Antitumor Agents

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Thermoresponsive Chlorambucil Derivatives for Tumour Targeting

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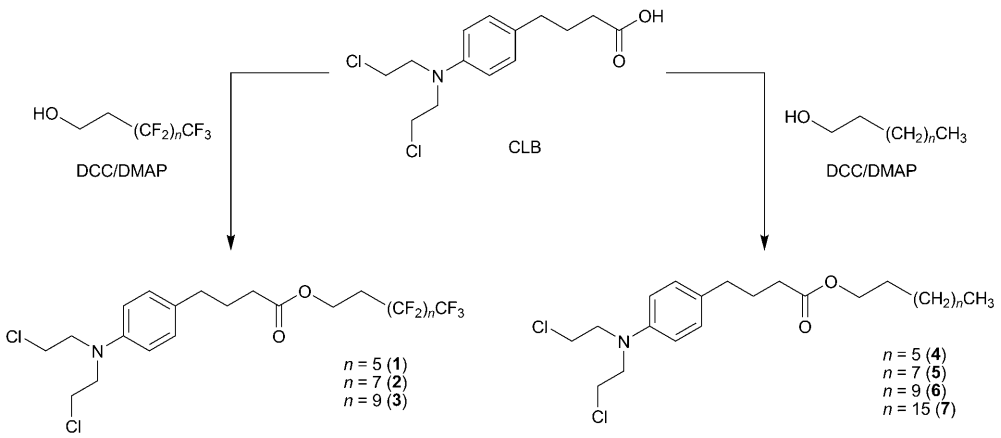
Many of the most widely applied anticancer agents result in severe side-effects, including, in extreme cases, secondary cancers that are only detected a long time after administration of the drug has ceased.[1] A promising approach to overcome nonselectivity relies on drug enhancement by the application of external techniques, such that the toxicity of the drug is low until it is activated at the tumor site. One such combination approach is to combine chemotherapy with hyperthermia at the tumor site.[2] Indeed, the cytotoxicity of some anticancer drugs is enhanced under mild hyperthermia (40–42 °C), even though the drugs also work under normal conditions, and were not intentionally designed for this application.[3] The thermosensitivity of small-molecule drugs can be enhanced by attaching them to thermosensitive macromolecules, for example, liposomal drug carriers,[4] that are insoluble at 37 °C and become soluble under hyperthermia and cross the cell membrane.[5] Other polymers that are soluble at 37 °C may aggregate in a heated tumor.[6] For example, ThermoDox is a drug delivery system based on a low temperature sensitive liposome (LTSL) containing doxorubicin. This system is currently in phase III clinical trials in combination with radiofrequency ablation (RFA) for the treatment of hepatocellular carcinoma (HCC).[7] When heated to 42 °C, the LTSL releases 100 % of the doxorubicin drug in less than 20 seconds.[8] However, the development of low-molecular-weight thermosensitive drugs that exclude macromolecular carriers would be attractive, and herein we describe rationally designed thermoactive molecular derivatives of chlorambucil (CLB);[9] these derivatives are essentially inactive at 37 °C, and are activated by mild hyperthermia (41 °C) in vitro. This behavior, which is referred to as thermoactivity, was achieved by covalently linking perfluorinated "pony tails" to CLB, as perfluorinated compounds were shown to be highly thermoresponsive in the field of catalysis.[10]

A series of perfluorinated CLB derivatives (**1**–**3**) and their hydrocarbon equivalents (**4**–**7**) were synthesized. CLB was coupled by an ester link with either a fluorinated alcohol or its hydrocarbon equivalent using *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP), as shown in Scheme 1 (see the Supporting Information for full details of synthesis and characterization). CLB derivatization with hydrophobic chains makes compounds **1**–**3** and **7** less soluble than CLB in water at 37 °C, with the solubility of **3** and **7** increasing rapidly with temperature. Every derivative has a higher partition coefficient ($\log P_{octanol/water}$) value (6.26–12.47) than CLB ($\log P_{o/w} = 3.92$), with the perfluorinated analogues being the most lipophilic (see the Supporting Information for temperature–solubility profiles in water and $\log P_{o/w}$ values).



Scheme 1. Synthesis of CLB derivatives **1**–**7**.

Consequently, the anticancer activity of CLB and its derivatives was initially evaluated in two human ovarian carcinoma cell lines (A2780 and its cisplatin-resistant variant A2780cisR) by using the MTT assay. IC_{50} values were obtained on cells treated with the compounds incubated for 72 h at 37 °C, and also under mild hyperthermia, that is, the cells were incubated for 4 h at 41.5 °C followed by 68 h at 37 °C (Table 1). At 37 °C, the hydrocarbon analogues **4** and **5**

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Supporting information for this article (detailed descriptions of the synthesis and characterization of all compounds, procedures for the cytotoxicity determination, the plasmid DNA interaction study, and the comet assays) is available on the WWW under http://dx.doi.org/10.1002/anie.201101133.

Table 1: Cytotoxicity of CLB and **1**–**7** in A2780 and A2780cisR cells at 37 °C and 41.5 °C.

Compound	IC_{50} in A2780 [μM]		IC_{50} in A2780cisR [μM]	
	37 °C	41.5 °C	37 °C	41.5 °C
CLB	12 ± 6	39 ± 14	43 ± 5	52 ± 1
1	26 ± 8	38 ± 9	65 ± 5	67 ± 2
2	38 ± 12	23 ± 7	25 ± 6	27 ± 6
3	> 200	37 ± 5	> 200	40 ± 4
4	6 ± 2	14 ± 7	16 ± 1	17 ± 7
5	9 ± 1	45 ± 7	19 ± 2	35 ± 6
6	40 ± 4	51 ± 10	45 ± 10	66 ± 2
7	> 200	51 ± 4	> 200	41 ± 5

are more cytotoxic than CLB. In contrast, **6** is less active than CLB in A2780, but has a comparable IC_{50} value to CLB in A2780cisR cell line at 37 °C. Compound **2** is more active than CLB at 37 °C, but only in A2780cisR cell line. Compound **2** is less cytotoxic than CLB in A2780 cells, as are **1**, **3**, and **7**, with the latter two compounds being inactive ($IC_{50} > 200$ μM) at 37 °C. Under mild hyperthermia, CLB and its derivatives have equivalent cytotoxicity or are less cytotoxic relative to treatment at 37 °C. Compound **1** has the same cytotoxicity as CLB at 41.5 °C, and **6** appears to be less active than CLB in both cell lines.

Compounds **2** and **4** become even more active than CLB at 41.5 °C. On increasing the temperature to 41.5 °C, **3** and **7** become active in both cell lines. Notably, in the A2780cisR cell line at 41.5 °C, **3** and **7** are even more active than CLB at either 37 °C ($IC_{50} = 43$ μM) or at 41.5 °C ($IC_{50} = 52$ μM; Table 1). Compounds **1**, **3**, **6**, **7**, and CLB were further studied in five human cancer cell lines (HT-29 colon cancer cells, HT-1080 fibrosarcoma cells, THP-1 and U937 monocytic myeloma cells, and TK-6 lymphoblastoid cells) and HCEC human endothelial cells as a model for angiogenic cells at 37 °C and under mild hyperthermia that includes 2 h at 41 °C (Table 2).

In the HT-29 cell line, CLB and **1** are moderately toxic at 37 °C while **3**, **6**, and **7** are inactive with IC_{50} values above 100 μM. At 41 °C, CLB, **1**, **3**, and **6** have increasing cytotoxicity in the HT-29 cell line; CLB and **1** have comparable IC_{50} values while **3** becomes more active than CLB at 37 °C. Although **6** has a lower IC_{50} value at 41 °C, it is still essentially inactive. In the HT-29 cell line, only **7** becomes less active at the higher temperature. The compounds behave similarly in the HCEC cell line; CLB and **1** are slightly cytotoxic at 37 °C and both have increased cytotoxicity at 41 °C. Reasonable thermoresponsive behavior is observed for **3**, which is inactive at 37 °C and at higher temperature becomes as active as CLB at 37 °C. The IC_{50} values for **6** and **7** do not change significantly at 41 °C. In the HT-1080 cell line, CLB and **1** have comparable cytotoxicity at both 37 and 41 °C. CLB and **1** are less cytotoxic at 41 °C than at 37 °C, whereas the toxicity of **3**, **6**, and **7** increases from modest at 37 °C to moderate at 41 °C, with **3** becoming more active than CLB and as active as **1**. In the THP-1 cell line, apart from CLB that remains moderately cytotoxic at both temperatures, all compounds show an increase in their cytotoxicity with temperature, with **1** becoming as cytotoxic as CLB and **3** undergoing the largest change in IC_{50} values ($\Delta = 32$ μM) in this cell line. At 37 °C, CLB is the most cytotoxic of the series in the U-937 cell line, followed by **1**, while **6** and **7** are moderately toxic, and **3** is inactive. At 41 °C, CLB, **1**, **6**, and **7** are less cytotoxic and **1** becomes more cytotoxic than CLB. Compound **3** is the only derivative that exhibits an increase in cytotoxicity with temperature. In the TK-6 cell line at 37 °C, CLB and **1** are reasonably cytotoxic compared to the other compounds, and at 41 °C, CLB, **1**, and **3** become more toxic; **1** is as toxic as CLB at 37 °C. The IC_{50} values of **6** and **7** increase at higher temperature; **7** remains essentially unchanged and **6** becomes inactive.

Table 2: Cytotoxicity of CLB, **1**, **3**, **6**, and **7** on adherent (A) and non-adherent (B) cells at 37 °C and 41 °C.

A)	IC_{50} [μM] HT-29		IC_{50} [μM] HT-1080		IC_{50} [μM] HCEC	
	37 °C	41 °C	37 °C	41 °C	37 °C	41 °C
CLB	54 ± 3	44 ± 1	21 ± 1	35 ± 1	64 ± 4	54 ± 1
1	48 ± 2	44 ± 1	23 ± 2	32 ± 1	89 ± 4	49 ± 2
3	189 ± 4	50 ± 3	81 ± 2	32 ± 2	125 ± 1	64 ± 2
6	> 200	111 ± 2	64 ± 3	40 ± 2	99 ± 1	101 ± 1
7	120 ± 3	> 200	67 ± 2	52 ± 4	114 ± 3	109 ± 4

B)	IC_{50} [μM] THP-1		IC_{50} [μM] U-937		IC_{50} [μM] TK-6	
	37 °C	41 °C	37 °C	41 °C	37 °C	41 °C
CLB	25 ± 5	28 ± 1	4 ± 1	25 ± 1	22 ± 4	13 ± 1
1	35 ± 1	26 ± 2	6 ± 1	18 ± 2	31 ± 2	22 ± 5
3	84 ± 2	52 ± 2	> 200	62 ± 5	83 ± 4	60 ± 2
6	105 ± 1	77 ± 2	60 ± 1	81 ± 5	86 ± 1	174 ± 5
7	94 ± 4	82 ± 5	48 ± 3	59 ± 3	51 ± 2	58 ± 2

From the data provided in Table 2, compound **3**, which has the highest $\log P_{o/w}$ value (see the Supporting Information), is the only derivative with universal thermoactive behavior in all the tested cell lines. The extent of this behavior depends on the cell line with the best thermoactive effect in adherent cell lines (HT-29, HCEC, and HT-1080), in which **3** is as cytotoxic as CLB at 41 °C. Compound **7** does not show thermoresponsive behavior in U-937 and TK-6 cell lines. In general, **6** shows the same thermoactive behavior as **3** but to a lesser extent, that is, the difference in cytotoxicity at 37 and 41 °C is smaller, and in U-937 and TK-6 cells no temperature selectivity is observed, thus indicating the importance of perfluorinated chains over aliphatic chains to induce the thermoresponsive behavior. The short perfluorinated chain in **1** leads to a compound with an overall cytotoxicity that is comparable to that of CLB with only a modest thermoresponsive effect observed in the HT-1080 cell line.

Preliminary investigations into the mechanism of action of **3** with respect to the synergistic effect with hyperthermia were undertaken. Since CBL is a DNA alkylating agent,[11] the interaction of **3** with DNA was evaluated in vitro with pBR322 plasmid DNA, by incubation with **3** at various concentrations (50, 100 and 200 μM) for 24 h at 37 °C, or 1 h at 41 °C followed by 23 h at 37 °C. The effect on DNA was visualized by gel electrophoresis (Figure 1), which showed that **3** interacts slightly with DNA at 37 °C, as the amount of supercoiled DNA (SC) decreases with the formation of alkylated DNA. The exact nature of the damaged DNA is not

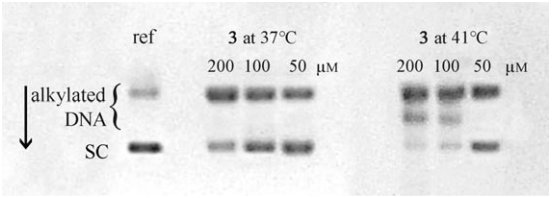

Figure 1. Agarose gels of plasmid DNA incubated at 37 °C for 24 h (left) or at 41 °C for 1 h and 23 h at 37 °C (right) with **3** at various concentrations. The first line (ref) shows the control DNA.

clear, but could correspond to alkylation that results in cross-linking,[12] although there is also evidence to suggest that CLB can induce strand breaks and produce open circular DNA.[13] Under hyperthermia, this transformation is increased and the appearance of a new band between the two reference bands is also observed. Therefore, hyperthermia helps to trigger the activity of **3** with DNA in vitro.

To evaluate the damage caused by **3** on cellular DNA, a comet assay[14] was performed on HT-29 and TK-6 cells. Cells were incubated with **3** (or CLB or **1** as controls; see the Supporting Information) for 24 h at 37 °C, or 2 h at 41 °C followed by 22 h at 37 °C. Damage was visually evaluated by using fluorescence microscopy (4′,6-diamidino-2-phenylindole (DAPI) staining) by classifying cells into five categories according to the comet tails (Figure 2 and Figure 3).[15] At 37 °C, **3** causes modest damage to cellular DNA. Under mild hyperthermia, however, the amount of cellular DNA damage is proportional to the concentration of **3**, whereas CLB shows

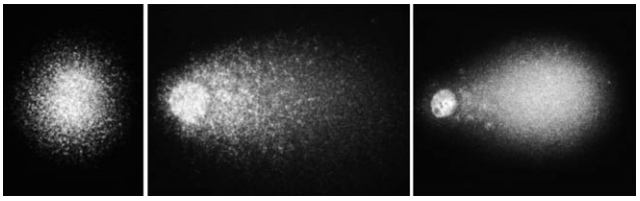

Figure 2. DAPI-stained nuclei after the comet assay at different damage stages: class 0 (left, intact nucleus, untreated cell), class 2 (center, medium-sized tail, cell treated with 50 μM of **3** at 37 °C), class 4 (right, damaged nucleus, 100 μM of **3** under hyperthermia).

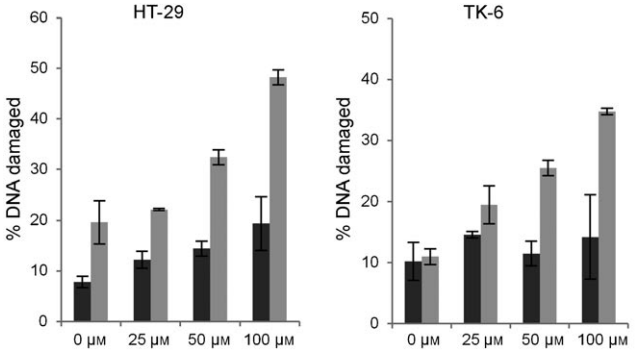

Figure 3. Percentage of damaged cellular DNA incubated with various concentrations of **3** at 37 °C for 24 h (dark gray) or at 41 °C for 2 h followed by 22 h at 37 °C (light gray).

no difference in DNA damage at 37 and 41 °C, and **1**, which has a short perfluorinated chain, gives rise to a slight thermoresponsive effect (see the Supporting Information). These data are in excellent agreement with the universal thermoactive behavior of **3**, that is, hyperthermia alone does not have any effect on TK-6 cells and is slightly toxic for HT-29 cells, and in the presence of **3** little change is observed at 37 °C, whereas under mild hyperthermia the comet assay shows a significant increase in DNA damage.

In conclusion, we have shown that an organic anticancer drug modified with a perfluorinated chain can exert thermoresponsive behavior to target tumor tissue. The concept uses hyperthermia to trigger drug cytotoxicity inside heated tumor cells. The fluorinated CLB derivative **3** has shown thermoactive behavior in the eight tested cell lines, and, under mild hyperthermia, is as cytotoxic as the parent drug, but does not show significant toxicity at 37 °C. This discovery is a first step toward the rational design of other thermoactive anticancer drugs.

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