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Targeted Prodrug Design for the Treatment of Malignant Melanoma

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Abstract

Malignant melanoma is a serious health problem once the current chemotherapy exhibits resistance to traditional drugs. Several challenges must be overcome during drug design in order to increase the efficacy and safety of the new drugs. Therefore, the specific chemical release of cytotoxic agents near to the target is an attractive approach to improve the anticancer activity and reduce the systemic toxicity. Herein, this review article describes the advances in the development of targeted prodrugs for the treatment of malignant melanoma.

Keywords

Prodrug, Skin cancer, Melanoma

Introduction

Advanced malignant melanoma is a highly aggressive form of skin cancer, characterized to be one of the toughest treatments among human tumors [1,2]. In 2016, it has been estimated 76,000 new cases, responsible to cause 10,000 deaths only in the United States [3].

An increased number in resistance cases by malignant cells against the current therapy justifies the discovery of new drugs [4,5]. Dacarbazine was one of the first drug approved by US Food and Drug Administration (FDA) for melanoma treatment. This drug is used alone or in combination with other cytostatic agents and exhibits response rates ranging from 30-40% [6,7].

Nowadays, the current chemotherapy includes drugs, such as: a) temozolomide - used alone or through two different combinations [cisplatin, vinblastine and dacarbazine (CVD) or cisplatin, dacarbazine, carmustine, and tamoxifen (Dartmouth regimen)]; b) carboplatin and paclitaxel (sometimes combined with sorafenib); and c) immunotherapeutic drugs (ipilimumab, trametinib, pembrolizumab, vemurafenib, dabrafenib, peginterferon alfa-2b) [8]. In addition, targeted therapies for advanced melanoma involving BRAF kinase inhibitors and anti-cytotoxic T-lymphocyte antigen-4 (CTLA-4) antibodies have gained particular interest of researchers [9].

The molecular modification has demonstrated to be a promising approach to discover new anticancer drugs. Specifically, the development of prodrugs is an interesting strategy to overcome adverse effects and increase the drug specificity against malignant cells [10].

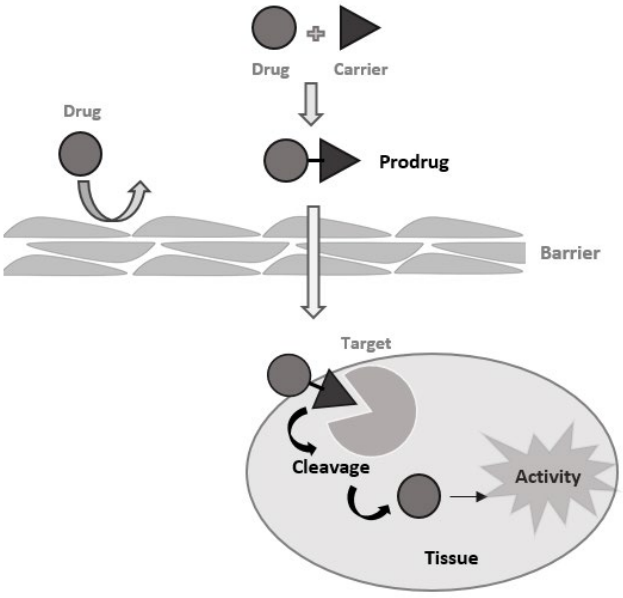

Figure 1: Prodrug strategy. The drug is released after chemical or enzymatic activation proving the pharmacological effect near to the site of action.

The term prodrug was first defined in 1958 by Adrien Albert [11] as an inactive compound that need an enzymatic or chemical biotransformation *in vivo* to release the active drug. By analogy, this strategy can be compared to a "Trojan horse" in that, the drug is a non-toxic compound *per se*; however, after chemical or enzymatic bioconversion, the active drug is released near and/or at the site of action (Figure 1) [10].

There are two main categories of prodrugs: classical prodrugs and bioprecursors. For classical prodrugs, the active drug is linked to a carrier (small molecules or macromolecules) through a bioreversible covalent linkage [12]. Prodrug specificity can be obtained using carriers or linkers that can be identified by the target (membrane receptor or enzyme). This mechanism increases the drug efficacy and decrease in toxicity [12-19].

Capecitabine (**1**), an antineoplastic prodrug of 5-fluorouracil (5-FU) (**2**), is an example of classical prodrug (Figure 2). The active drug

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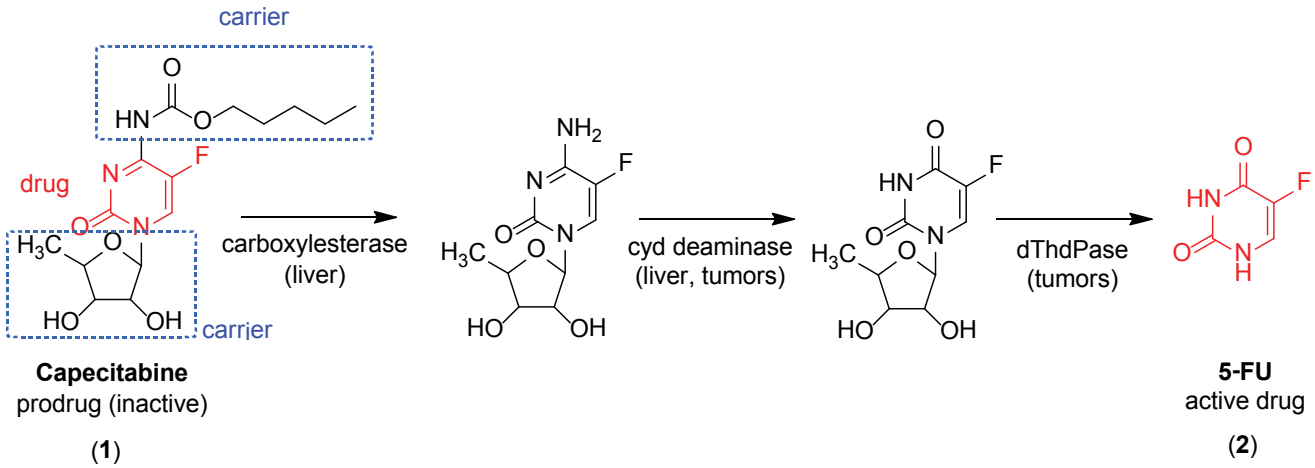


Figure 2: Structure and metabolic pathway of capecitabine (**1**). Cyd deaminase = cytidine deaminase; dThdPase = thymidine phosphorylase.

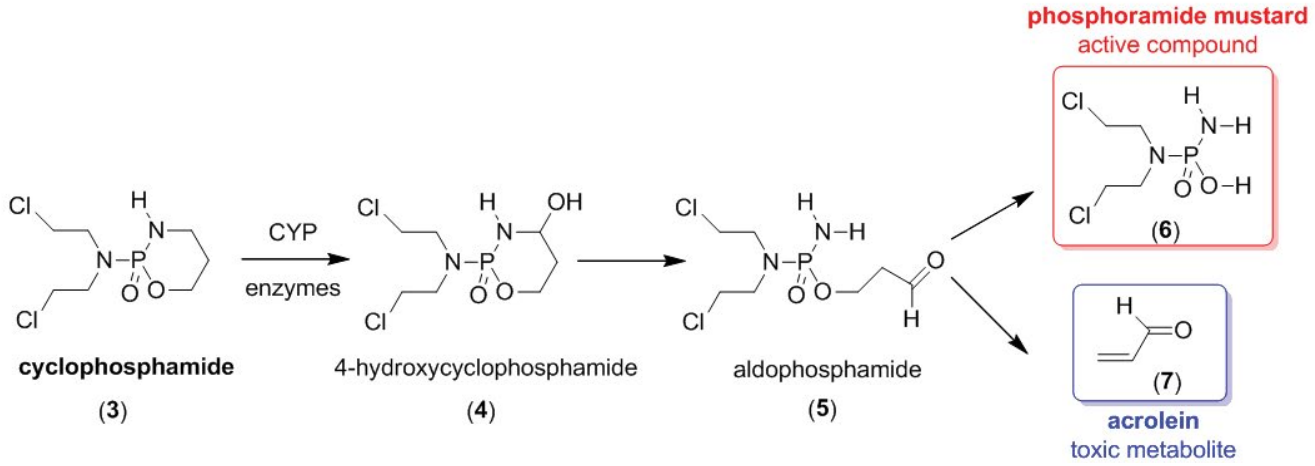


Figure 3: Activation of cyclophosphamide (**3**), an antitumor drug, to its active and inactive metabolites.

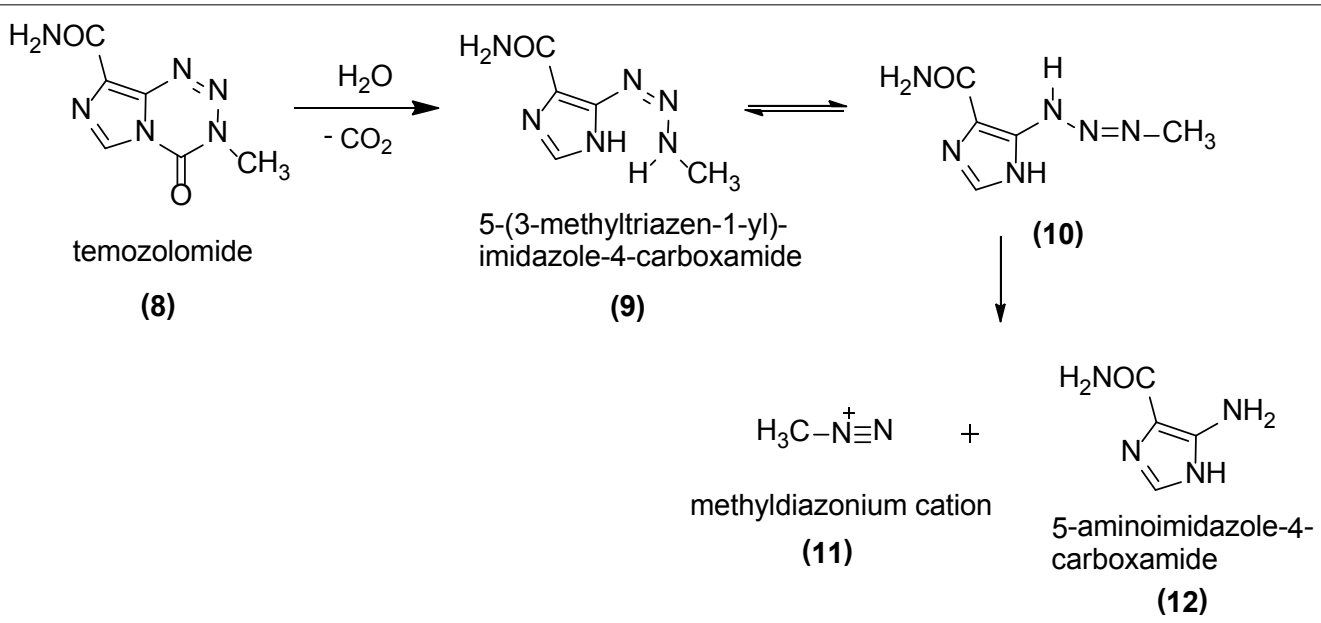


Figure 4: Temozolomide (8) bioactivation. Adapted from [22].

(5-FU) is released selectively into tumors cells by bioconversion of three enzymes (carboxylesterase, cytidine deaminase and thymidine phosphorylase) [20].

On the other hand, bioprecursors prodrugs also need activation by metabolic processes *in vivo;* however they do not contain a carrier. Examples of bioprecursors include: statins (e.g., simvastatin), antiviral drugs (e.g., zidovudine and acyclovir) and chemotherapeutic drugs (e.g., tirapazamine) [12]. Figure 3 shows the chemical structure of cyclophosphamide (**3**), an antitumor bioprecursor that is metabolized *in vivo* by cytochrome P450 enzymes into active and inactive metabolites. For this example, the phosphoramide mustard (**6**) is the active compound with cytotoxic activity [21].

Temozolomide (**8**) shown in Figure 4 as well as cyclophosphamide is an alkylating agent bioprecursor prodrug used in melanoma treatment (but not exclusively). The mechanism of action is to release a highly reactive methyldiazonium cation (**11**) that methylate DNA at *N7* positions of guanine (preferentially), *N3* adenine and *O6* guanine residues [22-24].

In this review article, our aim is to describe the use of the prodrug approach as a tool to discover new drugs for malignant melanoma. Therefore, it will be discussed the current advances and the strategies reported in the literature in the last years.

Prodrugs Useful to Treat Malignant Melanoma

Several endogenous enzymes have shown ability to activate prodrugs with antitumor activity. These enzymes can be exclusively expressed in tumors tissues or only overexpressed in malignant cells [12].

Tyrosinase, for example, is an enzyme found in melanocytes that shows an important function in melanogenesis. Mutant melanocytes demonstrate increased levels of tyrosinase compared to normal cells. Therefore, this enzyme can be explored as high selective *in-situ* tool for the activation of anti-melanoma prodrugs [1,2].

Researchers have used tyrosinase substrates as chemotherapeutic drug carriers during the drug design of the prodrug MDEPT (Melanocyte-directed Enzyme Prodrug Therapy) [6,25]. The prodrug molecule was designed using a cytotoxic agent (drug) linked to a tyrosinase substrate (carrier). Inside the melanoma, after bioaction by tyrosinase, the active drug is released through an intramolecular reaction (Figure 5) [1]. Furthermore, additional mechanisms involving the formation of radical oxygen species (ROS) and glutathione reductase (GSH) depletion potentiates the drug toxicity inside the melanoma cells.

Jordan and co-workers [25] synthesized a series of prodrugs for MDEPT using as cytotoxic agents phenyl mustard (**13a-13f**), bisethyl amine mustard (**14a-14c**) and daunomycin (**15**). For all those compounds, it was used a carbamate, urea or thiourea linker (Figure 6). These compounds were activated by tyrosinase in the same way as described.

In order to evaluate the ability of all compounds to be activated by tyrosinase, the researchers used the enzyme purified of mushrooms. The reason for that is due to structural similarity since the active sites of both contain a common binuclear copper centre. In addition, human tyrosinase is not commercially available and several studies have reported mushroom's tyrosinase as a suitable and accurate model in earlier studies [26]. In this study, the experiments measured the rate of oxidation by tyrosinase (R). The maximum value of R (Rmax) correlates with the ability to release of the drug after oxidation.

Interestingly, it was observed a decreased rate in tyrosinase oxidation when the carbamate group (**13c**) is replaced by thiocarbamate (**13d**). The results also showed that compound **14a** (R max = 20 nanomol/min) displayed superior oxidation rate compared to tyrosine methyl ester (R max = 17.5 nanomol/min), a natural substrate for tyrosinase. Moreover, compounds **13a**, **13b** and **14c** (Figure 6) exhibited similar oxidation to tyrosine methyl ester; while **13e** and **13f**, containing a heteroatom insertion, were less effective in this experiment (Rmax = 7.5 nmol/min) [19].

Knaggs *et al.* [27] obtained prodrugs (**16a-16f**) derived from 6-aminodopamine and 4-aminophenol as candidates for MDEPT (Figure 7). The compounds **16a-16d** were oxidized at levels ranging from 70-78% of the rate compared to *L*-tyrosine (Rmax = 17 nmols/min), a natural substrate for tyrosinase. Compounds **16e** and **16f** have shown low rate of oxidation at levels of 50% and 25%, respectively. Drug release from the urea-linked prodrugs (compounds **16a** and **16c)** was more effective, suggesting that urea linked prodrugs were better candidates for MDEPT than thiourea analogs.

The 1-aryl-3,3-dimethyltriazenes are also known antineoplastic drugs, however, this class of compounds exhibited low selectivity for cancer cells. In order to solve this problem, new triazene prodrugs for

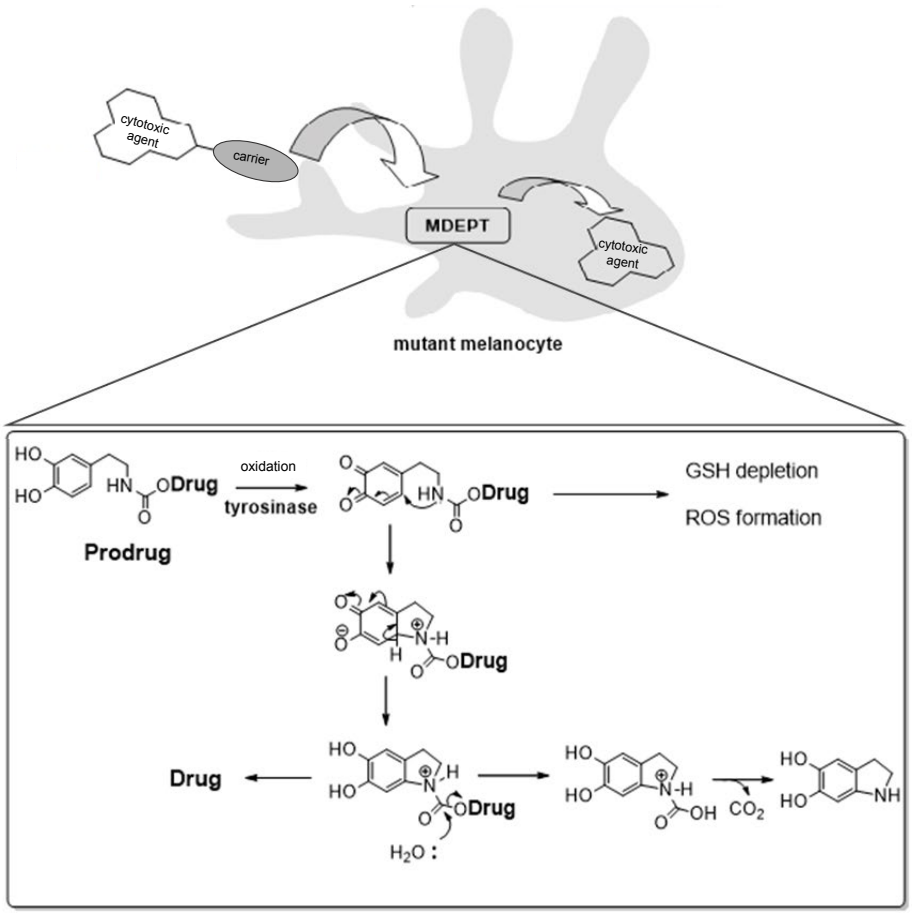


Figure 5: *In vivo* bioactivation action of MDEPT.

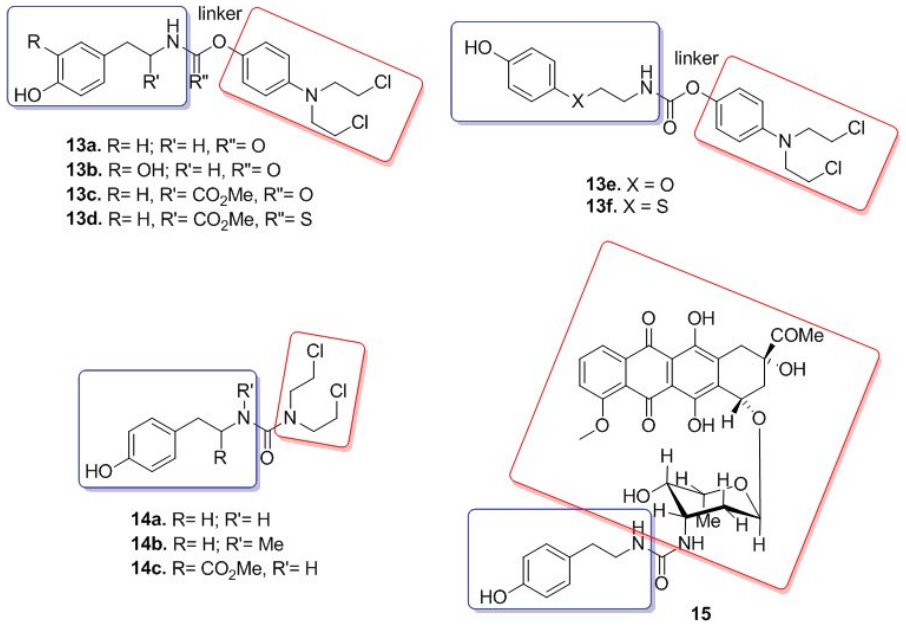


Figure 6: Phenyl mustard prodrugs (**13a-13f**), bis-(2-chloroethyl)amine urea mustards (**14a-14c**) and daunomycin prodrug (**15**). In blue are represented chemical groups that act as tyrosinase substrates. After enzymatic activation, the active drugs (red) are released in order to kill the tumor cells.

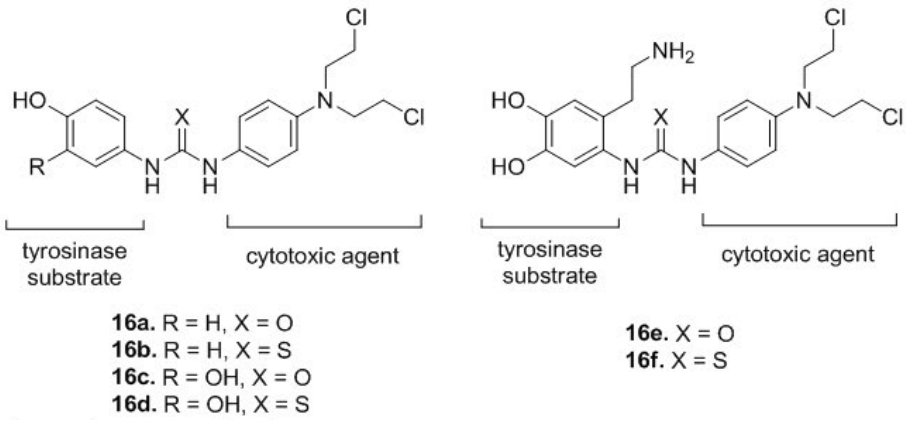
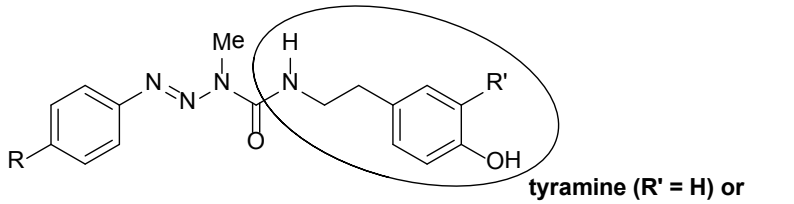


Figure 7: Chemical structures of prodrugs **16a-16f.**

Table 1: Half-lives ($t_{1/2}$) of triazene prodrugs (**17a-17g**) in PBS buffer, 80% human plasma and in the presence of tyrosinase (100 U/mL) at 37ºC.



(17)

Compound	R	R'	$t_{1/2}$		
			7.4 buffer (h)	80% human plasma (h)	Tyrosinase (min)
17a	CH_3CO	H	[a]	[b]	13.0
17b	CO_2Et	H	[a]	71.5	12.9
17c	CN	H	[a]	[b]	11.8
17d	Me	H	364	[b]	18.2
17e	CH_3CO	OH	15.1	9.30	9.9
17f	CO_2Et	OH	20.8	14.7	6.1
17g	Me	OH	[b]	5.66	8.4

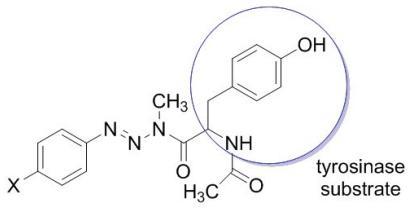
*substrate concentration 10^{-5} M; ** substrate concentration 5 x 10^{-5} M.

[a]Stable for 15 days; [b]Stable for 2 days.

MDEPT were synthesized by Perry and co-workers [1]. In this work, tyramine (**17a-17d**) or dopamine (**17e-17g**) subunits were selected as substrates for tyrosinase (Table 1).

These prodrugs **17a-17g** were very stable using phosphate buffer and human plasma. In addition, they were substrate of the enzyme tyrosinase. All compounds have exhibited short half-lives that ranged from 6.1 min - 18.2 min.

Table 2: Half-lives ($t_{1/2}$) of triazene derivatives (**18a-18f**) in PBS buffer, 80% human plasma and in the presence of tyrosinase (300 U/mL) at 37ºC.

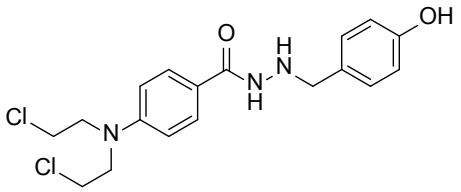


(18)

Compound	X	$t_{1/2}$		
		7.4 buffer (h)	80% human plasma (h)	Tyrosinase (min)
18a	COMe	56.6 ± 1.0	1.41 ± 0.13	19.9 ± 1.2
18b	CO_2Et	70.3 ± 0.8	5.32 ± 0.08	18.0 ± 1.2
18c	CN	42.7 ± 0.5	1.23 ± 0.17	15.6 ± 1.8
18d	Br	[a]	6.80 ± 0.01	8.9 ± 0.10
18e	$CONH_2$	40.6 ± 0.6	5.19 ± 0.08	17.4 ± 0.42
18f	CO_2Me	75.1 ± 1.7	2.80 ± 0.26	18.3 ± 0.56

Substrate concentration 10^{-5} M.

[a]Stable for 15 days.



(19)

Figure 8: Chemical structure of (N-{4-[bis-(2-chloroethyl)amino]benzoyl}-N0-(4-hydroxybenzyl)hydrazine) prodrug (**19**).

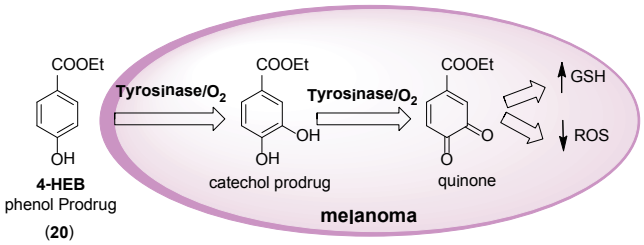
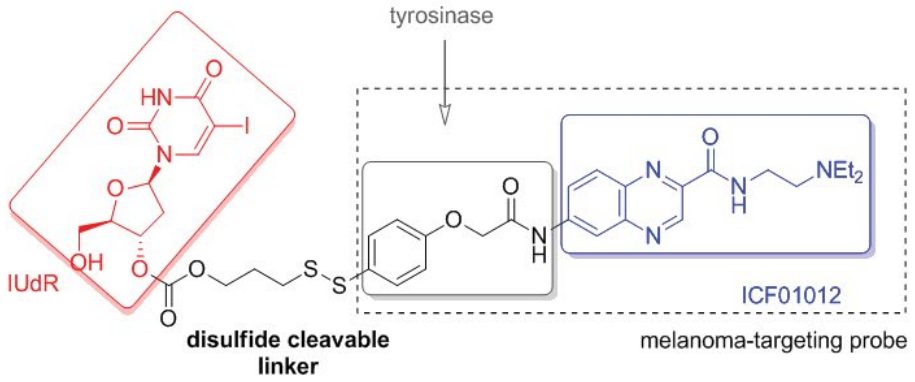


Figure 9: Mechanism of ethyl 4-hydroxybenzoate (4-HEB) (**20**) bioconversion by tyrosinase (Adapted from [30]).



(21)

Figure 10: Structure of disulfide prodrug IUdR-SS-MTP (**21**).

Monteiro *et al.* [28] obtained new triazene derivatives (**18a-18f**) for MDEPT with good results (Table 2). The half-lives in the presence of the enzyme ranged from 9 (**18d**) to 20 min (**18a**). The compound **18c** displayed the high cytotoxicity against three different melanoma cell lines. Moreover, this compound (**18c**) demonstrated an increased specificity for tyrosinase when compared to temozolomide, a commercially available prodrug with tyrosinase-independent toxicity.

Gasowska-Bajger and Wojtasek [6] have described that not only carbamate or urea linkers, but also hydrazine linkers and phenolic substrates (4-tert-butylcatechol or *N*-acetyl-*L*-tyrosine) can be used in drug design for MDEPT. Therefore, they synthetized an aniline mustard prodrug (**19**) containing a hydrazine linker and a phenolic activator (Figure 8) [29]. The prodrug (*N*-{4-[bis-(2-chloroethyl) amino]benzoyl}-*N*-(4-hydroxybenzyl)hydrazine) (**19**) showed *in vivo* low specificity for the melanoma cells and weak activity to reduce melanoma solid tumor size compared to melphalan (cytostatic agent) at doses of 100 µg.

Vad *et al.* [30] used the same approach to characterize the oxidation and toxicity against five melanocytic melanoma cell lines for 24 phenolic compounds. The phenol prodrug is recognized by the melanoma tyrosinase. After oxidation, it is converted into catechol prodrug that is recognized by tyrosinase being then transformed into aquinone specie. The quinone derivative promotes the decrease of GHS and increase of ROS (Figure 9). The 4-HEB derivative (**20**) (IC_{50} = 75 µM) exhibited high selective activity against cell lines, which expressed functional tyrosinase.

These authors also extended the study for acetaminophen [31] and acetylsalicylic acid [32] and reported that both are tyrosinase substrates. This is an interesting point to be explored in the future for prodrug design. They suggested that the mechanism of toxicity of drugs is due to o-quinone formation, ROS formation, intracellular GSH depletion and mitochondrial toxicity.

Glutathione (GSH) is also overexpressed in tumor cells at levels 1000times superior to that of the blood plasma [29]. Based on this

knowledge, Aissi and co-workers (2015) designed the prodrug (**21**) based on a melanin-targeting derivative N-(2-diethylaminoethyl)-6-iodoquinoxaline-2-carboxamide (ICF01012). The ICF01012 was conjugated with a self-immolated disulfide linker with the chemotherapeutic IUdR (idoxuridine), an antimetabolite drug. The compound (21) (Figure 10) was able to release IUdR under disulfide reductive conditions. This data suggests that this compound has potential effectiveness to be activated into the tumor cell [33].

Mittal and co-workers [34] identified using bioinformatics tools that the enzyme prolidase could be an interesting target to development new selective prodrugs for melanoma treatment. The levels of prolidase in malignant cells are high. In addition, this enzyme has high substrate specificity for dipeptides containing proline at the carboxyl terminus [35]. Then, the researchers designed new L- and D- proline prodrugs of melphalan (**22a-22b**), named as prophalan-L and prophalan-D, respectively (Figure 11).

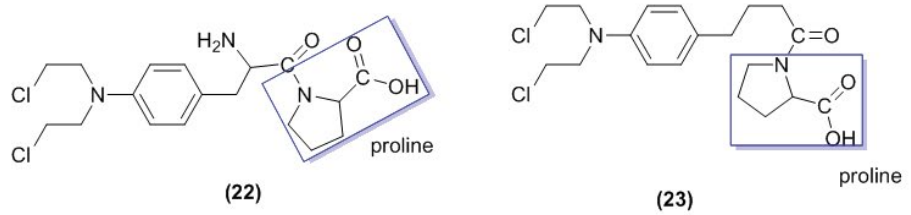
They also reported the bioconversion and antiproliferative activity of these derivatives against melanoma cell line (SK-MEL-5). The results showed that prophalan-L was 7-times more susceptible to the action of prolidase than prophalan-D. The cytotoxic activity of prophalan-L (measured as growth inhibition; GI_{50} = 74.8 μM) was slightly weaker than that of melphalan (GI_{50} = 57.0 μM). In this assay, prophalan-D was ineffective, suggesting the necessity of activation of the prodrug by prolidase.

The same research group synthesized the prodrug of chlorambucil (**23**), mustard structurally similar to melphalan. Unfortunately, this compound exhibited low hydrolysis when incubated with porcine kidney prolidase [5].

Chrzanowski *et al.* [36] reported similar coupling of proline moiety to melphalan with few difference in the mode of linkage. In this study, the drug released is the *N*-substituted melphalan. Despite of the good results, the prodrug (**24**) (Figure 12) displayed similar susceptibility to the action of prolidase compared to glycyl-proline, an endogenous substrate for prolidase.

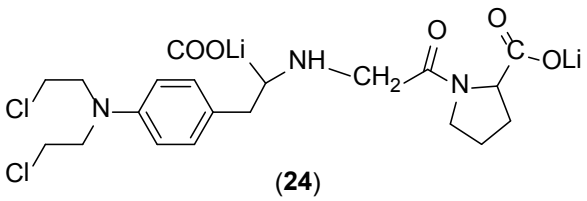
Matrix metalloproteinases (MMPs) are a group of zinc dependent enzymes essential for diverse physiopathological events including regulation of adhesion, migration and growth of cancer cells. MMP-2 and MMP-9 were found to be overexpressed in melanoma tumor cells. This data motivated Mansour and co-workers [37] to explore the protease activity of these enzymes in order to release the antineoplastic agent doxorubicin from a macromolecular carrier (serum albumin) (Figure 13). *In vivo* data reveals that prodrug (**25**) demonstrated 4-times superior efficacy than parental drug doxorubicin in mice in the A375 melanoma model.

Bisphosphonates are useful to treat osteoporosis and malignant bone disease. These drugs also are inhibitors of adhesion, invasion and growth of cancer cells. Heikkila *et al.* reported the inhibition of MMP by nitrogen containing bisphosphonates [38]. However, the low cell permeability limits its clinical use. In order to improve the pharmacokinetic profile of these class, Webster *et al.* [39] synthetized clodronate (**26a**) and methylene (**26b**) bisphosphonate prodrugs (Figure 14). All compounds were evaluated against different cancer cells, including melanoma. The compound **26a** were able to inhibit different tumor growth cells. This prodrug also displayed selective cytotoxicity against melanoma, with IC_{50} values of 7 ± 1 μmol/L and 11.7 ± 3 μmol/L at 72 hours against two melanoma cell lines, SK-MEL-5, and UACC-62 respectively.



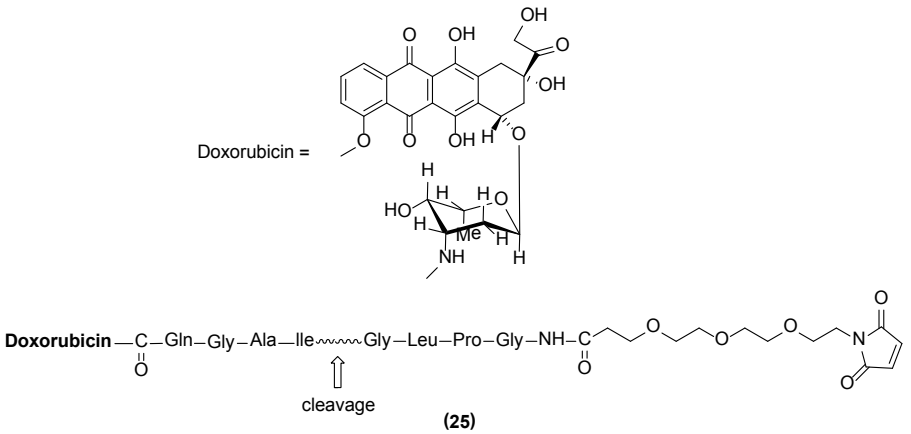
(22) (23)

Figure 11: Chemical structure of prophalan, a prodrug of proline and melphalan (**22a=** L-prophalan, **22b=** D-prophalan) and chlorambucil prodrug (**23**).



(**24**)

Figure 12: Chemical structure of prodrug (**24**) proposed by Chrzanowski *et al.* (2001).



(**25**)

Figure 13: Chemical structure of the albumin-binding doxorubicin prodrug (**25**).

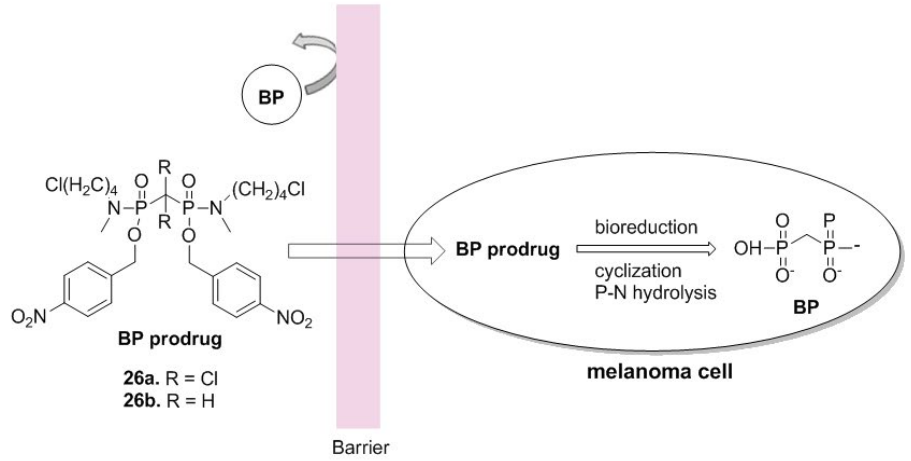


Figure 14: Schematic representation of the bisphosphonate prodrug activation (compounds **26a-26b**) (Adapted from [39]).

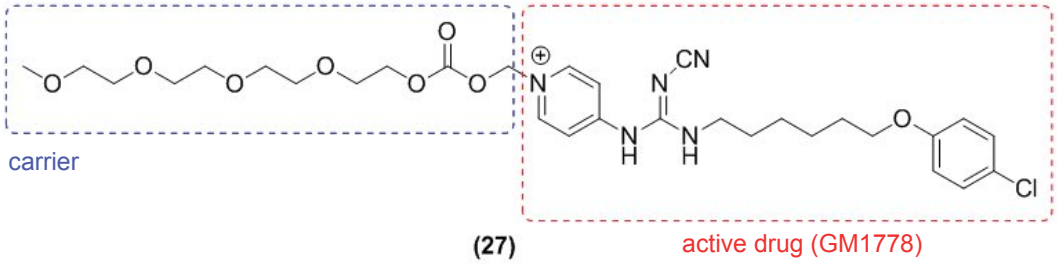


Figure 15: Chemical structure of GMX1777 (**22**).

Nicotinamide adenine dinucleotide (NAD) has an important role in several cell functions, including DNA repair [40]. The prodrug (**27**) GMX1777, Gemin X) (Figure 15) is an inhibitor of the enzyme nicotinamide phosphoribosyl transferase (NAMPT). This enzyme is crucial for the synthesis of NAD^+ being overexpressed in cancer cells [41,42]. In previous studies, the compound cyanoguanidinopyridine GMX1778 (active form of the prodrug GMX1777) showed potent anti-tumor activity in several *in vivo* tumor models as IM-9 (multiple myeloma), HCT-116 (colon carcinoma) and SHP-77 (SCLC) [41,42].

The first results about clinical trial with GMX1777 in patients with advanced malignancies have shown moderate clinical effect after administration of ascending doses (24-hour IV infusion for 21 days). The researches concluded that GMX1777 can be used in combination with other drugs to treat malignant melanoma [43]. Based on these preliminary results, a new phase I/II study of GMX1777 in combination with temozolomide was initiated in patients with metastatic melanoma with a 3-hour infusion for 5 consecutive days every 4 weeks. However, this study was terminated prematurely due to financial constraints [44].

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Conclusion

Over the past few decades, the skin cancer is dramatically increasing worldwide. The malignant melanoma is one of the most aggressive melanocytes neoplasm whose treatment is still limited. The current therapy has several limitations that include high toxicity. Therefore, the search of new effective and safe drugs is urgent. Herein, we demonstrated that the prodrug approach is a useful tool to discover new chemotherapeutic agents more selective and less toxic. Metabolic differences between healthy and tumor cells, including the specific enzymes can be explored to get drug selectivity for melanoma. The knowledge of overexpressed enzymes in melanoma tumor such as tyrosinase, prolidase, glutathione, metalloproteases represents an advance in this field that can be explored using prodrug approach. It has been shown that although the studies are still embryonic, the use of targeted prodrug approach point out to clinical success in near future.

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