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Small-Molecule based Delivery Systems for Alkylating Antineoplastic Compounds

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Alkylating agents are a major class of anticancer drugs for the treatment of various cancers including hematological malignancies. Targeting alkylating moieties to DNA by attachment of a DNA minor groove binding carrier such as distamycin, netropsin, or Hoechst 33252 reduces the loss of active drug due to reaction with other cell components and makes it possible to direct the alkylation both sequence specifically and regiospecifically. We reported the synthesis and structure–activity studies of amidine analogues of alkylating antineoplastic compounds, which appeared to be a new class of cytotoxic minor groove binders and topoisomerase II inhibitors. Another approach to overcome the toxicity of alkylating agents to normal tissue is to construct a prodrug with lower hydrophobicity and cytotoxicity but is preferentially activated in cancer cells. Overexpression of prolidase in some neoplastic cells suggests that the proline analogue of alkylating agents may serve as a prolidase convertible prodrugs. We have compared several aspects of pharmacological actions of proline analogues of chlorambucil and melphalan in breast cancer cells. The results suggest that prolidase could serve as a target enzyme for the selective action of anticancer agents.

Introduction

DNA alkylating agents have played an important part in cancer chemotherapy since the introduction of the nitrogen mustards more than fifty years ago. The first nitrogen mustard used in therapy was mechlorethamine, and the related compounds chlorambucil, melphalan, and cyclophosphamide remain in use today. A drawback common to all DNA alkylating agents is their high chemical reactivity.[1] This can result in loss of drug by reaction with other cellular nucleophiles, particularly proteins, and low-molecular weight thiols. This makes them vulnerable to cellular resistance mechanisms such as increased levels of glutathione. Other limitations, discussed particularly for mustards, are a lack of intrinsic DNA binding affinity of the core *N*,*N*-bis(2-chloroethyl)amine pharmacophore, and a requirement for bifunctional cross-linking of DNA to be fully cytotoxic. These characteristics lower their potency and the observed high ratio of genotoxic monoadducts to cross-links (up to 20:1) contributes to their known carcinogenicity. There is also evidence that the major guanine N7 adduct formed by mustards and other "simple" alkylators is readily repaired, which may also result in lower cytotoxicity.[1] For these reasons there has been much interest in the concept of specifically targeting "simple" mustards and other alkylators to DNA by attaching them to DNA affine carrier molecules, as this could in principle address these limitations. Increasing the concentration of drug in the vicinity of the DNA would mean less chance of losing active drug by reaction with other cell components. Additionally, the use of DNA-affine carriers with their own defined binding geometry makes it possible to alter both the region and sequence specificity of alkylation compared with that of the "simple" mustards (or other alkylators).[2]

Distamycin and netropsin based ligands

The minor-groove binding molecules, such as distamycin A and netropsin were used as DNA minor groove sequence-selective vectors of alkylating functions. A number of X-ray crystallographic and NMR studies have shown that these molecules bind into the minor groove of B-type DNA duplexes with high selectivity for AT-rich sequences containing at least four adenine–thymine base pairs. Van der Waals forces and hydrogen bonding play the key role for the DNA binding, whereas hydrophobic interactions and electrostatic binding components from the cationic end stabilize the complex.[3] Distamycin A (Figure 1) was used as the DNA minor groove sequence-selective vector of alkylating functions, leading to compounds endowed with relevant cytotoxic and antitumor activity in comparison to that, very weak activity, of distamycin itself.[4] The main representative of this class that was clinically tested in the recent past is tallimustine, a benzoic acid nitrogen mustard derivative of distamycin.[5] Tallimustine (Figure 1) showed cytotoxicity against L1210 murine leukemia more than two orders of magnitude higher then distamycin and more than one order of magnitude higher then classical nitrogen mustard melphalan (Figure 1). This compound is a very sequence and regiospecific alkylator, reacting only by monoalkylation at the N3 position of the 3′-adenine in the sequence 5′-TTTTGA-3′.

Whereas the cytotoxicity of tallimustine is related to the ability to form interstrand cross-links in DNA with consequent inhibition of DNA replication and transcription, the mechanism of antitumor action of tallimustine, although it is not yet fully

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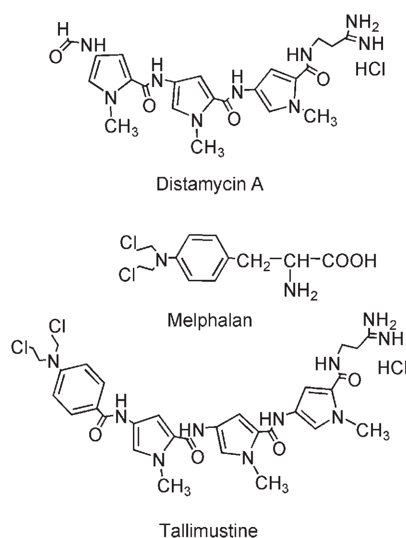

Figure 1. Structure of distamycin A, melphalan, and tallimustine.

elucidated, may be due to its ability to inhibit the binding of some transcription factors to their consensus sequences in DNA. The cell cycle phase perturbations caused by tallimustine and melphalan were different and can be related to the different DNA damage done by these two drugs.[5] Unfortunately, tallimustine showed a severe myelotoxicity that probably impaired the achievement of effective therapeutic doses and its Phase II clinical development was discontinued.

In the course of our investigations of minor groove-binding drugs, carbocyclic analogues of netropsin and distamycin **1**–**4** were synthesized and tested for DNA-binding properties.[6] The mustard function was introduced by treatment of the acyl chloride of chlorambucil with the amines in the presence of DMAP in pyridine (Figure 2).

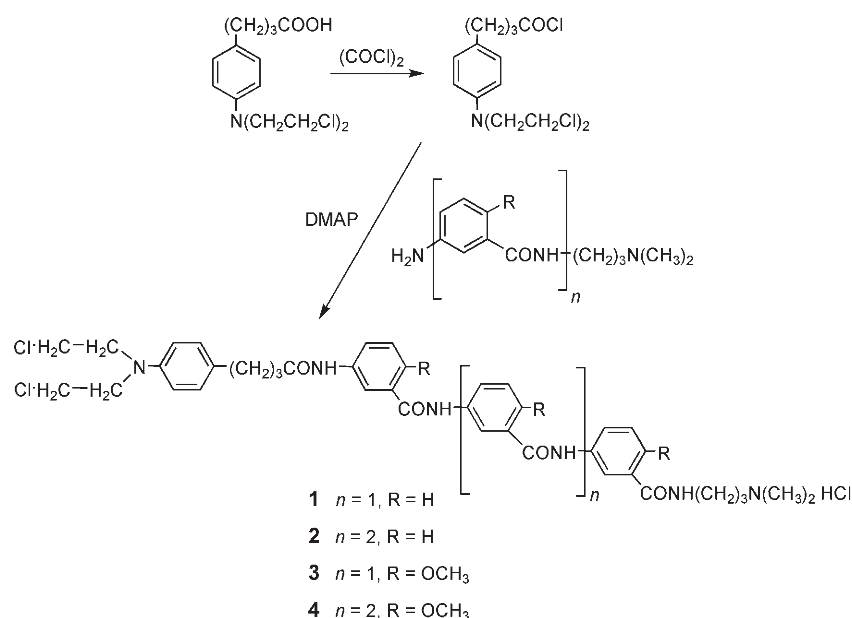

Figure 2. Synthesis of compounds **1**–**4**.

The compounds **1**–**4** bind to AT sequences more weakly than the extensively studied minor-groove binders such as netropsin and distamycin. However, these compounds show sequence selectivity.[6] It is worth noting that the carbocyclic analogues of netropsin and distamycin are readily available, can be modified easily, and are stable under most experimental conditions. All of the compounds **1**–**4** showed cytotoxic effects in cultured breast cancer MCF-7 cells, but they failed to present practical significant advantages in terms of activity compared to chlorambucil (Table 1).[6]

Table 1. In vitro activity of **1**–**4** against breast cancer MCF-7 cells.

Compd.	IC_{50} [μM]
Chlorambucil	103.70 ± 0.4
1	85.69 ± 0.3
2	104.06 ± 0.3
3	96.89 ± 0.4
4	98.62 ± 0.4

Bisbenzimidazole base ligands

Bisbenzimidazoles are well-defined reversible minor groove binding ligands. Several X-ray crystallographic and NMR studies on complexes of Hoechst 33258 with A/T-containing oligonucleotides have shown that the drug is bound in the minor groove, with the planar benzimidazole groups oriented parallel to the groove direction and each inner-facing nitrogen atom hydrogen bonding in a bifurcated manner to a pair of adjacent hydrogen-bond donors on the edge of the A/T base pairs.[3] The bulky piperazine ring has been located in most structures bound in a G/C region, with the minor groove observed to be wider at this sequence.[3] Hoechst 33258 is cytotoxic in its own right, and has undergone Phase I clinical evaluation as an anticancer agent. Studies on bisbenzimidazoles where the mustard was directly attached to the phenyl ring, but the benzimidazole DNA binding chromophores were altered by changing the heteroatoms, showed that analogues retaining DNA-affine H-bonding moieties had higher reversible binding and faster kinetics of alkylation.[7] These compounds alkylated mainly at guanine residues, particularly at 5′-GGTT sequences. Whether this was in the major or minor groove was not established, but it was noted that alkylation was inhibited by the reversible minor groove binder distamycin.[7] Overall, despite the highly se-

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quence-selective reversible binding of the bis(benzimidazole) chromophore itself, mustard analogues of this carrier have not shown the degree of sequence-and regioselective alkylation that was expected.

In the course of our investigations of minor groove binding drugs, we reported a cytotoxicity and DNA-binding ability of carbamate derivatives of Hoechst 33258 with chloroalkyl and bromoalkyl moieties (Figure 3).[8] These new compounds (**5**–**8**)

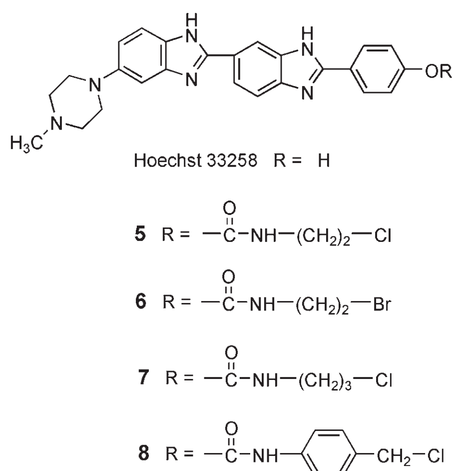

Figure 3. Structure of Hoechst 33258 and compounds **5**–**8**.

were readily prepared in good yields by addition of chloroethyl, bromoethyl, chloropropyl, or 4-(chloromethyl)phenyl isocyanates to Hoechst 33258. Their cytotoxic activity was evaluated on human breast cancer MCF-7. Compounds **5**–**8** were more cytotoxic than Hoechst 33258. In particular derivative **8**, the most active of the series, is up to three times more potent than Hoechst 33258 (Table 2). The DNA-binding ability of these compounds was evaluated by an ultrafiltration method using calf thymus DNA. These data showed that in broad terms the cytotoxic potency of **5**–**8** in cultured breast cancer MCF-7 cells increases, in accord with their increases in DNA affinity (Table 2).[8]

Table 2. In vitro activity of Hoechst 33258 and 5–8 against human breast cancer MCF-7 cells.

Compd.	IC_{50} [μM]	Binding Constant (10^3 M^{-1})
Hoechst 33258	>100	7.2
5	74.9±2	5.5
6	64.6±2	6.6
7	55.8±2	7.6
8	28.6±2	8.9

Amidine analogues of chlorambucil and melphalan

Our group has also reported the design, synthesis, and biological evaluation of amidine analogues of chlorambucil.[9] We started from the 5-[4-(*N*-alkylamidino)phenyl]-2-furancarboxylic acids, which were prepared by the general method described previously by Bielawski et al.[9] The amidino acids were conjugated with ethylenediamine in the presence of *N*,*N*′-carbonyldiimidazole as a condensing agent to give the compounds **9**–**14** in good yields (Figure 4). The compounds **9**–**14** are able to

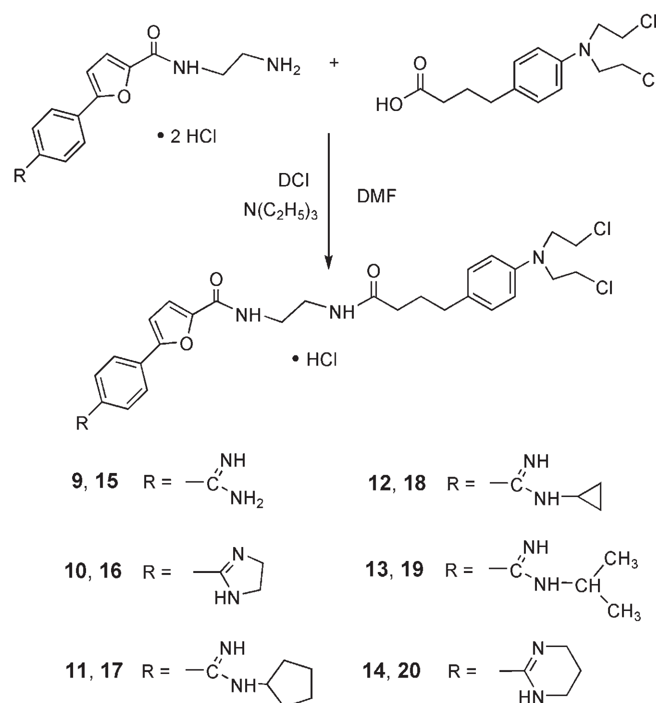

Figure 4. Synthesis of amidine analogues of chlorambucil **15**–**20**.

bind to double stranded DNA preferentially at AT base pairs along the minor groove by formation of hydrogen bonds. The final incorporation of the alkylating unit into the DNA-binding moieties was achieved using the classical coupling procedure. These new amidine analogues of chlorambucil **15**–**20** differ by the nature of their terminal basic side chains and were isolated as the hydrochloride salts.[9] A feature of these compounds is the presence of an amidino moiety that, because of its strong basic nature (its calculated pKa should be about 12), exhibits a complete protonation in any biological condition, and may play a key role both in the DNA binding and cell or tissue bioavailability.

We studied the effect of compounds **15**–**20** and chlorambucil on cell viability of breast cancer cells. All of the tested compounds showed concentration dependent activity, yet with different potency (Figure 5). The values of IC_{50} were relatively higher for **16** and **15** which possess a cationic 4,5-dihydro-1*H*-imidazol and an amidine function, respectively. Compound **16**, the most active of the series, is approximately five times more potent than chlorambucil.[9] The compounds of series **15**–**20** exhibited a positive correlation for decreasing cytotoxic potency as the size of N-terminal amidine group increases. These data suggest that steric factors associated with substituents at the N-terminal position may substantially influence the activity of the amidine analogues of chlorambucil.

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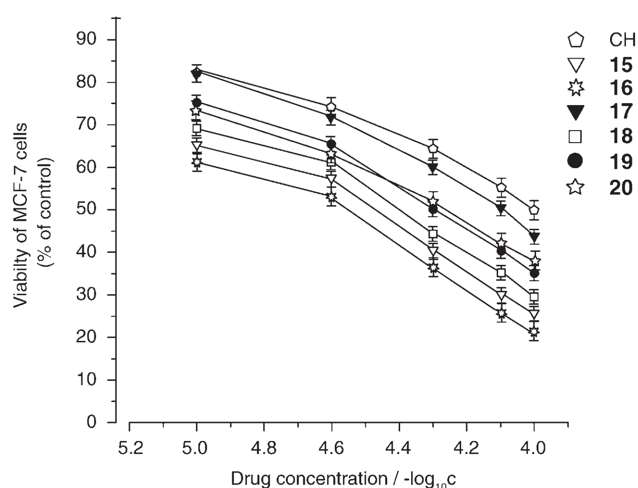

Figure 5. Viability of MCF-7 cells treated for 24 h with different concentrations of compounds **15**–**20** and chlorambucil (CH).

The homopolymer DNA-binding data reported in Table 3 characterizes the affinity of the compounds **15**–**20** for a more limited set of DNA-binding sites and can give an indication of base-sequence specificity for DNA-binding molecules.[9] The compounds **15**–**20** were shown to have very moderate binding affinities for DNA in ethidium bromide displacement assay when compared with the extensively studied minor groove binders such as netropsin and distamycin (Table 3). The increase in the size of the N-alkyl terminal amidine substituents for the series of **15**–**20** decreased the binding affinity, which suggested that steric hindrance might be responsible for a negative role of the substituents to the DNA-binding ability. DNA binding suggests that the combined effect resulting from alkylation and DNA minor groove binding might be in part responsible for the cytotoxic activity of **15**–**20**.

The binding of **15**–**20** in the minor groove of DNA may prevent binding of regulatory proteins or transcription factors to DNA promotors, as has been shown for other amidines.[6,7] It is well known that sites of topoisomerase II mediated DNA cleavage are not randomly distributed in DNA fragments, hence this enzyme seems to recognize specific sequences in a given substrate. This topological enzyme binds at least in part to AT-rich sequences in the minor groove of B-DNA. The ability of compounds **15**–**20** to inhibit topoisomerase II activity was quantified by measuring the action on supercoiled DNA substrate as a function of increasing concentration of the ligands by the use of agarose gel electrophoresis.[9] Chlorambucil as a control was, as expected, ineffective in this assay. These results demonstrated that **15**–**20** have topoisomerase II (Topo II) inhibitory activity with 50% inhibitory concentrations (IC_{50}) ranging from 5 to 70 μм (Table 3).[9] We were unable to establish a quantitative relationship between potency of enzyme inhibition and cytotoxicity.

We obtained also series of amidine analogues of melphalan differing by the nature of terminal basic side. The synthetic route followed by us is outlined in Figure 6. The amidino acids were conjugated with a melphalan methyl ester in the presence of *N*,*N'*-carbonyldiimidazole (DCI) as condensing agent in DMF at 0 °C to give the compounds **21**–**26** in good yields. Compound **24**, the most active of the series, is approximately two times more potent than melphalan.[10]

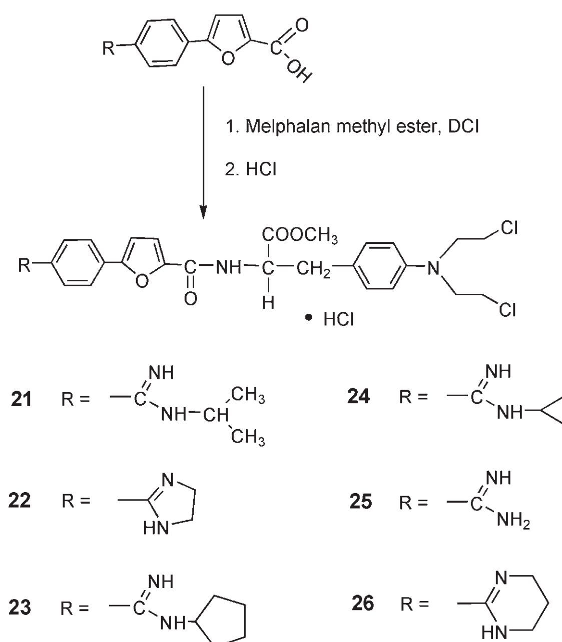

Figure 6. Synthesis of amidine analogues of melphalan **21**–**26**.

An attempt has also been made to correlate the observed biological activity with topoisomerases inhibitory properties and the DNA-binding properties of selected compounds. The cytotoxic properties of the amidine analogues of melphalan towards cultured human breast cancer cells correlate with topoisomerase II inhibitory properties but not with DNA-binding properties.[10]

A molecular mechanics and molecular dynamics approach was used to examine the structure of complex formed between the $d(CGCGAATTCGCG)_2$ duplex and compound **21**. It is

Table 3. DNA binding and topoisomerase II inhibitory effect of netropsin, distamycin, and compounds **15**–**20**.

Compd.	ct DNA[a] (K_{app}×10^5 м$^{-1}$)	poly(dA-dT)$_2$[a] (K_{app}×10^5 м$^{-1}$)	poly(dG-dC)$_2$[a] (K_{app}×10^5 м$^{-1}$)	Inhibition of Topo II [μм]
netropsin	8.7 ± 0.2	875.0 ± 0.2	2.5 ± 0.2	5
distamycin	7.5 ± 0.2	340 ± 0.2	2.0 ± 0.2	3
15	1.8 ± 0.2	3.2 ± 0.2	0.4 ± 0.2	10
16	2.6 ± 0.2	3.8 ± 0.2	0.4 ± 0.2	5
17	1.6 ± 0.2	2.8 ± 0.2	0.3 ± 0.2	15
18	1.4 ± 0.2	2.6 ± 0.2	0.4 ± 0.2	30
19	1.2 ± 0.2	2.2 ± 0.2	0.3 ± 0.2	40
20	0.9 ± 0.2	0.8 ± 0.2	0.3 ± 0.2	70

[a] The error for netropsin, distamycin, and compounds **15**–**20** is ±0.2×10^5 м$^{-1}$.

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predicted that compound **21** should have a decreased affinity for the minor groove of AT-rich regions in comparison to netropsin and furamidine. From the energetic analysis it appears that van der Waals and electrostatic interactions are more important than specific hydrogen bonds in stabilizing the ligand–duplex.

The results of our studies suggest that amidine analogues of melphalan may have other consequences for the metabolism of breast cancer cells. We have found that compound **21** is a more potent inhibitor of collagen biosynthesis than the parent drug, melphalan.[11] Decreased amounts of collagen in extracellular matrix is known to enhance mobility and invasion of neoplastic cells, but it also contributes to inhibition of cell growth and induction of apoptosis. The phenomenon was related to the inhibition of β_1-integrin and IGF-I receptor mediated signaling caused by **21**.[11]

We also demonstrated that melphalan for 24 h did not affect the expression of proteins involved in the signaling cascade activated by β_1-integrin receptor. In contrast, compound **21** inhibited expression of Shc and MAP-kinases in both cell lines. Decreased expression of FAK-kinase was found only in MDA-MB 231 cells.[11] Another important benefit evoked by the compound **21** seems to be inhibition of phospho-ERK's activation.[11] Upregulation of those kinases was found in various breast cancers.[12] Blocking these kinases was found to have proapoptotic and antiproliferative effect on MDA-MB 231, that indicates a new target in the treatment of breast malignancies.[13]

These results and other recent studies indicate that the amidine analogues of melphalan represent multifunctional inhibitors of breast cancer cells growth and metabolism. These results also indicate the different properties of the amidine analogues of alkylating agents and conventional ones.

Prolidase-convertible prodrugs

One approach to overcome the toxicity of alkylating agents to normal tissue is to construct a prodrug with lower hydrophobicity and cytotoxicity but preferentially activated in cancer cells. Prodrugs have been traditionally used to increase oral bioavailability, but recently prodrug strategies have also been employed to achieve drug targeting. The recent anticancer agents capecitabine and imatinib, selectively target cancer cells by exploiting the differences between normal and cancerous cells.[14] Enzymes that are differentially expressed in disease states are possible targets since enzymes comprise 30% of all drug targets. Of these, hydrolases such as esterases and peptidases are of special importance as prodrugs containing ester or amide linkages are quite common.[15] Of several possible enzymes that were so identified, prolidase was found to be overexpressed in breast cancer MDA-MB 231 cells, lung adenocarcinomas, and melanoma cancer cell lines.[16–18]

Prolidase is a 493 amino acid protein, and the subunit of this enzyme is a homodimer around 110 kDa. Prolidase has unique substrate specificity as it acts on dipeptides with proline at the carboxy terminus, and is a metalloenzyme requiring manganese (Mn^{2+}) as a cofactor for optimum catalytic activity.[19] The primary biological function of prolidase involves the metabolism of collagen degradation products and the recycling of proline from imidodipeptides for collagen synthesis. It is evident that an absence of prolidase will severely impede the efficient recycling of collagen proline. On the other hand, enhanced liver prolidase activity was found during the fibrotic process. It suggests that prolidase, by providing proline for collagen biosynthesis may regulate turnover of collagen and may be a rate-limiting factor in the regulation of collagen production. Prolidase deficiency is a rare autosomal recessive disorder characterized by massive imidodipeptiduria, skin lesions, recurrent infections, mental retardation, and elevated proline-containing dipeptides in plasma.[19] In fibroblast cultures from prolidase deficient patients an increase in the rapidly degraded collagen and decrease in proline pool has been found.[19,20]

Prolidase catalyses the final step in collagen degradation which completes the recycling of proline. The efficiency for proline recycling is about 90%. The best and most abundant substrate for prolidase is glycyl-L-proline (Gly-L-Pro). Collagen which accounts for about one third of total body proteins represents polypeptide containing the highest amount of imidobonds compared to all known proteins. In $\alpha1$ chains of type I collagen, Gly-L-Pro occurs 25 times.[21]

It has also been observed that aminopeptidase P, the closest relative in substrate specificity to prolidase, hydrolyzes tripeptides, or higher peptides but does not hydrolyze dipeptides. Thus, prolidase is one of the very specific peptidases for proline-containing dipeptides. However, when methionyl group or haloacetylprolines replace its amino group in imidodipeptides, good substrates result, suggesting that an α-amino group is not an absolute specificity requirement for prolidase.[22]

We synthesized proline prodrugs of chlorambucil and melphalan (Figure 7). Compound **27** was synthesized by using the carbodiimide coupling method. The protecting benzyl group was removed by catalytic hydrogenation at room temperature and atmospheric pressure gave the desired compound. Compound **28** was obtained in four steps from starting material L-proline benzyl ester. Compound **28** was isolated as a dilithium salt.[23]

Compound **28** shows susceptibility to the action of breast cancer MDA-MB 231 cells prolidase, compared to standard prolidase substrate glycyl-L-proline (Gly-L-Pro) and about sixfold higher susceptibility, compared to another of its substrate glycyl-L-hydroxyproline (Gly-L-Hyp) (Figure 8). The proline analogue of chlorambucil (**27**) shows susceptibility to the action of prolidase in a range similar to that observed in the case of glycyl-L-hydroxyproline. In the presence of prolidase inhibitor, Z-Pro, cell homogenate looses the ability to convert proline analogues of chlorambucil and melphalan (Figure 8). It suggests that **27** and **28** may represent prolidase-convertible prodrugs. Proline analogues of chlorambucil and melphalan evoked slightly higher cytotoxicity for MDA-MB 231 cells, compared to the parent drugs.[16,17]

As imido-bound proline can be reused for collagen synthesis, we have compared the effect of melphalan and **28** on synthesis of this protein in breast cancer MDA-MB 231 cells.[17] Both drugs inhibited collagen biosynthesis during the course

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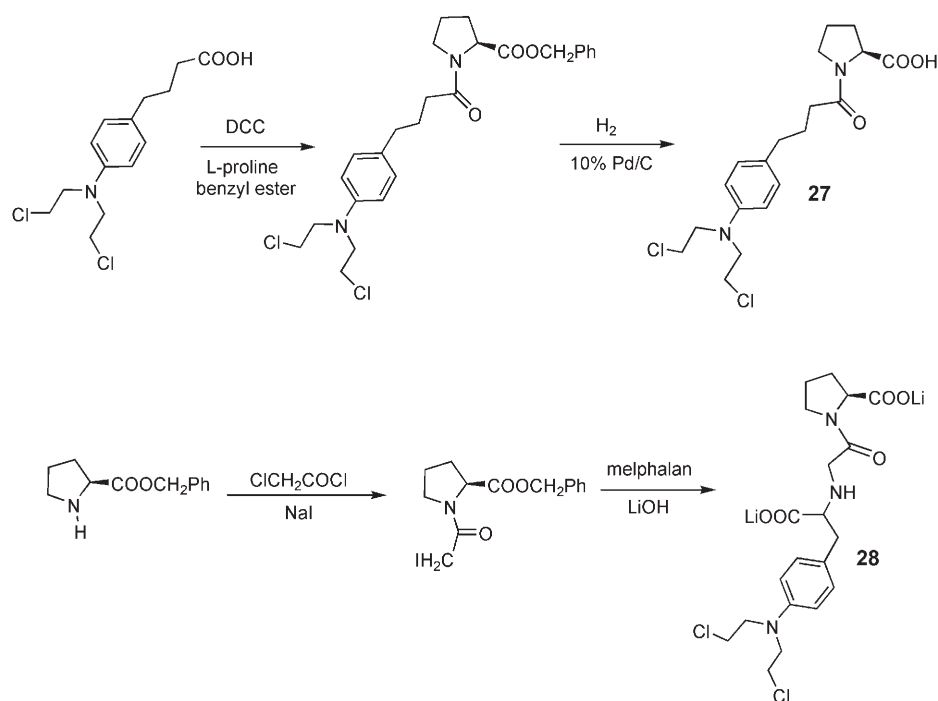

Figure 7. Synthesis of proline analogues of alkylating agents.

It suggests that the inhibitory action of Melphalan and **28** on collagen biosynthesis in MDA-MB 231 cells may occur by different pathways. Although, the mechanism of this process is unknown it suggests that this feature of **28** may be of benefit from the point of its potential application in pharmacotherapy of neoplastic diseases, as IGF-I receptor is also involved in stimulation of cell division.

Mittal et al. obtained prodrugs of melphalan that comprised of linkage of the carboxy terminus of the L-phenylalanine moiety of melphalan to the N terminus of L and D stereoisomers of proline (Figure 9).[18] The specific activity of kidney prolidase for the D analogue was roughly 100-fold lower compared to the L-proline analogue, suggesting substrate specificity of prolidase.

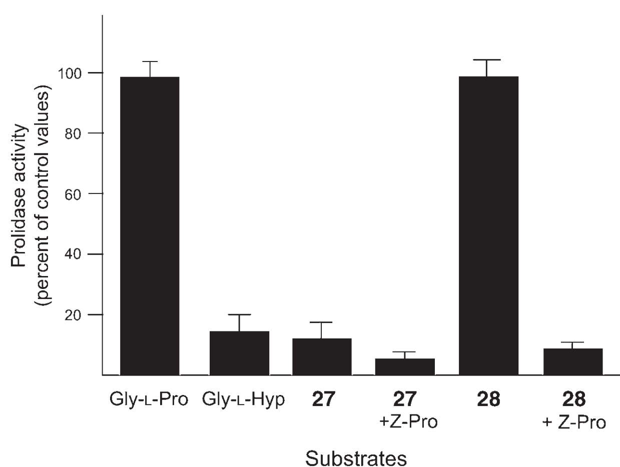

Figure 8. Susceptibility of proline analogue of chlorambucil (**27**), Gly-L-Pro, Gly-L-Hyp and proline analogue of melphalan (**28**) to the action of prolidase.

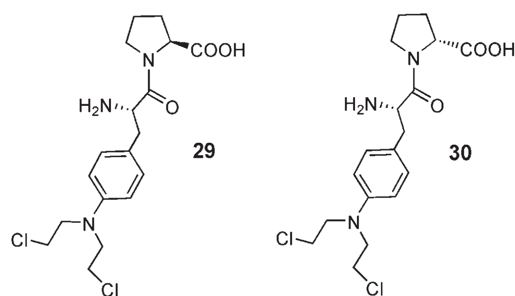

Figure 9. Structure of proline analogues of melphalan.

of the experiment. However, the cells incubated with compound **27** for 24 and 48 h produced much more collagen than the cells incubated with melphalan. Compound **27** also evoked lower inhibitory effects on collagen biosynthesis in breast cancer MCF-7 cells, compared to the free drug.[24] This phenomenon may be due to its delivery of proline substrate for collagen biosynthesis into the cells and lack of its effect on prolidase activity inhibition, compared to the free drug.

It is known that IGF-I is the main stimulator of collagen biosynthesis. Its actions is regulated by IGF-I receptor expression that induces downstream signaling through the MAP kinase pathway.[25] Interestingly, melphalan did not affect the expression of both IGF-I receptor and MAP kinases, whereas compound **28** distinctly reduced the expression of these proteins.

These two melphalan prodrugs, prophalan-L (**29**) and prophalan-D (**30**) were evaluated for their antiproliferative activity in SK-MEL-5 melanoma cells along with the parent drug melphalan. Prophalan-L exhibits antiproliferative action similar to that of the parent drug whereas the D analogue, prophalan-D, was relatively ineffective at comparable concentrations. The roughly sixfold lower antiproliferative activity of prophalan-D compared to prophalan-L is quite consistent with its sevenfold lower hydrolysis rate in SK-MEL-5 cell homogenates compared to prophalan-L.[18]

The results of these studies support the proposition that prolidase could serve as a target enzyme for the selective action of anticancer agents. However, to demonstrate selective delivery of the prodrugs to tumor tissues, it would be necessary to examine potential bioactivation of the prodrugs by prolidase expressed in normal tissues and in organs such as the liver and kidney.

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Keywords: alkylating agents · cancer · DNA binding · prodrug · prolidase

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