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Original article

Synthesis and evaluation of *N*-acylamino acids derivatives of triazenes. Activation by tyrosinase in human melanoma cell lines

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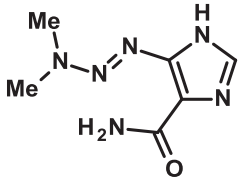
ABSTRACT

In this research work we report the synthesis of a new series of triazene prodrugs designed for Melanocyte-Directed Enzyme Prodrug Therapy (MDEPT). These compounds are derived from the *N*-acyltyrosine amino acid – a good enzyme substrate for the tyrosinase enzyme, which is significantly overexpressed in melanoma cells. We analysed their chemical stability and plasma enzymatic hydrolysis, and we also evaluated the release of the antitumoral drug in the presence of the tyrosinase. Subsequently, we performed the evaluation of the prodrug cytotoxicity in melanoma cell lines with different levels of tyrosinase activity. Prodrug **5c** showed the highest cytotoxicity against melanoma cell lines, and this effect correlated well with the tyrosinase activity suggesting that prodrug cytotoxicity is tyrosinase-dependent.

1. Introduction

Disseminated melanoma is a highly metastatic malignancy which is generally lethal. Systemic chemotherapy is often the only recourse, but to date the results have been very disappointing and the lack of selective cytotoxicity often leads to intolerable side effects. Triazenes are a well-known class of anticancer drugs used in the treatment of melanoma, which have a N=N–N structure type generally close to an aromatic ring. Dacarbazine (DTIC) (**1**) and Temozolomide (**2**) are the only triazenes in current chemotherapy [1,2]. The mechanism of their antitumour activity works *via* the alkylation of the DNA, through *in vivo* generation of methyldiazonium cations. Both temozolomide and DTIC are prodrugs of the active alkylating agent 5-(3-methyltriazen-1-yl)imidazole-4-carboximide – MTIC (**3**). Unlike DTIC, which requires metabolic activation by cytochrome P450, temozolomide spontaneously converts to **3** under physiologic conditions [3–7].



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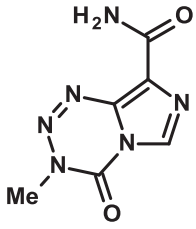

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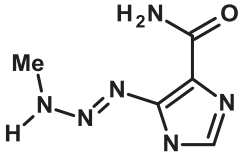

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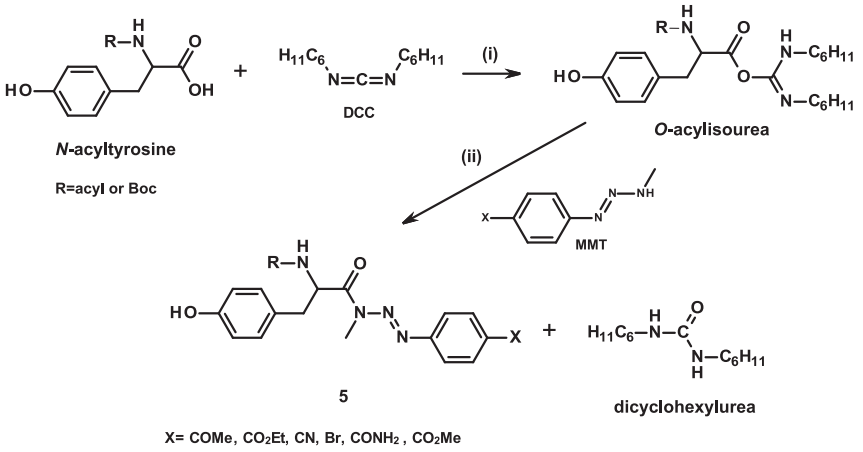


Scheme 1. Synthesis of triazene prodrugs **5**. (i) Tetrahydrofuran (THF), 1 h, rt; (ii) Triethylamine (TEA), NaH, THF, 48 h, rt.

Tyrosinase (EC 1.14.18.1), is an enzyme which abounds in melanoma cells and is considered an ideal molecular target for the development of anti-melanoma drugs [8]. This enzyme catalyzes the oxidation of L-tyrosine into the corresponding *o*-quinone, dopaquinone, which is the first step in melanin pigment biosynthesis. The degree of malignancy of pigment cells was shown to correlate with tyrosinase activity [9]. Melanocyte-Directed Enzyme Prodrug Therapy (MDEPT) is an acronym coined for a prodrug strategy whose target is the treatment of melanoma: since tyrosinase is naturally overexpressed in the tumour, and virtually absent from the other cells, it provides an in-built drug delivery mechanism that will be selective of melanoma tumours [10]. The efficacy of the MDEPT strategy relies on the fact that tyrosinase activity is aberrantly high in melanoma tumour cells. In this tyrosinase mediated drug delivery system the release of the cytotoxic agent from prodrugs is a consequence of the recognition and oxidation of the promoiety of prodrugs. Our group had previously investigated the structure–activity relationship of a series of tyramine and dopamine derivatives of triazenes. We synthesized and evaluated a series of prodrugs to be activated by tyrosinase [11]. The release of the monomethyltriazene (MMT) was based on an intramolecular Michael addition reaction. Unfortunately, we had to conclude that this process required a much better nucleophile than the urea nitrogen atom [12].

Following the same strategy, with the purpose of developing more potent and better targeted compounds for melanoma, we developed new triazene derivatives **5a**–**f** with an amide linker between the cytotoxic unit, the triazene, and the carrier, *N*-acyltyrosine. The use of amino acids as promoieties offers several advantages: (a) a number of structurally diverse amino acids (aliphatic, aromatic, acidic, basic, neutral, etc.) are commercially available, (b) there are fewer safety concerns regarding the use of amino acids as promoieties, (c) amino acid and peptide chemistries are well established, and (d) amino acid prodrugs can potentially target carrier-mediated transporters for their transport across cell membranes [13]. The aim of our analysis is to find out whether or not the triazene prodrugs synthesized **5a**–**f** show the hoped for tyrosinase dependent cytotoxicity and specificity. To achieve this goal we performed assays of activation of prodrugs by mushroom tyrosinase and cytotoxicity studies in human melanoma cell lines.

2. Results and discussion

2.1. Synthesis

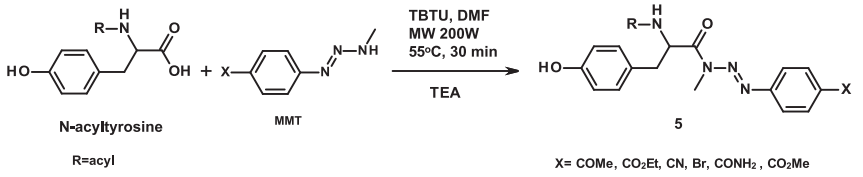
The synthesis of aminoacylderivatives of triazenes **5a**–**f** was based on the coupling of the *N*-acyltyrosine with the triazene. The synthetic methodology implies the use of a coupling activator such as Dicyclohexylcarbodiimide (DCC), due to the mild conditions under which it can be used, and which are important for the triazene stability. The first step is the addition of the carboxy group of *N*-acyl or *N*-Boc-L-tyrosine to the carbodiimide functionality, to produce rapidly an *O*-acylisourea which is a potent acylating agent. In the subsequent step, the addition of the nucleophile triazene leads to the final prodrugs *N*-acyltyrosine triazene derivatives **5a**–**f**, and *N*-Boc-L-tyrosine triazene derivatives **6a**,**b** with the concomitant formation of dicyclohexylurea (Scheme 1). This urea is only partially soluble in most solvents but in our case it gave rise to purification problems. Another drawback of this methodology is that the reactions are not complete and sometimes, depending on the triazene, the yields are low.

To improve the coupling rates and yields we also applied microwave assisted synthesis (MAS), which functions as an alternative to the traditional amide coupling described in Scheme 1. The syntheses were found to be achievable, since we were concerned with the stability of the triazenes obtained by MAS. The results reveal a much more rapid and cleaner reaction, and – to our knowledge – these results provide the first demonstration of MAS applied to MMT reactivity (Scheme 2). However, the final yields obtained were found to be of the same order of magnitude as the ones obtained by the DCC methodology.

2.2. Kinetic study

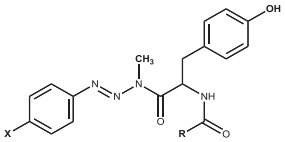
2.2.1. Stability in phosphate buffer

The α-(acyltyrosine)acyltriazenes **5a**–**f** hydrolysis in an isotonic phosphate buffer generates the corresponding 3-methyltriazenes



Scheme 2. Synthesis of prodrugs **5** by MAS.

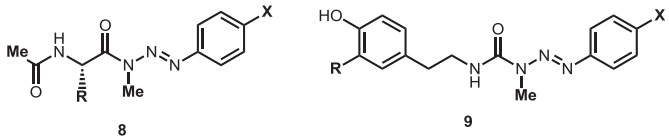
Table 1
Log *P* and half-lives of prodrugs, $t_{½}$, in PBS buffer, 80% human plasma and in the presence of tyrosinase (300 U/mL) at 37 °C.

Compound	X	R	Log P_{exp}	$t_{1/2}$		
				pH 7.4 buffer (h)	80% Human plasma (h)	Tyrosinase (min)
5a	COMe	Me	2.33 ± 0.02	56.6 ± 1.0	1.41 ± 0.13	19.8 ± 1.2
5b	CO_2Et	Me	2.96 ± 0.02	70.3 ± 0.8	5.32 ± 0.08	18.0 ± 1.2
5c	CN	Me	2.14 ± 0.09	42.7 ± 0.5	1.23 ± 0.17	15.6 ± 1.8
5d	Br	Me	3.36 ± 0.07	a)	6.80 ± 0.01	8.9 ± 0.10
5e	CO NH_2	Me	1.27 ± 0.02	40.6 ± 0.6	5.19 ± 0.08	17.4 ± 0.42
5f	CO_2Me	Me	2.12 ± 0.07	75.1 ± 1.7	2.80 ± 0,26	18.3 ± 0.56
6a	COMe	$OC(Me)_3$	3.85 ± 0.05	72.2 ± 0.8	9.54 ± 0.86	b)
6b	CN	$OC(Me)_3$	3.90 ± 0.09	48.5 ± 0.7	8.10 ± 0.62	b)

a) stable for 15 days.
b) unchanged over 1 h.
[Concentration of the substrate] = 10^{-5} M.
All the experiments were performed in triplicate and the data given are the corresponding average values.

which get spontaneously decomposed into the corresponding aniline. These reactions were easily followed by HPLC, monitoring both the loss of starting material and the formation of products.

Table 1 show that with a physiological pH these compounds get decomposed with half-lives over 41 h emphasizing their high level of chemical stability. A comparison between the half-lives of prodrugs **5a**–**f** and the half-lives of several α-(acylamino)acyltriazenes **8a**–**h** (Fig. 1) shows that these derivatives revealed a higher chemical stability [14]. In fact, in PBS, *N*-acyltyrosine derivatives are four times more stable than their analogues derived from *N*-acylalanine **8a**–**e** or the analogues derived from *N*-acylglycine **8f** and *N*-acylphenylalanine **8g**. However, it seems that these derivatives are less stable than the prodrugs containing tyramine **9a** or dopamine **9b** (Fig. 1) promoiety coupled with the cytotoxic triazene *via* a urea functional group [11,14]. Another feature affecting the relative magnitude of the $t_{½}$ values for the compounds **5** is the influence of the substituent, –X, present in the aryl group. Inspection of the table enables us to assert that electron-withdrawing groups increase the rate of hydrolysis (these compounds present a Hammett correlation with a ρ value of +0.92; $r^2 = 0.95$; $n = 4$). The same was also previously observed in triazene prodrugs with peptide and urea linkage [14].



8

8a - R=Me; X= COMe
8b - R=Me; X= COOEt
8c - R=Me; X= CN
8d - R=Me; X= Br
8e - R=Me; X=$CONH_2$
8f - R=H; X=CN
8g - R=CH_2Ph; X=CN
8h - R=$CH(CH_3)_2$; X=CN

9

9a - R=H
9b - R=OH

Fig. 1. Chemical structures of α-(acylamino)acyltriazenes **8a**–**h** [14] and of urea triazene derivatives **9a**,**b** [11].

2.2.2. Stability in human plasma

Blood serum and plasma contain a range of enzymes that catalyse the hydrolysis of amides [15]. Consequently, we were interested in examining the stability of our triazene derivatives since they contain an amide link.

The results in Table 1 show that in human plasma all the compounds are substrates for the plasma enzymes with half-lives ranging from 1.23 to 6.8 h. *N*-acyltyrosine derivatives hydrolyse in 80% human plasma containing 20% of isotonic phosphate buffer to the corresponding MMT, which further break down into the corresponding anilines (Fig. 2). The hydrolysis rates are 8–40 times faster than with phosphate buffer but still quite stable in human plasma. Analysis of $t_{½}$ for compounds **5a**–**f** reveals that the rate of reaction is influenced by the nature of the substituent –X in the triazene aryl group, the more reactive compounds being those containing electron-withdrawing substituents. There is a correlation between log $t_{½}$ values and the Hammett ρ_p values yielding a ρ value of 1.9 ($r^2 = 0.80$; $n = 6$). When we compared the half-life

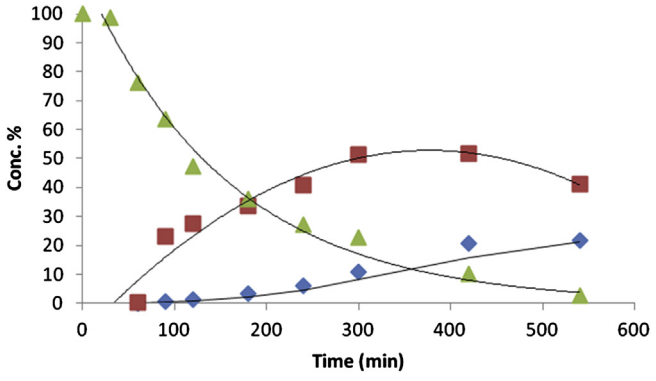


Fig. 2. Time course for the hydrolysis of **5c** in 80% in human plasma at 37 °C: ▲, **5c**; ◆, corresponding aniline; ■, corresponding MMT.

values of our *N*-acyltyrosine derivatives **5a**–**f** in human plasma with the correspondent values obtained for derivatives **8a**–**h** we observed that for the recognition and hydrolysis promoted by plasma enzymes, the compounds behaved the same way, that is, presented the same easiness of hydrolysis, so the type of *N*-acylaminoacid seems to have little effect in this medium.

Comparison with prodrugs **9** (tyramine and dopamine triazene derivatives) show that compounds **5** are more susceptible to plasma enzymes although prodrug present an adequate plasma stability for MDEPT strategy.

The partition coefficients between octanol and pH 7.4 phosphate buffer at 25 °C for compounds **5** (Table 1) give values of log *P* ranging from 1.27 to 3.36. Our prodrugs **5** proved to be more lipophilic than their analogues **8**. There is a correlation between prodrug stability in human plasma (log $t_{1/2}$) and lipophilicity (log *P*) (Fig. 3) with the more lipophilic prodrugs being the more stable. Substrate lipophilicity is one of the main features that influence esterase activity – decreasing enzyme activity being associated with increasing lipophilicity as we observed in our study [16].

$$\log t_{1/2} = 4.19\log P - 7.33\left(n = 5,\ r^2 = 0.88\right) \quad (1)$$

2.2.3. Triazene derivatives as substrates for tyrosinase

Triazene prodrugs **5a**–**f** were studied as mushroom tyrosinase substrates by HPLC. Since purified human tyrosinase is not commercially available, in this study we used a mushroom tyrosinase enzyme *in vitro* assays. For each compound, the half-lives in the presence of the enzyme, were determined (Table 1) and the products of activation identified and analysed. Prodrugs are oxidized by tyrosinase into the quinone intermediate. However, after a 15 min reaction it is possible to quantify the cytotoxic monomethyltriazene. Actually it is present along the total reaction time but in a very small proportion (below five per cent) (Fig. 4). A complete mass balance for the reaction is not possible since the aromatic amines (the final product of the reaction) are as also tyrosinase substrates and are concomitantly oxidized by the enzyme [17].

The results show that the *N*-acyltyrosine triazene derivatives **5a**–**f** are excellent tyrosinase substrates with half-lives ranging from 9 to 20 min (Table 1). However, other molecule functionalities seem to play a role in the recognition by the enzyme. In fact, compounds like **6a** and **6b** that contains the *N*-Boc group are not a substrate for the enzyme. The catalysis of the reaction by tyrosinase depends on the capacity of the substrate to bind to a tyrosinase active site. If the substrate binds tightly to the active site, tyrosinase will oxidize the substrate efficaciously. To investigate the role of a

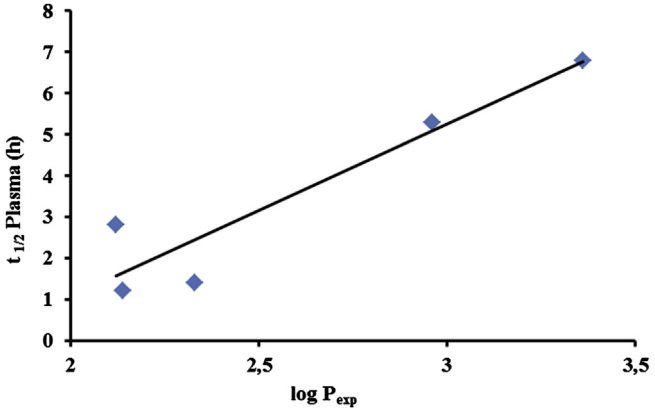


Fig. 3. Plot of the plasma half-lives ($t_{½}$) versus lipophilicity for compounds **5**.

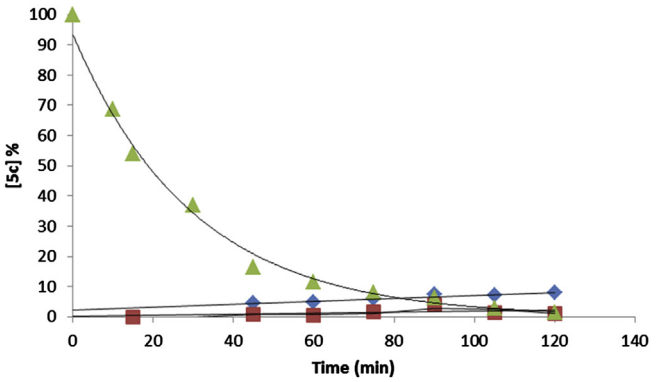


Fig. 4. Time course for the hydrolysis of **5c** in the presence of tyrosinase at 37 °C: ▲, **5c**; ♦, corresponding aniline; ■, corresponding MMT.

phenolic group as an essential functional group in the chemical structure of the prodrugs, we synthesized the phenylalanine derivative **8g**. This compound is not a substrate for tyrosinase since in the presence of the enzyme the half-life remains the same as in PBS.

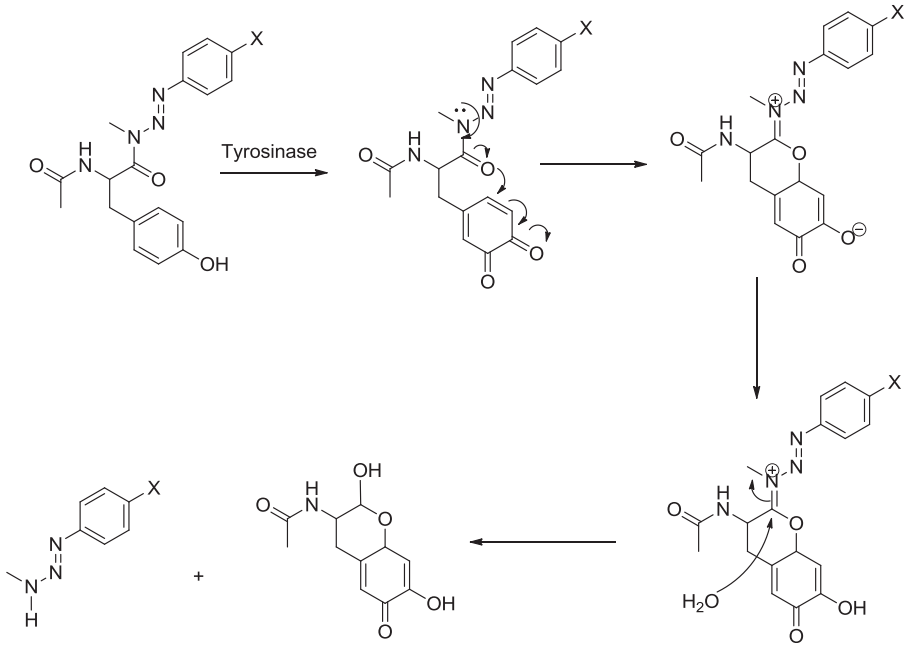
Presented in Table 2 are the kinetic parameters (K_m, V_{max}, k_{cat}, and k_{cat}/V_{max}) as well as the van der Waals volume calculated for compounds **5c**, **5f** and **6a**. The evaluation of comparable kinetic parameters is crucial to obtain a more thorough understanding of the reactivity of different tyrosinase substrates. The K_m values for compounds **5c** and **5f** compare favourably with other phenolic substrates including L-tyrosine, tyramine and dopamine (K_m = 0.25 ± 0.03 mM, 0.54 ± 0.05 and 0.28 ± 0.01 mM, respectively) indicating that they have structures that can bind to the active site in a very effective way, and consequently, should oxidize very easily [18,19]. On the contrary, tyrosinase could not efficaciously oxidize compound **6a** and **6b**, probably due to unexpected steric interactions between the *N*-Boc group and the tyrosinase binding motif, which results in an unfavourable alignment and position of **6a** and **6b** on the active site. From what is known about the substrate specificity of tyrosinase, the access to the active site is quite limited, and the presence of the bulky *tert*-butyl group in the lateral chain of substituted phenols or in the lateral chain of sterically hindered amino acids phenylhydrazides is limitative of any oxidation reaction [20–22].

The K_{cat}/K_m ratio – the catalytic efficiency – that determines the rate of reaction at low substrate concentrations, is significantly greater than those of tyramine or dopamine triazene prodrugs (1.9 and 8.5 respectively) suggesting a better affinity for these new substrates [11]. Comparing with our previous triazene prodrugs, derived from tyramine and dopamine, these new derivatives present the great improvement of liberating the cytotoxic monomethyltriazene through the action of the enzyme. These experimental results led us to propose a mechanism for the release of MMT based on the intramolecular cyclization of the quinone

Table 2
Kinetic parameters for the oxidation of acyltyrosine derivatives by mushroom tyrosinase.

Prodrug	K_m (mM)	V_{max} mM/min	k_{cat} (s^{-1})	K_{cat}/K_m ($M^{-1}s^{-1}$)	van der Waals volume (Å^3)
5c	0.20 ± 0.09	$5.4 \times 10^{-6} \pm 5.3 \times 10^{-7}$	0.03	151.2	329
5f	0.22 ± 0.04	$9.7 \times 10^{-6} \pm 3.9 \times 10^{-7}$	0.05	246.5	374
6a	–	–	–	–	388

Values reported are the average ± one standard deviation for the three independent determinations. The van der Waals volumes were calculated according to the literature [23].



Scheme 3. Proposed mechanism for MMT release after tyrosinase oxidation.

intermediate and the formation of a hemiaminal/iminium ion, which subsequently suffers a chemical hydrolysis producing the MMT triazene and a cyclic hemiacetal (Scheme 3).

Cyclization by nucleophilic attack of the amide nitrogen of *N*-acyltyrosine to the quinone intermediate is less probable as previous works have demonstrated. *N*-acyltyrosine is considered a non-cyclizing phenolic substrate of mushroom tyrosinase since the presence of the acetyl substituent in nitrogen inhibited cyclization (Scheme 4) [24].

2.3. Determination of prodrug cytotoxicity in melanoma cell lines: tyrosinase activity dependence

To investigate the cytotoxic effect of the prodrugs, three different human melanoma cell lines, MNT-1, SKMEL-30 and M8, were used as *in vitro* models.

Both MNT-1 and SKMEL-30 cell lines displayed a relevant level of tyrosinase activity, namely 5.30 and 4.13 mU/mg respectively, contrary to M8 cell line whose tyrosinase activity was undetected (Fig. 5) and was therefore used to represent a tyrosinase-absent cell line.

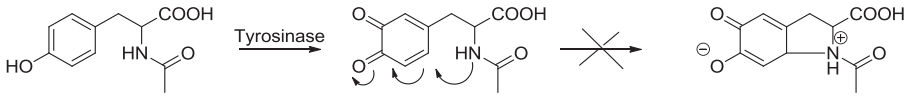
The triazene prodrugs **5a**, **5b** and **5c** were selected because of their partition coefficients (Table 1), which provide an estimate of their permeability across the membrane.

The working concentration range of the prodrugs was first determined by both the colorimetric (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay, which detects metabolic active but not inactive cells, and by microscopic observations with trypan blue dye exclusion assay (data not shown). The concentration of 50 and 100 μM was then selected to analyse further the percentage of apoptotic/necrotic cells derived after 48 h exposure of each cell line to the prodrugs.

All the prodrugs tested led to a dose-dependence increase in the number of apoptotic/necrotic MNT-1 cells, confirming their cytotoxic effect (Fig. 6). Compared to non treated cells, the percentage of apoptotic/necrotic cells increased by 7.88 and 11.23% respectively in the presence of 50 and 100 μM of prodrug **5a**, 17.46 and 21.23% respectively in the presence of 50 and 100 μM of prodrug **5b** and finally 10.67 and 29.36% respectively in the presence of 50 and 100 μM of prodrug **5c** (Fig. 6A). The cytotoxicity of prodrug **5c** was next analysed in another tyrosinase rich cell line (SKMEL-30), and a tyrosinase-absent (M8) cell line. Exposure to 50 and 100 μM of prodrug **5c** led to a significant increase of 8.50 and 17.01% respectively, in the percentage of MNT-1 apoptotic/necrotic cells and 10.41 and 12.47% respectively in the percentage of SKMEL-30 apoptotic/necrotic cells (Fig. 6B). On the contrary, M8 cells exposed to 50 and 100 μM of prodrug **5c** showed no significant alteration in the percentage of apoptotic/necrotic cells. Interestingly the percentage of apoptotic/necrotic cells obtained after prodrug **5c** exposure showed a direct correlation ($r = 0.9045$, $p = 0.0132$) with the tyrosinase activity presented by the cell lines. Moreover, we compared the cytotoxicity/specificity of prodrug **5c** and Temozolomide, a commercially available prodrug with tyrosinase-independent toxicity used for the treatment of melanoma. Interestingly, M8 cell line appeared to be more sensitive to Temozolomide than MNT-1 and SKMEL-30 cell lines – as illustrated – with an increased susceptibility at the highest prodrug concentration (Fig. 6C).

3. Conclusions

In conclusion, most of the derivatives tested in this study underwent metabolic oxidation by tyrosinase with the



Scheme 4. Oxidation of *N*-acetyltyrosine by tyrosinase.

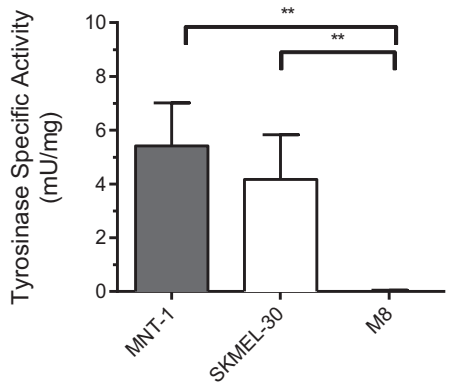


Fig. 5. Tyrosinase activity in melanoma cell lines. Tyrosinase activity was determined in the MNT-1, SKMEL-30 and M8 cell lines by measuring the formation of dopachrome as referred in the Experimental section. Data represent the averages ± SD of three different experiments each carried out in duplicate. The statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$) refers to the differences compared with the M8 cell line in which no relevant tyrosinase activity was detected (0.00 mU/mg).

correspondent liberation of triazene, the antitumoral agent. These compounds prove to be extremely stable in a pH 7.4 buffer and in human plasma and simultaneously are good substrates for tyrosinase. The cytotoxicity assays also show that the all-newly-synthesized prodrugs have a cytotoxic effect being the prodrug **5c** the highest cytotoxic. The correlation between this cytotoxicity and the tyrosinase activity suggests that the prodrug effect is tyrosinase dependent. When compared with Temozolomide prodrug **5c** showed an increased specificity for tyrosinase expressing cells, suggesting its strong therapeutic potential for melanoma treatment.

4. Experimental

4.1. General information

All the triazenes used in this study should be considered as mutagenic and/or carcinogenic and appropriate care should be taken in order to handle them safely.

Microwave heating was carried out with a single-mode cavity Discover Microwave Synthesizer (CEM Corporation, NC) producing continuous irradiation at 2455 MHz.

Melting points were determined using a Kofler camera Bock-Monoscop "M" and were not corrected. IR spectra were recorded as KBr discs using a Perkin Elmer 1310 spectrophotometer. ^{1}H and ^{13}CNMR spectra were recorded in $CDCl_3$, MeOD or DMSO-d_6 solutions using a Brucker AM 400 WB spectrometer. Chemical shifts, δ, are reported as p.p.m. from Me_4Si, and coupling constants, J, in Hz. Mass spectra were recorded in a Micromass Quattro Micro API benchtop, Waters mass spectrometer. All chemicals were reagent grade except those for kinetic studies and HPLC, which were of analytical or LiChrosolv® (Merck) grade. 1-Aryl-3-methyltriazenes were synthesized by previously published methods [25]. The (*S*)-*N*-acetyltyrosine and (*S*)-*N*-Boc-tyrosine were purchased from Sigma and Mushroom tyrosinase (5350 U/mg) was purchased from Sigma–Aldrich.

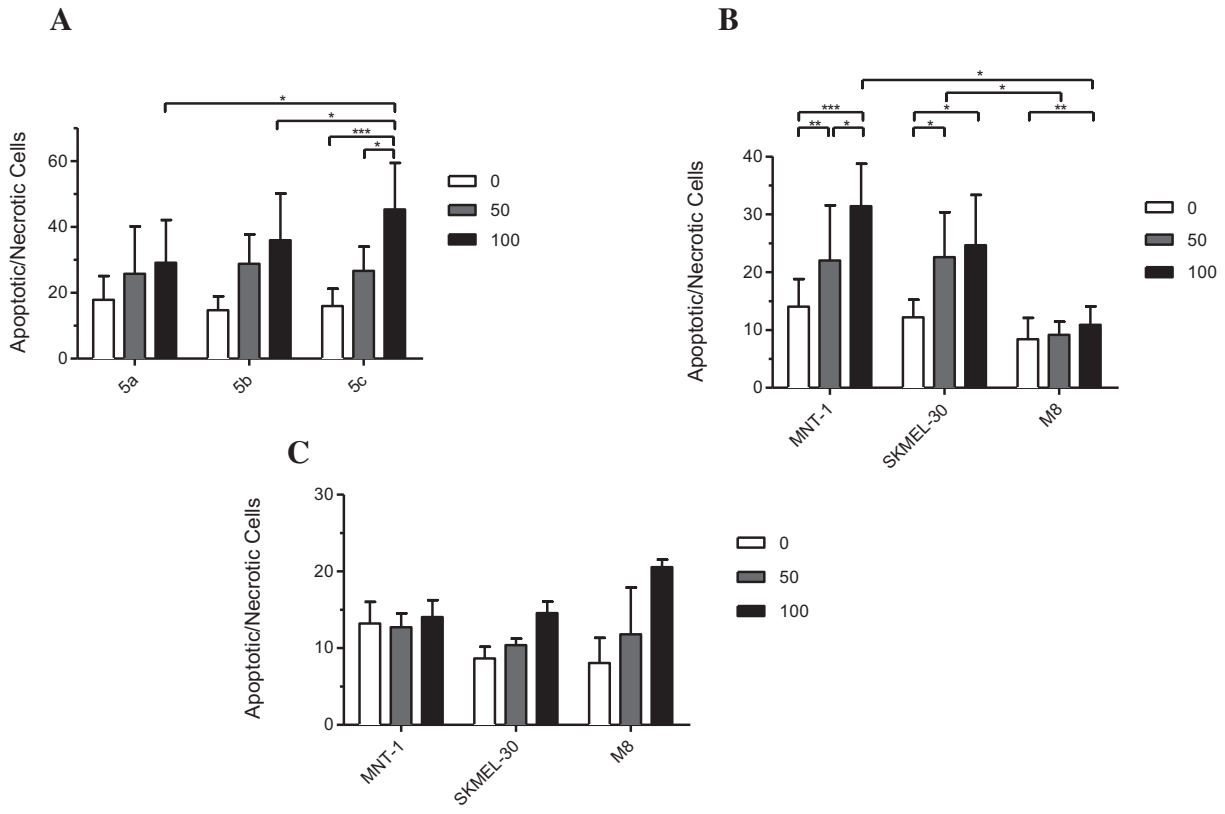


Fig. 6. Drug cytotoxicity on melanoma cell lines. The percentage of apoptotic/necrotic cells was estimated by flow cytometry after 48 h exposure to 0, 50 or 100 μM of the prodrugs. Cells were double-stained with APC-labelled annexin V and 7-AAD as mentioned in the Experimental section. A: Effect of prodrugs **5a**, **5b**, **5c** on MNT-1 cell line. B: Effect of prodrug **5c** on MNT-1, SKMEL-30 and M8 melanoma cell lines. C: Effect of Temozolomide on MNT-1, SKMEL-30 and M8 melanoma cell lines. The values represent the averages of at least 3 independent assays. The degree of significant difference is represented by $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.005$.

4.2. Synthesis

4.2.1. General procedures for the synthesis of prodrugs **5a**–**f** and **6a**, **b**

N,*N*′-Dicyclohexylcarbodiimide (2.4 mmol) was added to a solution of the *N*-acyltyrosine (1.9 mmol) in tetrahydrofuran (THF, 10 mL). After 60 min at room temperature under stirring, triethylamine (0.19 mmol) and the appropriate 1-aryl-3-methyltriazene (1.9 mmol) previously activated with NaH (1.9 mmol) in THF (5 mL) were added. After 48 h at room temperature, the *N*,*N*′-dicyclohexylurea by-product was removed by filtration and the solvent removed under reduced pressure. The crude residue was subjected to chromatography (silica gel 60, 70–230 mesh ASTM, Merck) using one of the following systems: diethyl ether: methanol (for **5a**), dichloromethane: methanol (for **5b**, **c**, **6a**, **b**), ethyl acetate: methanol (for **5d**, **5e**), ethyl acetate: dichloromethane (for **5f**) (see Supporting information, Table S1). The organic layers correspondents to the pure compounds were filtered after being dried on $MgSO_4$ before evaporation under vacuum. The isolated *N*-(acyltyrosine)acyltriazenes were subsequently recrystallized from ethyl acetate/n-hexane (for compounds **5a**, **5b**, **5e**, **6a**, **6b**, 17:3) and dichloromethane/*n*-hexane (**5c**, **5d**, **5f**, 17:3). For the synthesis of compounds **6** we used *N*-Boc-L-tyrosine as the starting material.

4.2.2. General procedure for the synthesis of α-(acyltyrosine) acyltriazenes by microwave synthesis

A 10 mL reaction vessel containing a small magnetic stir bar was load in ***N*,*N*,*N*′,*N*′-Tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate** (TBTU, 1 mmol), *N*-acyltyrosine (1 mmol), Dimethylaminopyridine (DMAP, 1 mmol) and the appropriate 1-aryl-3-methyltriazene (1 mmol). The reagent mixture was dissolved with 2 mL of dimethylformamide (DMF) and the reaction was carried in the presence of triethylamine (0.1 mmol). The tube containing the reaction mixture was sealed with an ActiVent cap and then it was exposed to microwave irradiation (200 W) for 30 min at a temperature of 55 °C. The buildup of pressure in the closed reaction vessel was carefully monitored. After the irradiation, the reaction tube was cooled with high pressure air through an inbuilt system inside in the instrument until the temperature had fallen below 25 °C (ca. 2 min). The reaction mixture was diluted with ethylacetate and the mixture was washed by 5% citric acid solution (10 mL), 5% $NaHCO_3$ (10 mL) and saturated NaCl solution (10 mL). The organic layer was dried over anhydrous magnesium sulphate and the solvent removed under reduced pressure. The residue was purified by column chromatography (silica gel 60, 70–230 mesh ASTM, Merck). The isolated *N*-(acyltyrosine)acyltriazenes were subsequently recrystallized from ethyl acetate/hexane or dichloromethane/hexane.

4.2.3. 3-[2-(Acetylamino)-3-(4-hydroxyphenyl)propanoyl]-1-(4-acetylphenyl)-3-methyltriazene (**5a**)

Yield 38%, m.p. 199–201 °C. IR (cm^{-1}): 3318, 1709, 1683, 1641. ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 1.83 (s, 3H, NHCOC*H*$_3$), 2.64 (s, 3H, COC*H*$_3$), 2.84 (H_A, dd, *J* = 8.4 and *J* = 13.6 Hz, C*H*$_2$), 2.93 (H_B, dd, *J* = 6.4 and *J* = 13.6 Hz, C*H*$_2$), 3.30 (s, 3H, NC*H*$_3$), 5.58 (dd, *J* = 7.6 Hz, 1H, ArCH$_2$C*H*), 6.57–6.98 (dd, *J* = 8.4 Hz, 4H, HO*Ar*), 7.65–8.10 (dd, *J* = 8.4 Hz, 4H, *Ar*), 8.52 (d, *J* = 7.6 Hz, 1H, N*H*), 9.18 (s, 1H, O*H*). ^{13}CNMR (100 MHz, $CDCl_3$): δ ppm 22.7 (CO*C*H$_3$), 27.3 (CO*C*H$_3$), 28.3 (N*C*H$_3$), 37.0 (*C*H$_2$Ar), 52.6 (*C*HCH$_2$Ar), 115.4, 122.5, 127.5, 130.0, 130.3, 137.1, 151.7, 156.4 (*C*Ar), 169.8 (*C*OCH$_3$), 175.0 (N=N–N–*C*O), 197.7 (*C*OCH$_3$). EI: *m*/*z* 382.0 (M^+), 133.0 ($CH_3COC_6H_4N_2^+$), 119.1 ($CH_3COC_6H_4^+$) (see Supporting Information).

4.2.4. 3-[2-(Acetylamino)-3-(4-hydroxyphenyl)propanoyl]-1-(4-ethoxycarbonylphenyl)-3-methyltriazene (**5b**)

Yield 66%, m.p. 212–215 °C. IR (cm^{-1}): 3318, 1724, 1703, 1649. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 1.35 (t, *J* = 8.0 Hz, 3H, C*H*$_3$CH$_2$), 1.84 (s, 3H, COC*H*$_3$), 2.84 (H_A, dd, *J* = 8.4 and *J* = 13.6 Hz, C*H*$_2$), 2.92 (H_B, dd, *J* = 6.4 and *J* = 13.6 Hz, C*H*$_2$), 3.40 (s, 3H, NC*H*$_3$), 4.35 (q, *J* = 8.0 Hz, 2H, CH$_3$C*H*$_2$), 5.56 (dd, *J* = 7.2 and *J* = 7.6 Hz, 1H, ArCH$_2$C*H*), 6.56–6.97 (dd, *J* = 8.4 Hz, 4H, HO*Ar*), 7.64–8.09 (dd, *J* = 8.4 Hz, 4H, *Ar*), 8.53 (d, *J* = 7.6 Hz, 1H, N*H*), 9.18 (s, 1H, O*H*). ^{13}CNMR (100 MHz, DMSO-d_6): δ ppm 14.6 (*C*H$_3$CH$_2$), 22.70 (COC*H*$_3$), 28.3 (N*C*H$_3$), 37.0 (*C*H$_2$Ar), 52.7 (*C*HCH$_2$Ar), 61.4 (CH$_3$*C*H$_2$O), 115.4, 122.5, 127.5, 130.3, 130.3, 130.9, 151.9, 156.4 (*C*Ar), 165.6 (CH$_3$CH$_2$O*C*O), 169.8 (*C*OCH$_3$), 175.0 (N=N–N–*C*O). EI: *m*/*z* 413.4 (M + H)$^+$, 177.3 ($CH_3CH_2OCOC_6H_4N_2^+$), 149.1 ($CH_3CH_2OCOC_6H_4^+$).

4.2.5. 3-[2-(Acetylamino)-3-(4-hydroxyphenyl)propanoyl]-1-(4-cyanophenyl)-3-methyltriazene (**5c**)

Yield 38%, m.p. 196–198 °C. IR (cm^{-1}): 3327, 2226, 1702, 1634. ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 1.83 (s, 3H, COC*H*$_3$), 2.84 (H_A, dd, *J* = 8.0 and *J* = 13.6 Hz, C*H*$_2$), 2.91 (H_B, dd, *J* = 7.2 and *J* = 13.6 Hz, C*H*$_2$), 3.30 (s, 3H, NC*H*$_3$), 5.56 (dd, *J* = 7.2 and *J* = 7.6 Hz, 1H, ArCH$_2$C*H*), 6.54–6.95 (dd, *J* = 8.0 Hz, 4H, HO*Ar*), 7.67–8.00 (dd, *J* = 8.0 Hz, 4H, *Ar*), 8.53 (d, *J* = 7.2 Hz, 1H, N*H*), 9.16 (s, 1H, O*H*). ^{13}CNMR (100 MHz, $CDCl_3$): δ ppm 22.6 (*C*H$_3$CO), 28.5 (N*C*H$_3$), 37.0 (*C*H$_2$Ar), 52.6 (*C*HCH$_2$Ar), 11.6 (*C*N), 115.4, 119.0, 123.5, 127.4, 130.3, 134.2, 151.6, 156.4 (*C*Ar), 169.8 (*C*O), 175.1 (*C*O). EI: *m*/*z* 365.0 (M^+), 206 (AcNHCH(CH$_2$Ph)CO$^+$), 178 (AcNHCH$^+$(CH$_2$Ph)), 130 ($CNC_6H_4N_2^+$), 107 ($C_6H_5CH_2^+$), 102 ($CNC_6H_4^+$).

4.2.6. 3-[2-(Acetylamino)-3-(4-hydroxyphenyl)propanoyl]-1-(4-bromophenyl)-3-methyltriazene (**5d**)

Yield 15%, m.p. 218–221 °C. IR (cm^{-1}): 3327, 3215, 1709, 1651. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 1.87 (s, 3H, COC*H*$_3$), 2.82–2.90 (2dd, *J* = 8.0 Hz, 2H, C*H*$_2$Ar), 3.27 (s, 3H, NC*H*$_3$), 5.67 (dd, *J* = 6.8 Hz, 1H, ArCH$_2$C*H*), 6.53–6.87 (dd, *J* = 8.0 Hz, 4H, HO*Ar*), 7.39–7.51 (dd, *J* = 8.0 Hz, 4H, *Ar*), 8.02 (d, *J* = 8 Hz, 1H, N*H*COCH$_3$), 8.81 (s, 1H, ArO*H*); ^{13}CNMR (100 MHz, DMSO-d_6): δ ppm 22.8 (COCH$_3$), 27.9 (NCH$_3$), 37.8 (CH$_2$Ar), 52.2 (*C*HCH$_2$Ar), 115.3, 123.9, 130.0, 132.2 (*C*Ar), 147.4 (Br–*C*Ar), 156.2 (Ar*C*–OH), 170.0 (NCOCH$_3$), 174.5 (N=N–N–*C*O); EI: *m*/*z* 419.3/421.3 (M^+); 185.0/183.0 ($BrC_6H_4N_2^+$); 157.0/155.0 ($BrC_6H_4^+$).

4.2.7. 3-[2-(Acetylamino)-3-(4-hydroxyphenyl)propanoyl]-1-(4-aminocarbonylphenyl)-3-methyltriazene (**5e**)

Yield 38%, m.p.216–217 °C. IR (cm^{-1}): 3334, 3301, 3251, 1738, 1698, 1657. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 1.83 (s, 3H, COC*H*$_3$), 2.82 (H_A, dd, *J* = 8.4 and *J* = 13.6 Hz, C*H*$_2$), 2.93 (H_B, dd, *J* = 8.4 and *J* = 13.6 Hz, C*H*$_2$), 3.30 (s, 3H, NC*H*$_3$), 5.57 (dd, *J* = 7.6 and *J* = 8.0 Hz, 1H, ArCH$_2$C*H*), 6.57–6.98 (dd, *J* = 8.4 Hz, 4H, HO*Ar*), 7.50 (s, 1H, N*H*$_2$), 7.60–8.01 (dd, *J* = 8.4 Hz, 4H, *Ar*), 8.10 (s, 1H, N*H*$_2$), 8.52 (d, *J* = 7.6 Hz, 1H, N*H*), 9.20 (s, 1H, O*H*). ^{13}CNMR (100 MHz, DMSO-d_6): δ ppm 22.1 (COCH$_3$), 27.6 (NCH$_3$), 36.3 (CH$_2$Ar), 52.1 (CHCH$_2$Ar), 114.8, 121.4, 1276.7, 128.6, 129.6, 134.3, 149.9, 156.1 (*C*Ar), 167.0 (NH$_2$*C*O), 169.2 (*C*OCH$_3$), 174.4 (N=N–N–*C*O). EI: *m*/*z* 384.3 (M + H)$^+$, 148.1 ($NH_2COC_6H_4N_2^+$), 120.1 ($NH_2COC_6H_4^+$).

4.2.8. 3-[2-(Acetylamino)-3-(4-hydroxyphenyl)propanoyl]-1-(4-methoxycarbonylphenyl)-3-methyltriazene (**5f**)

Yield 24%, m.p. 195–197 °C. IR (cm^{-1}): 3381, 3296, 1728, 1692, 1655. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 2.05 (s, 3H, COC*H*$_3$), 3.01 (H_A, dd, *J* = 6.8 and *J* = 13.8 Hz, C*H*$_2$), 3.12 (H_B, dd, *J* = 6.4 and *J* = 13.6 Hz, C*H*$_2$), 3.44 (s, 3H, NC*H*$_3$), 5.98 (q, *J* = 6.8 Hz, 1H, ArCH$_2$C*H*), 6.36 (d, *J* = 8.4 Hz, 1H, N*H*), 6.63–6.95 (dd, *J* = 8.4 Hz, 4H, HO*Ar*), 7.64–8.13 (dd, *J* = 8.4 Hz, 4H, *Ar*), ^{13}CNMR (100 MHz, DMSO-d_6): δ ppm 23.7 (COCH$_3$), 28.4 (NCH$_3$), 38.7 (CH$_2$Ar), 52.5 (CHCH$_2$Ar), 52.6 (OCH3), 115.8, 122.6, 126.7, 130.4, 130.7, 131.0 (*C*Ar), 151.9 (N–*C*Ar) 156.5 (Ar*C*–OH), 166.8 (*C*OCH$_3$), 170.2170.0 (NCOCH$_3$), 174.8 (N=N–N–*C*O). EI: *m*/*z* 399.3 (M + H)$^+$, 163.1 ($CH_3OCOC_6H_4N_2^+$), 135.1 ($CH_3OCOC_6H_4^+$).

4.2.9. 3-[2-(N-BOC)-3-(4-hydroxyphenyl)propanoyl]-1-(4-acetylphenyl)-3-methyltriazene (**6a**)

Yield 20%, m.p. 195–197 °C. IR (cm^{-1}): 3358, 3330, 1713, 1674, 1626. ^{1}H NMR (400 MHz, CD_3OD): δ ppm 1.38 (s, 9H, COC(CH_3)$_3$), 2.63 (s, 3H, ArCOCH_3), 2.84 (dd, J = 16.0 and J = 8.0 Hz, 2H, CH_2Ar), 3.32 (s, 3H, NCH_3), 5.52 (t, J = 8 Hz, 1H, CHCH$_2$Ar), 6.50–6.92 (dd, J = 8.0 Hz, 4H, HO*Ar*); 7.65–8.08 (dd, J = 8.0 Hz, 2H, *Ar*). ^{13}CNMR (100 MHz, DMSO-d_6): δ ppm 27.0 (CH_3CO*Ar*), 28.4 (C(CH_3)$_3$), 33.7 (NCH_3), 36.6 (ArCH_2), 54.2 (ArCH$_2$*C*), 78.7 (*C*(CH_3)$_3$), 115.2, 122.7, 127.8, 129.9, 130.3, 137.1, 151.7, 155.8 (*C*Ar), 156.2 (NH*C*O), 175.3 (*C*ONCH$_3$), 197.6 (Ar*C*OCH$_3$). EI: m/z 463.4 (M + Na)$^+$, 147.2 ($CH_3COC_6H_4N_2^+$), 118.9 ($CH_3COC_6H_4^+$).

4.2.10. 3-[2-(N-BOC)-3-(4-hydroxyphenyl)propanoyl]-1-(4-cyanophenyl)-3-methyltriazene (**6b**)

Yield 10%, m.p. 95–98 °C. IR (cm^{-1}): 3350, 2228, 1703, 1680. ^{1}H NMR (400 MHz, CD_3OD): δ ppm 1.42 (s, 9H, COC(CH_3)$_3$), 2.98 (t, J = 8.0 Hz, 2H, CH_2–Ar), 3.39 (s, 3H, NCH_3), 5.30 (d, J = 8.0 Hz, 1H, N*H*), 5.62 (q, J = 8.0 Hz, 1H, CHCH$_2$Ar), 6.61–6.91 (dd, J = 8.0 Hz, 4H, HO*Ar*), 7.62–7.71 (dd, J = 8.0 Hz, 4H, *Ar*). ^{13}CNMR (100 MHz, CDOD): δ ppm 28.4–28.3 (C(CH_3)$_3$), 39.1 (HNCH*C*H$_2$), 53.2 (OC*C*–HNH), 115.3, 122.9, 130.3, 133.2 (*C*Ar).

4.3. HPLC analysis

The analytical high-performance liquid chromatography (HPLC) system included a Hitachi UV Detector L-2400 Elite LaChrom, Hitachi Pump L-2130 Elite LaChrom Intel Core2 Duo, a Samsung Scala Monitor, a manual sample injector module equipped with 20 μL loop, a Merck Lichrospher® 100 RP$_{18}$ 125 mm × 4.6 mm (5 μm) column equipped with a Merck Lichrocart pre-column (Merck, Germany). For compounds **5a**, **6a** and **6b** an acetonitrile/water (60:40 to 40:60) an isocratic solvent system was used. For compounds **5b**–**f** we used a gradient elution beginning with a mobile phase of 95% water/5% acetonitrile ramping to 40% acetonitrile/60% water over 15 min. The column effluent was monitored at 300 nm for all compounds and a flow rate of 1.0 mL/min was used.

4.4. Apparent partition coefficients

The apparent partition coefficients (log P_{exp}) were determined at room temperature using 1-octanol-pH 7.4 phosphate buffer. Each phase was mutually saturated before the experiment. The volumes of each phase were chosen so that the solute concentrations after distribution in the aqueous phase were readily measurable. The compounds were dissolved in 1-octanol and the 1-octanol-phosphate buffer mixtures were shaken for 30 min to reach the distribution equilibrium. Each phase was analysed separately by HPLC, but the organic phase was diluted in acetonitrile (1:10) before HPLC analysis. Partition coefficients, log P_{exp}, were calculated from the ratio of the peak area in octanol to the peak area in the buffer.

4.5. Hydrolysis in buffer solution

Reaction mixtures were analysed using HPLC. Usually, a 10 μL aliquot of a 10^{-2} M stock solution of prodrug **5** in acetonitrile was added to 10 mL of the appropriate thermostated buffer solution at 37 °C. At regular intervals, samples of the reaction mixture were analysed by HPLC along the following conditions: mobile phase, acetonitrile/water (the composition of which depended on the compound). The substrate reaction progress curve was analysed to obtain the reaction rate constants.

4.6. Hydrolysis in human plasma

Human plasma was obtained from the pooled, heparinised blood of healthy donors, and – prior to use – was frozen and stored at −70 °C. For the hydrolysis experiments, the substrates were incubated at 37 °C in human plasma that had been diluted to 80% (v/v) with a pH 7.4 isotonic phosphate buffer. At appropriate intervals, aliquots of 200 μL were added to 400 μL of acetonitrile in order both to quench the reaction and precipitate plasma proteins. These samples were centrifuged and the supernatant analysed by HPLC for the presence of substrate and products.

4.7. Tyrosinase degradation studies

Mushroom tyrosinase was used at a concentration of 300 U/mL in a solution of phosphate buffer (pH 7.4, 3.0 mL) at 37 °C. A solution of the prodrug under investigation (15 μL of 10^{-2} M solution) was added and the mixture incubated and gently stirred at 37 °C. Aliquots were collected at selected time intervals and quenched by addition of an organic solvent (acetonitrile). The disappearance of the prodrug, with the concomitant appearance of metabolites, was followed by HPLC for evaluation of the hydrolysis kinetics.

4.8. Determination of K_m and V_{max}

K_m and V_{max} values were determined by HPLC method as described in 4.3 and 4.7. The tyrosinase concentration was calculated taking the value of Mr as 120,000 Da. Protein content was determined by Bradford's method using bovine serum albumin as standard [26]. Each substrate was incubated with 15.0 units of mushroom tyrosinase in a water bath at 37 °C at six different prodrug concentrations (0–10 mM), following simple saturation kinetics. Initial rates were determined by the slope of the straight line of the initial reaction for decrease of the prodrugs concentration **5c** and **5f** versus time. Values are the mean of triplicates. Data were fit using a simple Michaelis–Menten equation to yield K_m and V_{max} values. Linear regression analysis was performed using GraphPad Prism 5, Graphpad Software Inc.

4.9. Melanoma cell lines

The human melanotic melanoma cell line SKMEL-30 was purchased from the Leibniz-Institut DSMZ (Germany), the MNT-1 cell line was kindly offered by Dr. José Ramalho (Faculdade de Ciências Médicas, Lisbon, Portugal) and the amelanotic melanoma cell line – the M8 – was kindly offered by Dr. Edgardo D. Carrosela (Institut Universitaire d'Hématologie, Paris, France). Cells were grown in Roswell Park Memorial Institute medium (RPMI), supplemented with 10% foetal bovine serum, 2 mM L-glutamine and 100 μg/mL penicillin/streptomycin.

4.10. Analysis of cell proliferation and viability

Cancer cell lines were seeded in 96-well microtitre plates at 3 × 10^5 cells/mL, and different concentrations of prodrugs were added. After 24 h and 48 h incubations, the effect on proliferation/viability were determined by using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay and by using the hemocytometer-based trypan blue dye exclusion cell quantitation and viability assay. Control assays were conducted in the absence of prodrugs and in the presence of similar amounts of organic solvent only.

4.11. Analysis of cell apoptosis

Cell lines were cultured for 48 h at 1.5×10^5 cells/mL in serum free medium, to synchronize cells at G0/G1 phase. After 24 h in complete medium, the cells were incubated for another 48 h with 50 or 100 μM of the prodrugs. Temozolomide (Sigma) and the medium alone (0 μM) were used respectively as positive and negative controls for apoptosis. The cells were stained with allophycocyanin (APC)-conjugated Annexin-V (BD Bioscience) and 7-AAD, as we described [27]. The percentage of apoptotic/necrotic cells was analysed by flow cytometry and data elaborated with Paint-a-Gate (BD Bioscience) software.

4.12. Tyrosinase activity

Tyrosinase activity was examined by measuring the rate of oxidation of L-DOPA. Cells were lysed with a 20 mM phosphate buffer (pH 6.8) containing 1% Triton X-100 and then disrupted by freezing and thawing. The total protein concentration was determined by the Bradford assay and the tyrosinase activity was then determined in a 100 μL reaction mixture containing 50 mM of phosphate buffer (pH 6.8), 2.5 mM of L-DOPA, and 1.8 μg of protein. The dopachrome formation was monitored at 37 °C, by measuring absorbance at 475 nm, during 60 min. One unit of tyrosinase activity was defined as the amount of enzyme protein (mg) that catalysed the formation of 1 mmol of dopachrome in 1 min. The amount of dopachrome in the reaction was calculated applying the Lambert–Beer Law using a molar extinction coefficient for dopachrome of 3600 M^{-1} cm^{-1}. 0.1 μg of mushroom tyrosinase protein was used as positive control.

4.13. Statistical analysis

Differences between the control and drug-treated groups were analysed with ANOVA using GraphPad Prism software. $p < 0.05$ was considered a statistically significant difference.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.09.040.

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