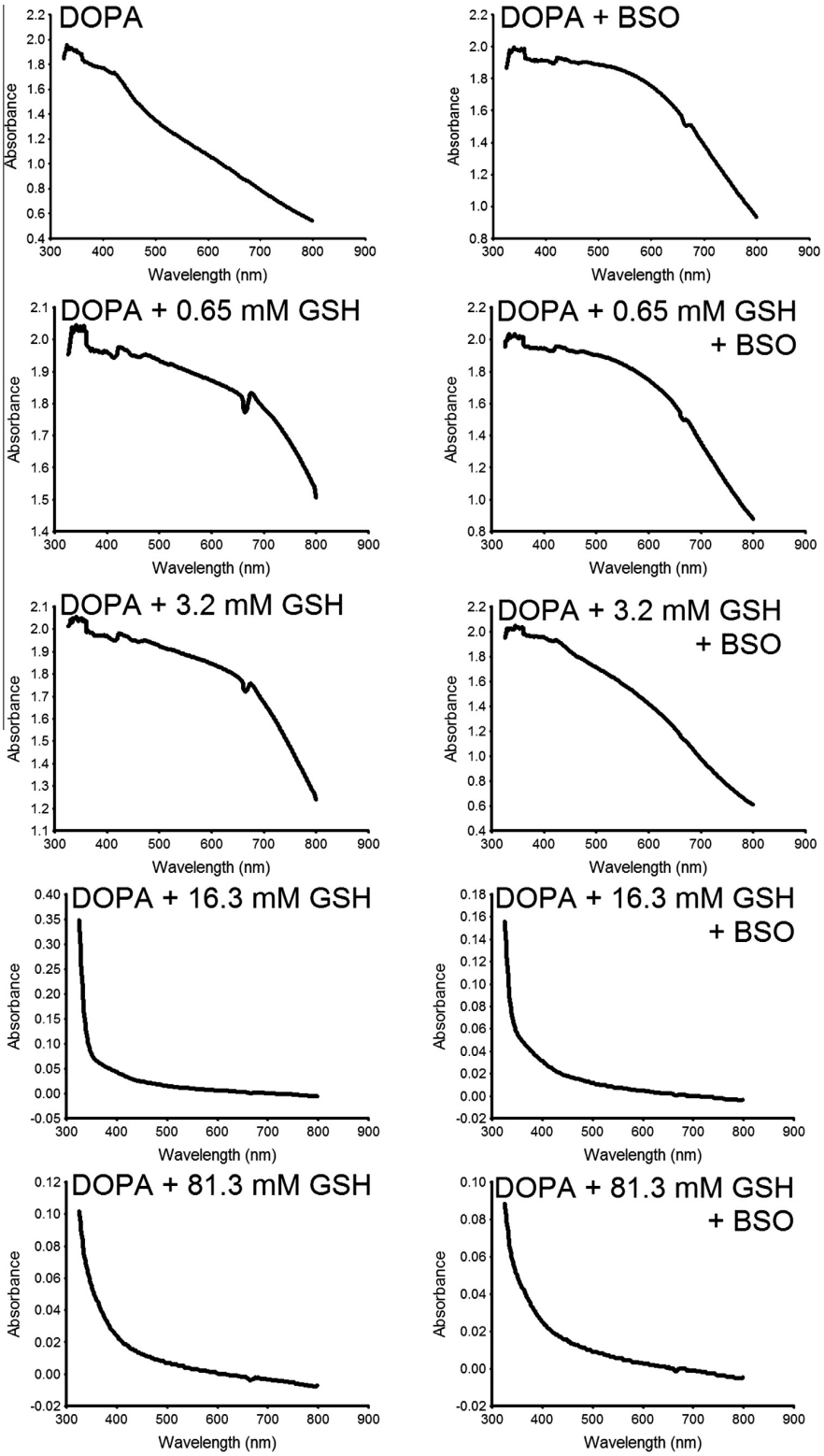

Figure 2. Absorbance spectra of the solutions resulting from the oxidation of L-DOPA by tyrosinase in the absence or presence of GSH and/or BSO. GSH was added at concentrations that were either lower (0.65 and 3.2 mM) or higher (16.3 and 81.3 mM) than the concentration of the substrate L-DOPA (10 mM). Each spectrum shows mean absorbance values of two duplicate solutions after 48 h at the conditions indicated.

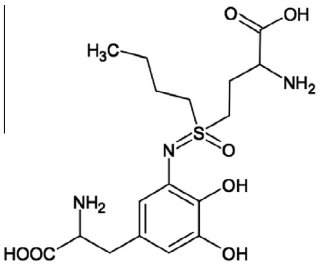

Figure 3. Postulated structure of the main BSO–DOPA conjugate formed by addition of BSO to dopaquinone.

Our results indicate that BSO reacts directly with some of the intermediate oxidation products of the melanogenesis pathway, diverting the synthesis of eumelanin and pheomelanin toward the production of more soluble pigments. BSO may therefore be a competitive inhibitor of melanogenesis, probably because its S=NH group reacts with dopaquinone to form BSO–DOPA conjugates (Fig. 3) that avoid the formation of dopachrome and cysteinyldopas and thus the synthesis of eumelanin and pheomelanin, respectively. This result is more marked for eumelanogenesis than for pheomelanogenesis, which may be due to a closer similarity between the hypothesized BSO–DOPA conjugates and cysteinyldopas (which are sulfur-containing like BSO) than between those conjugates and dopachrome (which does not contain sulfur), making that the properties of the final products of pheomelanogenesis in the presence of BSO are not significantly different from pheomelanins. However, the possibility that BSO competes with GSH for dopaquinone should not be discarded. This is likely, according to Figure 3. Future studies should determine the presence and structure of BSO–DOPA conjugates.

The impairment of the melanogenesis pathway suggests that BSO has depigmenting properties. Indeed, other depigmenting agents like lipoic acid derivatives exert their inhibitory effects on melanin synthesis by adding their sulfhydryl groups to dopaquinone to form derivatives that avoid dopachrome formation[14], similarly to the mechanism proposed here for the S=NH group of BSO (Fig. 3). There are at least two other compounds that are known to act as depigmenting agents by acting on dopaquinone during melanin synthesis: ascorbic acid, which reduces dopaquinone and blocks DHICA formation, and cysteamine, which favors pheomelanin synthesis by nucleophilic addition to dopaquinone.[23] Solano et al.[24] have already highlighted the hypopigmenting nature of BSO, but our study shows that this effect is not only mediated by an inhibition of GSH synthesis, but also by direct reactions of BSO with melanogenesis intermediates. This suggests that, if supplemented with GSH to counteract the inhibitory effect of γ-glutamylcysteine synthetase, BSO might have depigmenting effects without affecting the antioxidant capacity, with the corresponding relevance for cosmetic use. Indeed, it has already been shown that the oxidative damage due to BSO-induced depletion of GSH is avoided if BSO is administered with other antioxidants such as ascorbate.[25] BSO is widely used in pharmacology due to its specificity and efficiency in changing the metabolism of sulfur-containing amino acids,[26] so its pharmacological use as a depigmenting agent should be considered. The impairment of conventional pheomelanin synthesis by BSO may be particularly relevant to avoid the phototoxicity of pheomelanin derived from the generation of superoxide anion under exposure to ultraviolet (UV) radiation.[27] Lastly, it may be speculated that the impairment of melanin synthesis by BSO may contribute to avoid the resistance of melanoma to radiotherapy because of the high absorption of energy by eumelanin, which would add to the increase in radiosensitization of melanoma mediated by GSH depletion.[28] This may create synergies with other treatments against melanoma. Future studies should explore these possibilities.

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