Synthesis and in Vitro Evaluation of Quaternary Ammonium Derivatives of Chlorambucil and Melphalan, Anticancer Drugs Designed for the Chemotherapy of Chondrosarcoma

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To enhance affinity for malignant cartilaginous tumors (chondrosarcomas), quaternary ammonium (QA) conjugates of chlorambucil and melphalan were prepared by linking the QA moiety to nitrogen mustards via an amide bond. They exhibited closely similar and sometimes more favorable values than their parent compounds. In the cell lines tested, the two QA conjugates displayed appreciable cytotoxicity, the QA conjugate of chlorambucil even showing an enhanced efficiency against chondrosarcoma compared with chlorambucil.

Introduction

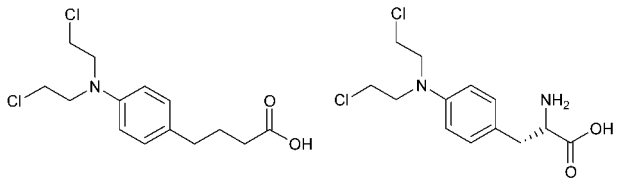
Previous works developed in our laboratory have demonstrated that molecules owning quaternary ammonium moieties exhibited a high affinity for cartilage.[1−5] This property can be used to specifically carry active principles, including anticancer agents, toward cartilaginous tissues. The malignant disease of cartilage, chondrosarcoma, is the second most common malignant tumor of bone after osteosarcomas.[6] Such tumors are generally radio-resistant and poorly respond to routine chemotherapy, which is also markedly toxic.[6−8] Our objective was to develop more selective anticancer drugs that would concentrate in the malignant cartilaginous tissue and so improve therapeutic index through a reduction of side effects. For this purpose, we have synthesized derivatives of chlorambucil (**1**) and melphalan (**2**) (Chart 1) linked to a quaternary ammonium (QA) moiety. We describe here the synthesis and characterization of two QA conjugates of chlorambucil and melphalan, where the QA entity was linked to the nitrogen mustard via the carboxylic function by an amide bond. We also evaluate several physicochemical and in vitro biological parameters and the cytotoxicity of the QA derivatives compared with their parent nitrogen mustards.

Chemistry

The QA derivative of chlorambucil (**4**) was prepared by the two-step procedure illustrated in Scheme 1. First, reaction of the primary amino group of 3-(dimethylamino)propylamine with the acid chloride of chlorambucil, generated from **1** and thionyl chloride, provided the butyramide **3**, which was then treated with methyl iodide to give the desired quaternary salt **4** in high yield.

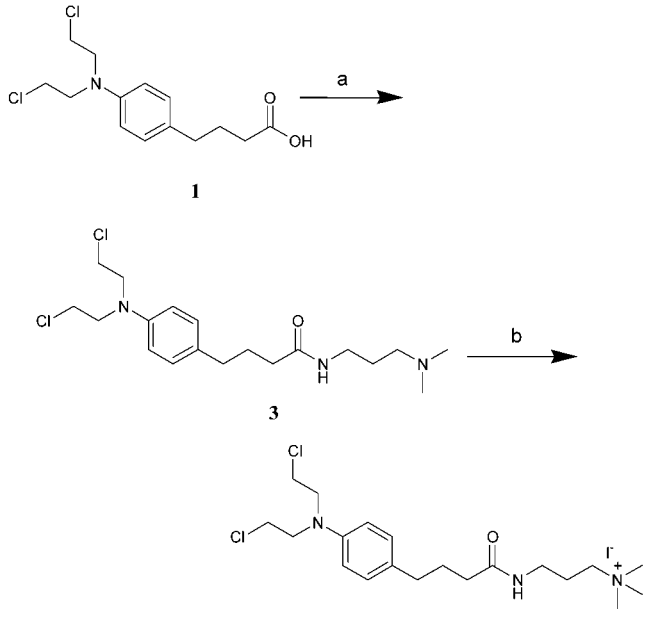
The synthetic sequence adopted for the preparation of the QA derivative of melphalan (**8**) was similar to that described above with, in addition, the protection−deprotection reactions for amino acids (Scheme 2). Melphalan (**2**), as its hydrochloride, was converted to its *N*-*tert*-butyloxycarbonyl (*t*-Boc) derivative **5** by treatment with di-*tert*-butyl dicarbonate ($(Boc)_2O$). Subsequently, reaction of crude **5** with 3-(dimethylamino)-propylamine, using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT) as condensing reagents, gave the amide **6** in moderate yield. Quaternarization of the tertiary amino function of **6** was carried out by

Chart 1



1, Chlorambucil **2, Melphalan**

Scheme 1[a]



[a] Reagents and conditions: (a) (1) thionyl chloride, CH_2Cl_2, 4 °C, overnight; (2) 3-(dimethylamino)propylamine, CH_2Cl_2, room temperature for 5 h; (b) methyl iodide, EtOH, room temperature for 3 h.

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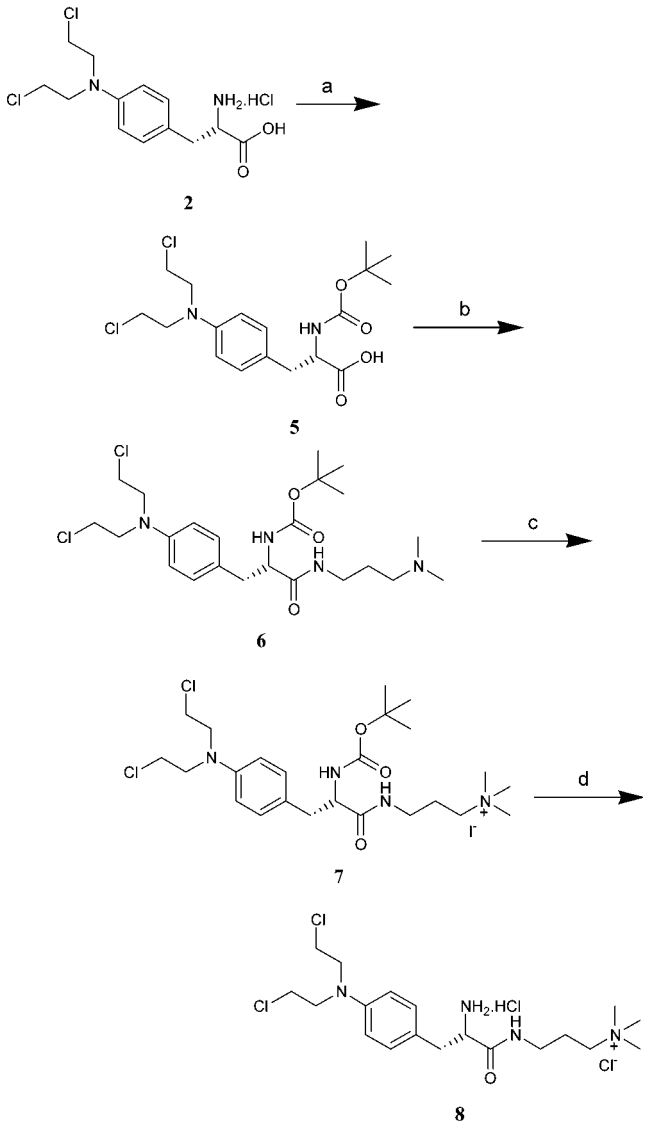
Table 1. Physicochemical Properties and Cellular Uptake Studies of **1**, **2**, **4**, and **8**

compd	hydrolysis rate[a] (%)	alkylating activity[b] $k' \times 10^3$	GSH activity[c] % of control	log P[d]	cellular uptake[e] pmol/μL M4 Beu	cellular uptake[e] pmol/μL H-EMC-SS
1	34.7 ± 0.3	30.2 ± 1.3	60.4 ± 3.7	0.75 ± 0.06	18.41 ± 0.20	2.73 ± 0.86
4	13.4 ± 1.1	40.4 ± 3.3	55.4 ± 3.2	0.32 ± 0.04	16.70 ± 1.21	2.97 ± 0.94
2	24.1 ± 0.4	10.3 ± 3.7	85.3 ± 1.3	0.11 ± 0.02	21.95 ± 4.64	2.88 ± 0.47
8	20.3 ± 0.8	15.7 ± 2.5	79.0 ± 2.3	−1.12 ± 0.14	6.82 ± 1.76	1.54 ± 0.74

[a] Determined in CH_3COCH_3/H_2O for 30 min at 66 °C. [b] Determined by the NBP assay at 570 nm. [c] Determined using DTNB and subsequent measurement of absorbance at 412 nm and expressed as percent of the control (no drug added). [d] Determined in octanol/phosphate buffer. [e] Values of cellular uptake at 1 h are expressed as picomoles per microliters cellular. All data are expressed the mean of three independent experiments ± standard deviation.

Scheme 2[a]



[a] Reagents and conditions: (a) di-*tert*-butyl dicarbonate, MeOH, room temperature for 30 min; (b) 3-(dimethylamino)propylamine, DCC, HOBT, room temperature for 5 h; (c) methyl iodide, EtOH, room temperature for 3 h; (d) 2 N ethanolic HCl, room temperature for 2 h.

treatment with methyl iodide, leading to **7**. Finally, acidolytic removal of the *t*-Boc protective group of **7** gave the target QA derivative **8** in excellent yield.

Results and Discussion

Binding of a quaternary ammonium moiety to chlorambucil and melphalan was undertaken to confer on these active principles an increased affinity for cartilaginous tissues, including chondrosarcoma. We performed experiments with a view to study the influence of such structure modification on physicochemical and biological properties of these nitrogen mustards, known to play, directly and indirectly, a role in their cytotoxic potency.[9−12] Table 1 shows the physicochemical properties and cell uptake studies performed on chlorambucil **1** and its QA derivative **4**, and melphalan **2** and its QA derivative **8**. In the case of nitrogen mustards, a high proportion of the drug is liable to be hydrolyzed before reaching DNA, leading to a loss of the cytotoxic activity.[9,10] The QA−chlorambucil conjugate **4** was found to be more stable than its parent compound, its hydrolysis rate being almost 3 times lower than that of **1**, whereas for the melphalan family, no striking difference was observed between the two types of compound. The measure of the alkylation rate of 4-(4-nitrobenzyl)-pyridine shows that the chlorambucil series alkylated NBP more rapidly than the melphalan series and the alkylation rate was a little higher for the QA derivatives **4** and **8** than for **1** and **2.** Our results also show that QA functionalization has no influence on reactivity toward glutathion. Finally, the in vitro evaluation of these three parameters does not reveal any striking differences between the parent nitrogen mustards and their QA conjugates but shows that the slight differences observed (weaker hydrolysis rates, little increasing of alkylating activity) are in favor of the QA conjugates.

A comparative cellular uptake study was performed on a melanoma cell line (M4Beu) and a chondrosarcoma cell line (H-EMC-SS). Our results clearly show that the uptake of the four tested products was markedly higher in melanoma cells than in chondrosarcoma cells. On the other hand, the uptake of compound **8** was significantly weaker than this of compound **2** for both cell lines. These results can be explained by the fact that chlorambucil is taken up by tumor cells by simple diffusion, while melphalan, because of its phenylalanine mustard structure, is taken up by cells by an active carrier-mediated process via amino acid transport systems.[13] These results can be also related to the lipophilicity of these compounds evaluated by the measure of their partition coefficient (log P). The introduction of the polar QA moiety leads to a decrease of the lipophilicity more important in the melphalan family, countering its ability to cross the cell membrane.

In Table 2 are given the results of the cytotoxic activity of compounds **1**, **2**, **4**, and **8**, determined in a panel of human and murine tumor cell lines including chondrosarcoma. According to the colony forming assay

Table 2. In Vitro Cytotoxic Activities of **1**, **2**, **4**, and **8**[a]

	IC_{50} (μM)				CC_{50} (μM)	
compd	M4 Beu[b]	M4 Dau[b]	B16[b]	MCF7[b]	H-EMC-SS	
1	5.1 ± 2.4	1.5 ± 0.9	5.1 ± 0.8	1.7 ± 0.2	3.5 ± 0.8[c]	2.7 ± 0.4[d]
4	5.1 ± 1.3	1.3 ± 0.4	8.6 ± 2.5	1.2 ± 0.3	0.7 ± 0.1[c]	0.6 ± 0.2[d]
2	1.2 ± 0.5	0.5 ± 0.1	2.2 ± 0.9	0.3 ± 0.1	1.6 ± 0.5[c]	0.9 ± 0.2[d]
8	14.4 ± 3.5	3.3 ± 0.8	19.5 ± 3.1	0.9 ± 0.02	1.8 ± 0.2[c]	1.2 ± 0.3[d]

[a] Data are the mean of at least three independent experiments ± standard deviation. [b] Corresponding IC_{50} values determined by the colony forming assay. [c] Value determined by Alamar Blue assay. [d] Value determined by Hoechst 33342 DNA fluorometric assay.

conditions,[14] the results obtained show, for the chlorambucil series, a similar antiproliferative activity between **1** and **4** whatever the cell line, these two drugs exhibiting closely similar IC_{50} values against M4 Beu, M3 Dau, B16, and MCF7 cells. In contrast, **8** was found to be less potent than **2**, the IC_{50} values obtained for the melphalan−QA conjugate being, depending on the cell line considered, 3−12 times higher than those observed for the parent mustard. Nevertheless, despite these less noteworthy results for the melphalan series, this study shows that **4** and **8** possess an antiproliferative potency, indicating that the introduction of the QA moiety into the structure of **1** and **2** does not abolish the in vitro antitumor potency, which is actually maintained in the case of the chlorambucil family. Surprisingly, the cytotoxic activity of the tested compounds against chondrosarcoma was found to be markedly different from that described when the other cell lines were studied, this difference being observed irrespective of the assay used (Blue Alamar or Hoechst) and the series tested. Two main observations can be made: (i) altough the cell uptake was weaker for chondrosarcoma, the cytotoxicity of **1** and **2** was close to that measured for the other cell lines; (ii) QA derivatives exhibited higher cytotoxic potency than their parent compounds, contrarily to the results obtained with the other cell lines. If these constatations seem to show that chondrosarcoma cells are more sensitive to the cytotoxic action of mustards, the increase of activity induced by the QA functionalization cannot be related to an increase of cell uptake. An explanation can be given by the fact that the chondrosarcoma cells synthesize proteoglycans able to bind QA derivatives through ionic interactions.[5,15] Although the uptake is not higher for QA derivatives than for the parent compounds, the ionic binding to proteoglycans possibly lengthens the residence time of the QA derivative into the cell, permitting an increase of the DNA alkylation potency.

Conclusion

The aim of this work concerning nitrogen mustards and chemotherapy of chondrosarcoma was to determine in vitro if the pharmacological activity known for the parent mustards was conserved and even improved for their QA conjugates. Clearly, the results reported here illustrate that the QA functionalization does not alter the cytotoxic potency in vitro but modifies the cell uptake by a decrease of lipophilicity. This phenomenon, which is a drawback for mammary and melanoma cells studied, is widely counterbalanced in chondrosarcoma cells by an important enhancement of the cytotoxic activity for QA derivatives. Binding of such molecules to intracellular proteoglycans, lengthening their residence time into chondrosarcoma cells, can explain this result. Given the fact that we have previously shown that QA derivatives exhibited in vivo an increased affinity for cartilage,[4] by concentration into the cartilaginous matrix, we can postulate that the functionalized mustards are able to follow this pathway and so increase the uptake in vivo compared to the nonfunctionalized molecules. On the basis of these promising in vitro data, the next step will consist in the investigation in vivo on rat model of the targeting of the QA derivatives on cartilaginous tissues. These experiments are currently in progress. Then, we expect obviously to evaluate the in vivo antitumor activity of these compounds on animals bearing human chondrosarcoma.

Experimental Section

Radioactivity cellular uptake was performed using ^{14}C-labeled molecules.[16] Colony forming and cytotoxicity assays were performed on different cell lines, human melanoma, mammary carcinoma, and chondrosarcoma cells, and on the murine B16 melanoma cells, using Alamar Blue and Hoescht 33342-DNA fluorimetric methods.[17−18]

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Supporting Information Available: General experimental procedure for preparation, physical and spectral characterizations ($^1H/^{13}C$ NMR, mass spectrometry, and IR data), and physicochemical parameters of all the synthesized compounds and general experimental procedure for cytotoxicity and cells uptake experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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