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Sulfur Analogues of Tyrosine in the Development of Triazene Hybrid Compounds: A New Strategy against Melanoma

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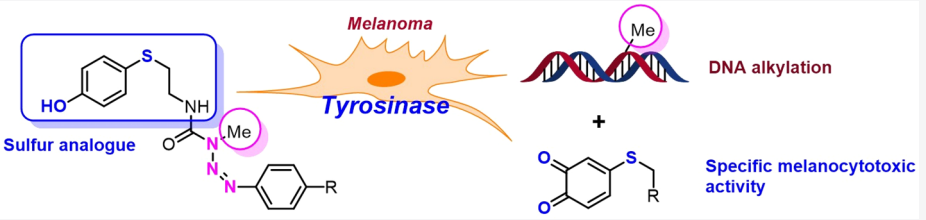

ABSTRACT: Malignant melanoma is the major cause of death from skin cancer. Treatment of metastatic melanoma remains an enormous challenge. In this study we developed hybrid compounds and studied their potential use in malignant melanoma chemotherapy. They were designed to act by a double mechanism of action, being composed of two pharmacophores: the tyrosine sulfur analogue 4-*S*-cysteaminylphenol (4-S-CAP, **10**), with immunomodulatory properties and specific melanocytotoxic activity, and triazene **4**, with DNA alkylating properties. The design of these compounds aims to achieve selective activation by the enzyme tyrosinase overexpressed in melanoma cells. Compounds **11a−e**, **13a**, and **13b** were found to be excellent tyrosinase substrates (0.5 min $\leq t_{1/2} \leq$ 3.7 min). Furthermore, derivatives **11** and **13** were evaluated for their molecular properties, hepatotoxicity, *in vivo* toxicity profile, and assessment of cytotoxic activity in melanoma and non-melanoma cell lines. The results were compared with those obtained for temozolomide, a triazene used in melanoma therapy. It was discovered that the hybrids are selective and effective drugs, representing a valuable model for the development of new multitarget melanoma therapy. In particular, compound **10** may be an important component for these strategies that use a metabolic pathway of melanin synthesis. Molecular hybridization of **10** with triazenes **4** renders the hybrids (**11** and **13**) unexpectedly devoid of hepatotoxicity while maintaining cytotoxic activity in malignant cells.

KEYWORDS: *Triazenes, melanoma, tyrosinase, hybrid compounds, target therapy*

Although malignant melanoma represents less than 5% of all malignant skin diseases, it is the major cause of death from skin cancer. Compared with other cancers, the incidence and mortality of melanoma have risen so rapidly in the last 50 years that melanoma has become a heavy health and economic burden.[1,2] In the ranking of the primary tumors that most easily metastasize in the brain, melanoma ranks third (after lung and breast cancers), greatly worsening the prognosis of patients with melanoma.[3]

Treatment of metastatic melanoma depends on the tumor stage at the time of diagnosis. Surgery remains the mainstay for local primary melanoma. Radiotherapy is a valid and effective therapeutic option especially in situations of medical inoperability or for most melanomas with few systemic options.[4] With regard to chemotherapy, dacarbazine (DTIC) is the only drug specifically approved by the U.S. Food and Drug Administration for the treatment of malignant melanoma. It is a bioprecursor of the DNA alkylating agent, 5-(3-methyltriazeno)imidazole-4-carboxamide (MTIC).[5] Unfortunately, DTIC is only able to promote medium survivals ranging from 6 to 11 months. The modest activity of DTIC in the treatment of metastatic melanoma patients has been attributed in part to the lower activity of cytochrome P450 (CYP450) in humans compared with rodents.[6] Another triazene, temozolomide (TMZ), approved for the treatment of anaplastic astrocytoma, has been used to treat brain metastases of melanoma since after oral administration it is able to cross the blood−brain barrier. Albeit

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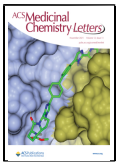

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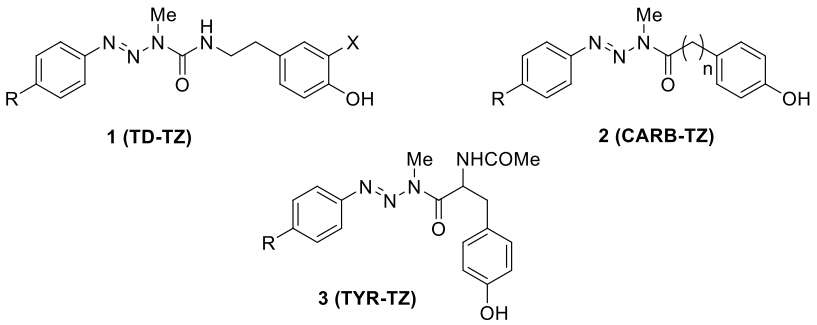
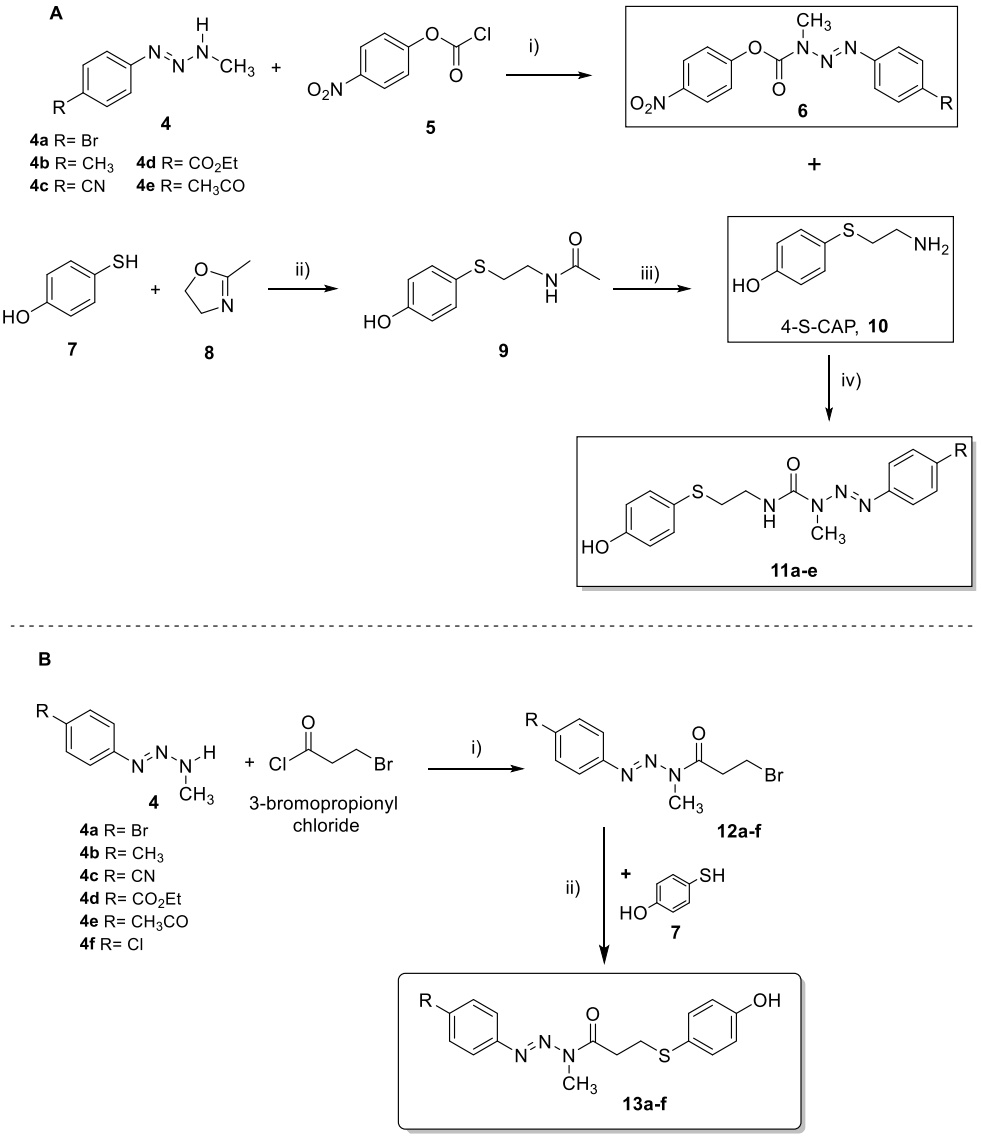


Figure 1. Previously synthesized hybrid drugs containing different phenols as tyrosinase substrates: TD-TZ, **1** (tyramine and dopamine); CARB-TZ, **2** (hydroxyphenyl carboxylic acids); TYR-TZ, **3** (*N*-acetyltyrosine).[13−15]

Scheme 1. Synthetic Pathways for (A) Triazene Derivatives 11 and 4-S-CAP (10)[20] and (B) Triazene Derivatives 13a−f[a]



[a]Reagents and conditions: (A) (i) CH_2Cl_2, pyridine; (ii) N_2 atmosphere, reflux (130 °C); (iii) conc. HCl, reflux (170 °C); (iv) THF, Et_3N, rt. (B) (i) CH_2Cl_2, pyridine; (ii) K_2CO_3, THF.

TMZ is also a prodrug, in the brain it is transformed into MTIC by chemical hydrolysis.[7]

Although not so widely used as chemotherapy and radiotherapy, immunotherapy is a therapeutic strategy that seeks to help the immune system fight cancer. The most commonly used immunostimulators for treatment of melanoma are interleukin (IL)-2, interferon (IFN)-α, thymosin α1, and monoclonal antibodies such as ipilimumab, prembolizumab, and nivolumab. Immunotherapy has contributed to improve the overall survival of patients at advanced melanoma stages.[2]

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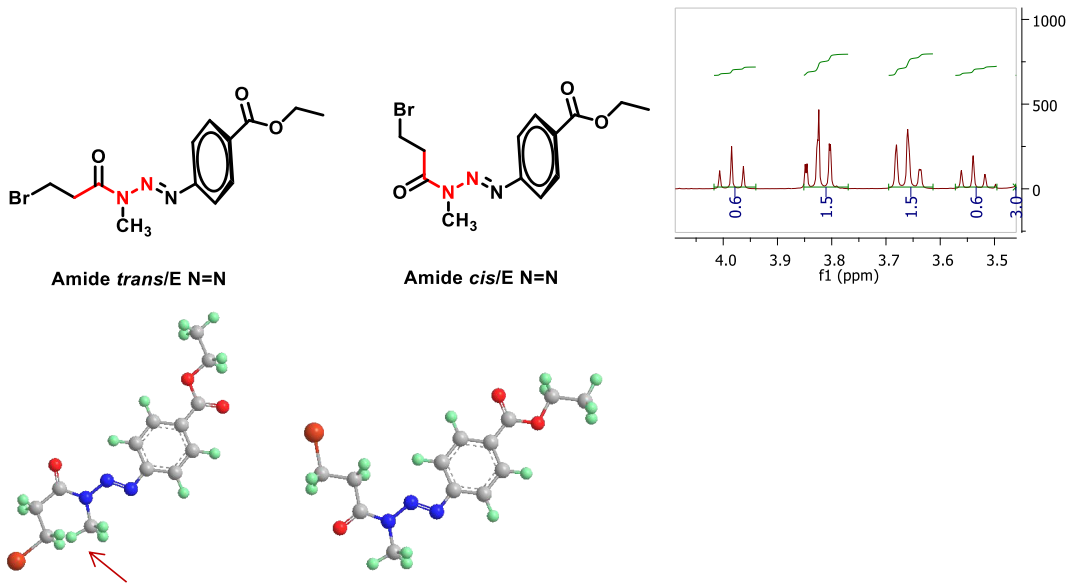

Figure 2. General structures of the *cis*- and *trans*-amide isomers for compound **12d**.

Targeted therapies namely, small-molecule drugs, are able to specifically act on particular mechanisms of melanoma occurrence and progression. These therapies include BRAF inhibitors, namely sorafenib (a multitargeted tyrosine kinase inhibitor), vemurafenib and dabrafenib, and/or MEK inhibitors such as binimetinib.[2,4]

Regrettably, current therapeutic strategies display limited clinical success, mainly because of the severe side effects, lack of specificity, and acquired resistance, which determine not only the high aggressiveness of the melanoma but also the high rate of tumor relapses.[2] To overcome some of these drawbacks, combination therapy is recommended in clinical practice.[2,4]

The design and synthesis of multitarget hybrid molecules has become a promising new strategy in anticancer therapy. Hybrid molecules, designed and synthesized with two or more different pharmacophore moieties that act simultaneously on the same or different targets at tumor cells, may be more potent and efficient than combination therapies.[8−12] In recent years, our team has designed hybrid molecules that combine an antitumor triazene and a substrate for the enzyme tyrosinase, which is overexpressed in melanoma cells (Figure 1).[13−15]

Tyrosinase (monophenol, 3,4-β-dihydroxy-L-phenylalanine oxygen oxidoreductase, EC 1.14.18.1) is involved in the biosynthetic pathways of melanin pigment. It catalyzes the two-step oxidation of phenolic compounds into the corresponding catechols (phenolase activity) and *o*-quinones (catecholase activity).[16] The tyrosinase overexpression and the high turnover rate of its catalytic activity render this enzyme an attractive molecular target for the development of melanoma-specific therapies.[8,9,11,12] In the present work, taking advantage of the same biosynthetic pathway that leads to the production of melanin, we assessed different phenols for tyrosinase-mediated selective release of cytotoxic triazenes. Oxidation of the phenol carrier leads to the formation of a corresponding *o*-quinone, and a subsequent cyclization-based mechanism finally allows the release of the cytotoxic triazene.[13−16]

Supported by our previous results, we envisioned a new strategy involving the design of hybrid compounds conjugating two pharmacophores with antimelanoma activity: aryltriazenes **4** and a sulfur analogue of tyrosine, 4-*S*-cysteaminylphenol (4-S-CAP, **10**), a substrate of melanoma tyrosinase that exhibits a significant *in vivo* depigmenting effect.[17]

4-S-CAP **10** was identified as a much better substrate for mushroom tyrosinase than L-tyrosine and also a good substrate for mammalian tyrosinase. Moreover, compound **10** is known to inhibit the growth of malignant melanoma and cause depigmentation of dark skin. One possible explanation for such activity is that in melanocytes this phenol is oxidized by tyrosinase to the corresponding *o*-quinone, which conjugates with sulfhydryl enzymes through cysteine residues, thus exerting cytotoxic effects.[17,18]

Herein we report the synthesis, stability studies in physiological conditions, toxicity evaluation, and *in vitro* antimelanoma activity of these hybrid molecules. For the design of these new hybrid molecules, two pharmacophore moieties, triazene **4** and 4-S-CAP **10**, were conjugated by insertion of urea or amide linkages. These two linkages were chosen because in general they are chemically stable, thus allowing the hybrids to reach tumor cells intact. Compounds **11** and **13** were aimed to be used in a targeted therapeutic strategy based on overexpression of the enzyme tyrosinase in malignant melanocytes. It was expected that the compounds would manifest cytotoxic activity only after tyrosinase oxidation, thus increasing the selectivity while minimizing side effects.

The synthetic approach used for the preparation of triazene hybrid compounds **11a−e** is shown in Scheme 1A. Key intermediates **6a−e** were synthesized by reaction of monomethyltriazenes (MMTs) **4a−e** with *p*-nitrophenyl chloroformate (**5**), allowing the insertion of urea functionality in moderate to good yields (43−91%).[19] The final hybrid compounds **11a−e** were obtained by coupling intermediates **6** with 4-S-CAP under basic conditions. These reactions also occurred in moderate to good yields (53−80%), and the compounds were fully identified and characterized by spectroscopic methods (Supporting Information, S3).

Compound **10** was synthesized by the reaction between 4-mercaptophenol (**7**) and 2-methyl-2-oxazoline (**8**) followed by acid-catalyzed hydrolysis of the amide bond, a method also called the Wehrmeister reaction (Scheme 1A). Compound **10**

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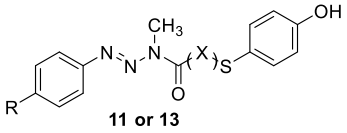
Table 1. Half-Lives ($t_{1/2}$) for Triazene Derivatives 11a−e and 13a−f in PBS or 80% Human Plasma at 37 °C, log *P* and clogP Values, and Ability to Act as Tyrosinase Substrates (100 units/mL)



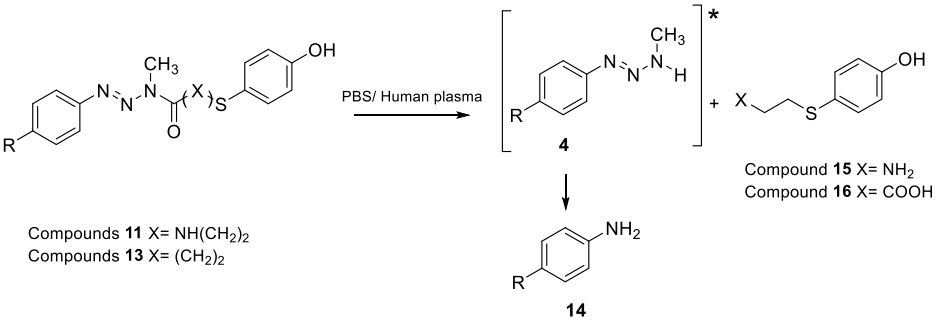
11 or 13

Compounds **11** X= $NH(CH_2)_2$
Compounds **13** X= $(CH_2)_2$

				$t_{1/2}$		
compound	R	log *P*	clogP[a]	PBS pH 7.4 (days)	80% human plasma (h)	tyrosinase (min)
11a	Br	3.95 ± 0.04	4.31	>10	104.6 ± 3.2	0.5 ± 0.2
11b	Me	4.21 ± 0.06	3.88	>10	88.5 ± 0.5	1.9 ± 0.1
11c	CN	3.49 ± 0.05	3.54	>10	74.0 ± 5.6	0.5 ± 0.1
11d	EtOCO	4.08 ± 0.10	3.88	>10	30.5 ± 2.9	3.7 ± 0.1
11e	MeCO	3.44 ± 0.05	3.51	>10	61.6 ± 11.9	1.0 ± 0.2
13a	Br	nd[b]	5.06	4.4 ± 0.4	8.3 ± 0.7	2.1 ± 0.1
13b	Me	nd	4.43	4.2 ± 1.1	8.7 ± 0.4	2.3 ± 0.2
13c	CN	nd	4.15	3.6 ± 0.2	2.99 ± 0.17	nd
13d	EtOCO	nd	4.68	5.4 ± 0.8	6.57 ± 0.41	nd
13e	MeCO	nd	3.99	5.8 ± 0.6	4.90 ± 0.33	nd
13f	Cl	nd	4.71	nd	nd	nd

[a]Calculated using Virtual Computational Chemistry Laboratory (VCCLAB) (http://www.vcclab.org, 2005). [b]nd: not determined.

Scheme 2. Decomposition Pathways in PBS and Human Plasma to Afford Hydrolysis Products of Hybrid Compounds 11 and 13



*Not detected in PBS.

was obtained in 70% overall yield, which is in accordance with previous published results.[20]

Amide-linked hybrids **13a−f** were synthesized by the reactions outlined in Scheme 1B. Intermediate amides **12a−f** were synthesized by the Schotten−Baumann reaction between MMTs **4** and 3-bromopropionyl chloride, followed by the reaction of these intermediates **12a−f** with mercaptophenol **7** in basic medium.[21] The intermediate amides **12** were generally obtained in low yields (15−35%), and the final hybrid compounds were obtained in moderate or good yields depending on the R group (41−75%). Compounds **13a−f** were also fully identified and characterized by spectroscopic methods (Supporting Information S7). Interestingly, in the ^{1}H NMR spectra, intermediate amides **12a−f** showed the presence of a mixture of *E* and *Z* diastereomers in the deuterated solvent solution (as shown for compound **12d** in Figure 2). In contrast to intermediate amides **12a−f**, *E*/*Z* diastereomers were not observed for hybrids **13a−f** in the same deuterated solvent solutions (Supporting Information, S6).

The existence of *E* and *Z* diastereomers is attributed to the partial double-bond character of the amide bond (O)C−N, which prevents the free rotation of atoms or groups of atoms around the axis of the bond. For asymmetric tertiary amides, the limited rotation implies the existence of nonisolable *E* and *Z* diastereomers. In the case of compound **12d**, the predominant diastereomer is possibly the *cis* one. In the 3D representations of the two isomers (Figure 2) a spatial repulsion between the close $-NCH_3$ and $-CH_2Br$ groups is observed (signaled in the figure with a red arrow), which probably shifts the balance to the *cis* isomer (1.5/0.5), as noticed in the spectrum integration values.

The stabilities of triazene derivatives **11a−e** and **13a−f** were studied both in phosphate buffered saline (PBS) and in 80% human plasma at 37 °C. These reactions were followed by HPLC by monitoring both the loss of compounds **11a−e** and **13a−f** and the formation of hydrolysis products. Half-lives for the hydrolysis of triazene derivatives in PBS and in human plasma are presented in Tables 1, S2, and S3.

These reactions followed pseudo-first-order kinetics and were monitored during at least 3 half-lives. Pseudo-first-order rate constants (k_{obs}) were calculated from the slopes of plots of ln(concentration) vs time (eq 1), and half-lives ($t_{1/2}$) were calculated using eq 2:

$$\ln(\text{concentration}) = -k_{obs}t + b \quad (1)$$

$$t_{1/2} = \frac{\ln(2)}{k_{obs}} \quad (2)$$

Analysis of the obtained results showed that hybrids **11a−e**, with the urea linker, are extremely stable in PBS, remaining undecomposed for more than 10 days. These results are in

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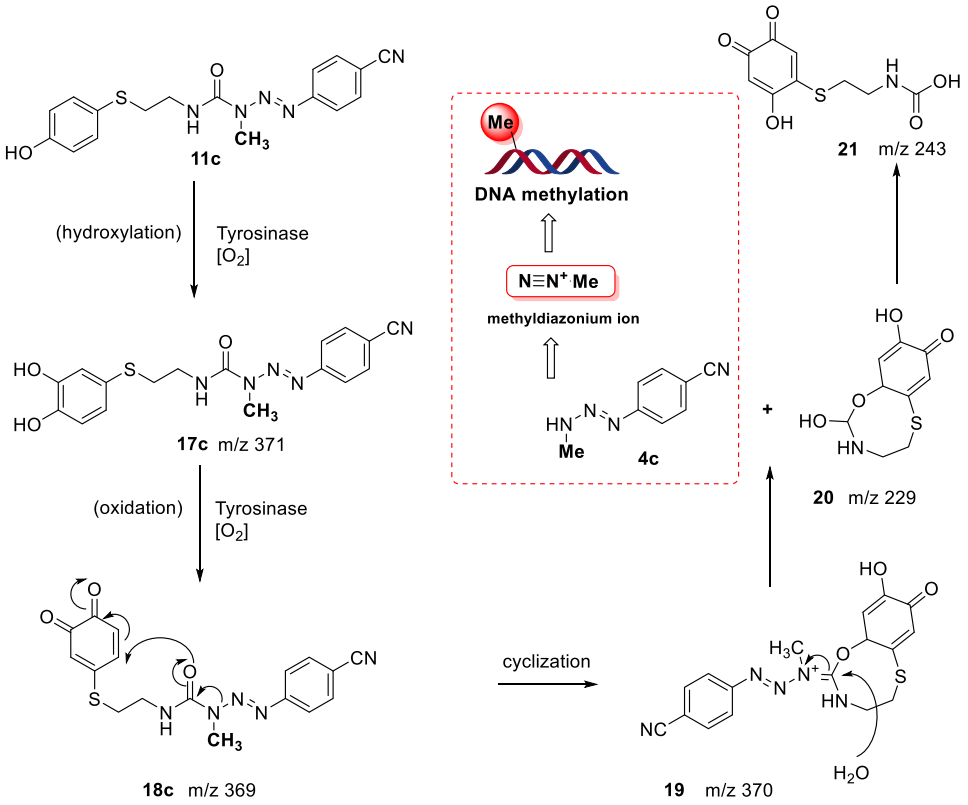
Scheme 3. Proposed Mechanism for MMT Release from Derivative 11c after Tyrosinase Activation



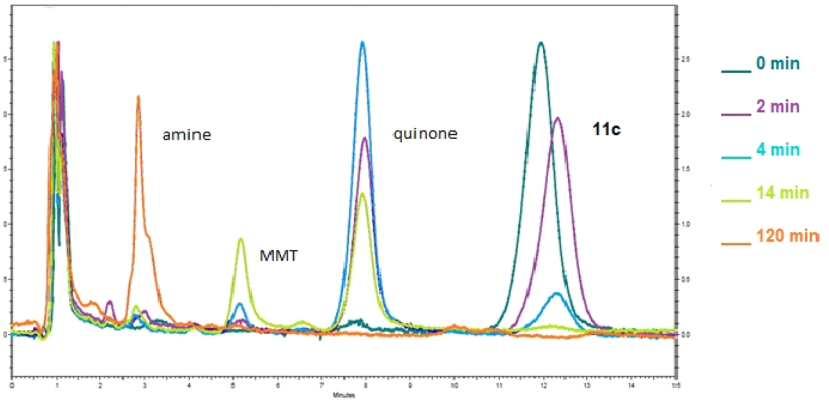


Figure 3. Overlapping chromatograms showing the disappearance of hybrid compound **11c** over time following tyrosinase oxidation, which results in formation of an intermediate quinone, that later degrades into MMT **4c** and the corresponding amine **14c**.

accordance with the previous ones obtained by our group for triazene derivatives of tyramine **1** (X = H), also conjugated through an urea linker, which were stable in PBS for more than 15 days.[13] However, the presence of a urea linkage is not a guarantee of stability since in the same study dopamine derivatives **1** (X = OH) proved to be much more unstable, with half-lives of around 15 to 20 h.[13]

Compounds **13a−f** with an amide linkage are also quite stable in PBS, with half-lives between 4 and 6 days at 37 °C. Hydrolysis of hybrids **13a−f** in this medium yielded the respective anilines (Scheme 2) in quantitative amounts. Again, these results are in line with the previous ones obtained by our group for compounds **2** (n = 2) with half-lives between 2.5 and 4 days. Amides **2** with longer alkyl chains separating the triazene and the phenol (n = 4) are more stable and remain undecomposed for more than 10 days in PBS.[15] Overall, comparing the reactivities of hybrids **11a−e** and **13a−f** confirmed that urea derivatives are more stable than amides in PBS at 37 °C.

Human plasma is rich in hydrolytic enzymes that can recognize ureas and amides and decompose hybrid compounds **11** and **13**. In fact, both types of compounds decompose in human plasma, but with a remarkable difference in reactivity. Indeed, ureas **11a−e** showed half-lives between 30 and 105 h, while amides **13a−f** presented half-lives between 3 to 9 h. Compound **11a** is 35 times more stable than compound **13c**, the most reactive. The products of reaction are MMTs **4** and the corresponding anilines **14** (Scheme 2).

The results for ureas **11a−e** are in line with our previous ones obtained for compounds **1** (X = H), highlighting the great stability of the urea bond in plasma. On the contrary, the amide bond is much more unstable, reflecting the higher concentration of amidases in plasma that, acting on the compounds, break

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down the linkage, which results in shorter plasma half-lives. It can be stated that urea derivatives **11a−e** are very stable in human plasma, and the potential hydrolysis promoted by plasma enzymes is not significant at 37 °C. Because they are poor substrates for the plasmatic enzymes, their premature hydrolysis is prevented, and the compounds can circulate freely through the body and reach tumor sites at high concentration. With regard to our original statement that hybrid compounds must target affected areas intact, the urea linker seems more adequate to accomplish this objective than the amide linker.

Next, derivatives **11a−e** and **13a−f** were evaluated as tyrosinase substrates. Looking at the half-lives in the presence of mushroom tyrosinase (Table 1), we can state that these hybrid compounds (**11** and **13**) are easily recognized and oxidized by the enzyme with half-lives between 0.5 and 4 min. In these assays, the first metabolite detected by HPLC is quinone **18** (Scheme 3). After the initial formation of these metabolites, we propose that they were decomposed into MMT **4** and cyclic compound **19**, although they were not detected by HPLC. Nevertheless, by analysis of the composition of the reaction mixtures from the tyrosinase assays by mass spectrometry, it was possible to observe the presence of peaks with m/z corresponding to compounds **17**, **18**, **19**, **20**, and **21**. The formation and complete disappearance of compound **18** after 2 h of reaction was also confirmed by HPLC. Moreover, at this time, the concomitant formation of a 100% yield of amine **14**, which results from decomposition of monomethyltriazene **4**, was detected. Figure 3 depicts the overlap of the chromatograms obtained from samples taken at different reaction time points. As an example, the disappearance of hybrid compound **11c** over time is shown, along with the appearance of intermediate quinone **18**, which later degrades into MMT **4c** and the corresponding amine **14c** (Figure S1).

For amides **13**, a parallel study involving mass spectrometry analysis of samples from the tyrosinase assays corroborated the presence of the corresponding dihydroxylated compound. For example, compound **13a** displayed two peaks at m/z 408 and 410 for the dihydroxylated compound ($M - H^+$), while compound **13b** presented one peak at m/z 344 ($M - H^+$) (Scheme S1 and Figure S2).

Evaluation of the kinetic parameters for hybrid compounds **11** showed that these compounds are very good substrates for mushroom tyrosinase with K_m values ranging from 0.12 to 0.064 mM. In fact, these new compounds exhibited a 2- to 4-fold higher affinity in comparison with the natural substrate L-tyrosine. This effect was not observed in our previous work on triazene derivatives[13−15] (Table S4).

The values of log P presented in Table 1 for compounds **11** ($3.51 < \log P < 4.31$) and **13** ($3.99 < \log P < 5.06$) demonstrate their lipophilicity. The presence of the linker amide (compounds **13**) increases the lipophilic character of the hybrids. With regard to compounds **11,** the experimental and calculated log P values are close. Comparison of compounds **13** with derivatives **2** shows that the introduction of sulfur in the ethyl chain increases the log P value by one unit.[15] Other molecular properties that must be contextualized with log P values are included in Table S5.

The cytotoxic activities of hybrid compounds **11** and **13** (Table 2) were also investigated. Compounds were screened in terms of antiproliferative properties following their incubation in two human melanoma cell lines (MNT-1 and A375), one murine cell line (B16F10), and a healthy human keratinocyte cell line (HaCaT). In general, the compounds were less cytotoxic to HaCaT cells, with a selectivity index ranging from 2 to 3. The hybrid compounds showed similar antiproliferative properties toward human and murine melanoma cell lines. Hybrid compound **11d** displayed the highest antiproliferative properties, with IC_{50} values ranging from 49 to 51 μM for melanotic cell lines (MNT-1 and B16F10) and 43.8 μM for the amelanotic cell line (A375).

Table 2. Inhibitory Effects of the Tested Compounds 11 and 13 after Incubation for 72 h with MNT-1 (Human Melanoma), B16F10 (Murine Melanoma), HaCaT (Human Keratinocyte), and A375 (Human Melanoma) Cells

	IC_{50} (μM)			
compound	MNT-1	B16F10	A375	HaCaT
11a	>75	>75	nd[a]	>150
11b	74.1 ± 2.9	78.3 ± 2.7	nd	>130
11c	>75	>75	nd	>150
11d	49.4 ± 1.6	51.0 ± 1.5	43.8 ± 3.3	>100
11e	>75	>75	nd	>150
13a	>75	>75	55.0 ± 3	nd
13b	Nd	>75	47.0 ± 1.6	45.8 ± 1.2
13c	>75	>75	>75	nd
13d	>75	>75	61.0 ± 4	nd
13e	>75	>75	>75	nd
13f	nd	>75	>75	>75
4a	>60	>60	nd	>60
4c	>60	>60	nd	>60
4d	>60	>60	nd	>60
4e	>75	>75	nd	>130
10 + 4e	37.1 ± 1.9	>75	nd	130 ± 8.7
10	42.8 ± 1.4	>75	nd	>125
TMZ	>75	>75	>75	>75

[a]nd: not determined.

Surprisingly, compounds **13a**, **13b**, and **13d** exhibited higher antiproliferative properties for the amelanotic A375 cell line. This cytotoxic activity probably does not depend exclusively on the presence of tyrosinase, in contrast to what was previously observed for compounds **1**, **2**, and **3** (without sulfur in their structure), in which the cytotoxic activity was slightly superior in melanotic cell lines.[14]

The individual cytotoxicities of the two pharmacophores in hybrids **11** were also determined. For that, 4-S-CAP **10** and MMTs **4a−e**, either separately or in a physical mixture at the same concentration as the one tested for hybrid compounds **11**, were evaluated in the same cell lines. MMTs **4a−e** alone did not show cytotoxicity in any of the cell lines under study. On the other hand, **10** exhibited greater cytotoxicity toward MNT-1 cells, with an IC_{50} value of 42.8 μM and a selectivity index of ≥3. The incubation of both compounds (**10** + **4e**) displayed cytotoxicity toward the MNT-1 cell line with a selectivity index of ≥3. The results of this study showed that the conjugation of the two pharmacophores in a hybrid molecule resulted in antitumor activity that depends mostly on the triazene structure. Furthermore, the type of linker between the two pharmacophores also influences the antitumor activity, since compounds with the amide linker showed cytotoxic activity only against the A375 cell line. Another important observation results from the data obtained for TMZ, the triazene compound used in the assay as a positive control (Table 2). Compound **11d** displayed superior antiproliferative activity compared with TMZ. Also, Figure 4 shows the results of cellular viability (%) for compounds **13b** and **13f** compared with the positive control.

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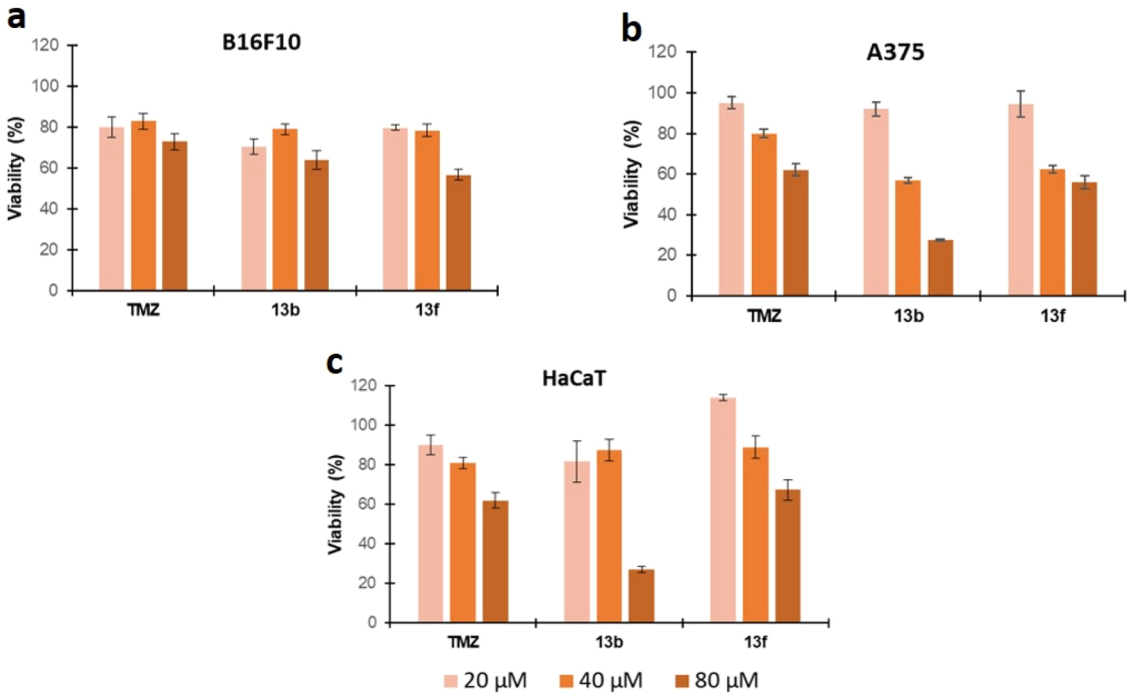

Figure 4. Cellular viability of the (a) murine melanoma cell line (B16F10), (b) human melanoma cell line (A375), and (c) human epidermal keratinocyte cell line (HaCaT) in the presence of two hybrid compounds, **13b** and **13f,** with potential antiproliferative properties. TMZ was used as a positive control. The compounds were incubated for 48 h, and the cell viability was determined by the MTS assay. The cell concentration was 1.5×10^3 cells/mL. The tested concentrations of all compounds were 20, 40, and 80 μM.

Again, compound **13b** exhibited higher cytotoxic properties than TMZ toward the A375 cell line. However, the selectivity index was <1.

Next, the hepatotoxicities of derivatives **11** were evaluated. Quinones have two chemical properties that must be addressed with regard to their potential overall toxicity: electrophilicity and oxidative stress promotion. As electrophiles, they can bind to sulfhydryl proteins, causing protein denaturation or enzyme inhibition. As oxidants, they can promote oxidative stress by generation of reactive oxygen radicals and the depletion of antioxidants from cells. Consequently, quinones are associated with the *in vivo* occurrence of acute cytotoxicity, immunotoxicity, or carcinogenesis.[22] In studies on the antimelanoma effect of 4-S-CAP **10** in *in vivo* models, the existing information on the metabolic fate of this compound is very scarce, namely, the *in vivo* formation of quinones as a result of oxidation by hepatic microsomes. Jimbow et al.[23] disclosed that in a female C57BL/6J murine melanoma metastases model possessing both B16F10 subcutaneous melanoma nodules and melanoma lung colonies, after a single intraperitoneal injection of radiolabeled phenolic thioether *N*-acetyl-4-*S*-cysteaminylphenol (NA-CAP), the radioactive material was cleared from the body and was not detectable in any normal organs except the lumen of the large intestine. On their interpretation, the significant detoxication of NA-CAP occurred in the liver and was followed by the excretion of NA-CAP metabolites into the bile. However, the metabolites that would have resulted from hepatic metabolism were not described or quantified. The authors suggested that reactive intermediates of NA-CAP (quinones or semiquinones) may result from the interaction of the compound with enzymes such as CYP450. Our previous studies regarding the potential hepatotoxicity of compounds **2** (derived from 4-hydroxypropanoic acid) revealed high concentrations of quinones and increased glutathione (GSH) depletion (35% to 64% in 180 min) in the incubation assays with rat microsomes, a common feature of 3-hydroxy- or 4-hydroxyphenyl compounds.[15,24] (Figure S3 and Table S6). Since the presence of a phenol group in hybrid compounds **11** raises a potential hepatotoxicity risk, we evaluated this risk by measuring the GSH depletion promoted by the compounds. In this study with rat microsomes we did not observe the depletion of GSH (Figure S4b). The study was complemented with HPLC analysis to search for *o*-quinones as CYP450 metabolites. These studies confirmed the absence of quinones (Figure S4a). The results allow us to claim that compounds **11** do not generate *o*-quinones in the presence of rat microsomes, giving them high therapeutic potential.

To provide further insights into the safety of the hybrid compounds, in a very preliminary *in vivo* study, the most promising compound, **11d**, was intravenously administered to BALB/c mice at a dose of 12 mg/kg of body weight 3 times *per* week for 1 week. The animals' behavior was monitored every day. Two days after the last injection, the mice were sacrificed, and blood and organs were collected and analyzed. The control group was constituted by mice that received only the vehicle for compound **11d** solubilization, a mixture of PBS and Cremophor (5% p/v). The tissue index was calculated according to eq 3:

$$\text{tissue index} = \sqrt{\frac{\text{organ weight}}{\text{animal weight}}} \times 100 \qquad (3)$$

The tissue index and hepatic biomarkers were assessed, as depicted in Table 3. Determination of the tissue index allows the evaluation of changes in the weights of specific organs that may occur relative to the whole body weight. Changes in organ weight have long been accepted as a sensitive indicator of chemically induced toxicity.[25] In particular, tissue indexes of the liver, kidneys, and spleen are considered the most valuable in different areas such as pharmaceutical, veterinary, chemical, and

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Table 3. Preliminary *In Vivo* Safety Assays of 11d: Tissue Indexes and γ-GT Values

Group of mice	Tissue Index					γ-GT (U/L)
	Liver	Spleen	Kidney	Lung	Heart	
Control	22±1	6±1	13±1	8±1	7±1	47±9
Compound 11d	22±1	6±1	13±1	8±1	7±1	57±4

food/nutritional/consumer.[25] While an increased tissue index indicates organ hypertrophy, congestion, or edema, a decreased ratio indicates organ atrophy and degenerative changes.[26]

In terms of tissue index, no changes for the analyzed organs were observed among the two groups of mice (Table 3). The γ-glutamyl transferase (γ-GT) blood levels were also determined, since these levels are used as an index of liver dysfunction and the enzyme plays an essential role in glutathione metabolism. No statistically significant differences between the control group and the animals that received compound **11d** were observed.

To complement the evaluation of the *in vivo* safety of compound **11d**, a hemolytic assay using human red blood cells was performed. The results showed that compound **11d** at concentrations ranging from 0.2 to 400 μM did not promote hemolysis (less than 1%) after 1 h of incubation at 37 °C.[27] Taken together, these results highlight the safety profile of compound **11d**.

In conclusion, the studies presented herein led to the synthesis and identification of novel hybrid compounds designed to be activated by tyrosinase, the enzyme overexpressed in melanoma tumor cells. Under physiological conditions, compounds **11** and **13** proved to be very stable, being slowly hydrolyzed by plasma enzymes. The parameters of enzymatic kinetics characterize these compounds as excellent substrates of tyrosinase, as confirmed by mass spectroscopy studies regarding the identification of the generated metabolites. Some of these compounds reveal an increased and selective potency toward melanoma cell lines. Cytotoxicity assays highlighted **11d** as having the highest antiproliferative properties (IC_{50} values of 49.4 and 51.0 μM for MNT-1 and B16F10, respectively). The hepatotoxicity and hemolysis studies for the most promising compound, **11d**, revealed a very good safety profile since the formation of oxidative quinones occurred only in melanoma tumor cells (promoted by tyrosinase) and not by hepatic microsomal oxidation (CYP450). In addition, preliminary safety *in vivo* assays with BALB/c mice confirmed the absence of chemically induced toxicity over vital organs and no toxic hepatic effects. On the other hand, the cytotoxic effect demonstrated by compounds **13** on the A375 amelanotic cell line opens new perspectives about other potential pathways involved in tumor cell death promoted by sulfur analogues. Currently, encapsulation studies in lipid-based delivery systems of the most promising compound, **11d**, are underway, as well as *in vivo* evaluation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.1c00252.

Experimental procedures for the synthesis and full NMR characterization of compounds **11**−**13**; description of stability studies under physiological conditions and in the presence of mushroom tyrosinase; general procedures for determination of apparent partition coefficients, cytotoxicity screening assays, and hepatotoxicity studies; and assay protocols for the *in vivo* toxicity profile in BALB/c mice for compound **11d** (PDF)

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Notes

The authors declare no competing financial interest.

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