Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 3551–3554
Bioorganic & Medicinal Chemistry Letters

Synthesis and biological activity of mustard derivatives of combretastatins

Beatrice Coggiola,[†] Francesca Pagliai,[†] Gianna Allegrone, Armando A. Genazzani and Gian Cesare Tron[*]

DiSCAFF, Università del Piemonte Orientale, Via Bovio, 6, 28100 Novara, Italy

Received 24 March 2005; revised 13 May 2005; accepted 17 May 2005
Available online 15 June 2005

Abstract—A series of chimeric compounds bearing the combretastatin and the nitrogen mustard cores were synthesized. All the compounds were cytotoxic and inhibited tubulin polymerization. When combretastatin was joined to chlorambucil via an ester linkage, the resultant compound proved to be significantly more potent than the two compounds put together. When combretastatin was joined to nitrogen mustard via an ether linkage or when a true hybrid was synthesized, loss of potency was observed. Nonetheless, these latter compounds appeared to be more efficacious and surprisingly were able to inhibit tubulin depolymerization at high concentrations.

1. Introduction

It is generally accepted that most anticancer therapies should be based on the use of more than one compound. Such a strategy could improve therapeutic benefits both because it reduces drug resistance and because it can increase efficacy while decreasing side effects. Furthermore, it has been shown that some antitumoral agents are not a substrate for multidrug resistance (MDR) proteins and therefore might be ideal for this drug cocktail approach. For instance, it has been shown that combretastatin A4 (CA-4, **1**) is able to act against MDR positive tumoral cell lines.[1] CA-4 acts on microtubules and prevents the polymerization of tubulin,[2] binding to the same site as colchicine on β-tubulin.[3] Indeed, CA-4 phosphate, its water soluble pro-drug, has recently entered clinical trials.[4] Although tubulin is a ubiquitous protein, it has been shown that **1** displays a remarkable preference for neovasculature, and therefore, besides being a cytotoxic agent, it can also be classified as an angiostatic/toxic drug. Nonetheless, although in preclinical studies, administration of CA-4 phosphate resulted in rapid necrosis of the tumor, it has also been shown that a resistant rim of cells remains after treatment. This effect is most likely due to the preponderant contribution of normal vasculature in the blood flow to the periphery of the tumoral mass.[5] Therefore, the tumoral mass is potentially capable of undergoing rapid re-growth upon discontinuation of the drug,[5,6] which suggests that a multi-pharmacy approach would be beneficial even with **1**.[2]

We hypothesized that the use of **1** in conjunction with a classical anticancer agent might overcome the multidrug resistance induced by the latter and the inability of the former to inhibit blood flow to the periphery of the tumoral mass,[5] thereby generating a more efficacious tool. As a proof of principle, we chose alkylating agents (i.e., nitrogen mustards) and decided to generate novel chimeras. It has been shown that isosteric substitution of a hydroxyl group in **1** with an amino group results in a compound (AC 7739, **2**) that displays an increase in cytotoxicity, while maintaining an identical pharmacodynamic profile.[7] Similarly, compounds with only a dimethylamino group in the *para* position of ring B of **1** maintain their cytotoxic potential, suggesting that the presence of a primary amino group is not fundamental.[8] We have therefore synthesized a nitrogen mustard derivative on the amino group of AC7739 **4**. Alongside, to explore further the potential of the combretastatin/nitrogen mustard combination, we have also synthesized

Keywords: Alkylating agents; Antiproliferative activity; Combretastatin A-4; Chlorambucil; Tubulin polymerization.
* Corresponding author. Tel.: +39 0321 375857; fax: +39 0321 357821; e-mail: tron@pharm.unipmn.it
† These authors contributed equally to this work.

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.05.052

two chimeras that bear the basic skeleton of **1** joined with 2-[bis(2-chloroethyl)amino]-1-ethanol via an ether linkage (**5**) or with chlorambucil via an ester linkage (**5**).

2. Chemistry

Compounds[9] were prepared starting from **1** and its amino derivative **2** (Scheme 1), synthesized as previously described.[10,11]

Compound **2** was double alkylated using chloro propanol to yield the desired diol **3**. Subsequent treatment with methansulfonyl chloride gave the dimesylate derivative. The latter was converted in situ to dichloride by treatment with lithium chloride in DMF to give **4**.

Compound **5**, characterized by the presence of an ethyl spacer between the phenolic ring and the N-mustard residue, was obtained, albeit in poor yield (10%), by reacting CA-4 with tris(2-chloroethyl)amine hydrochloride in DMF using potassium *tert*-butylate as base.

Compound **6** was obtained by coupling **1** with chlorambucil using the classical EDCI/DMAP protocol. The correct stereochemical assignment of the double bond was determined by ^{1}H NMR and, when required, UV spectra (data not shown).

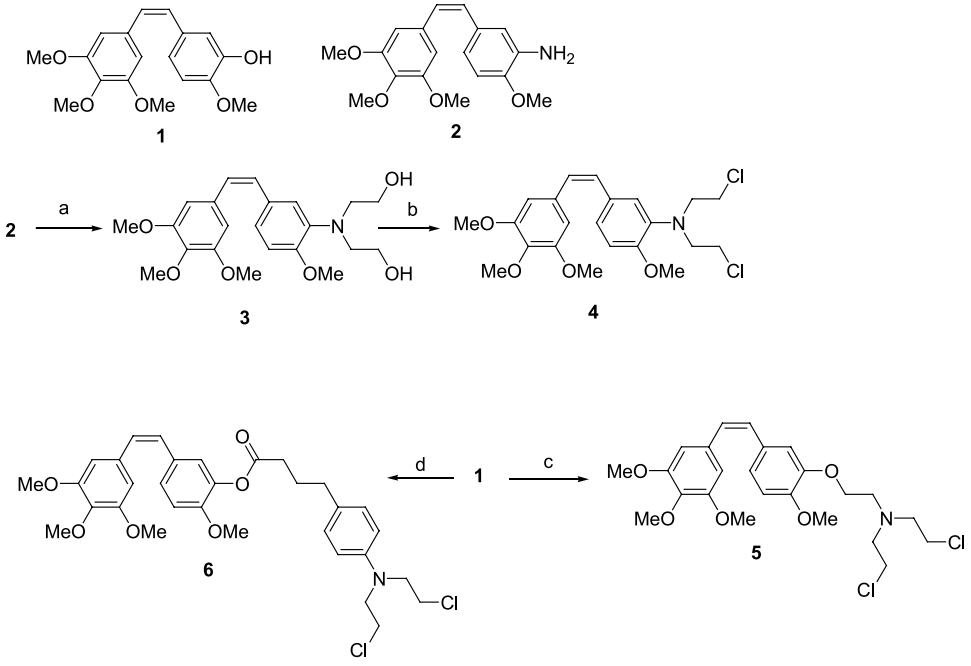
Attempts were also made to synthesize a chimeric derivative bearing only the amino group in the para position. The attempt to obtain an active molecule, though, failed since the compound displayed remarkable capacity to isomerize from cis to trans during the alogenation protocol.

3. Results and discussion

To analyze the relative cytotoxicity of the synthesized compounds, SH-SY5Y neuroblastoma cell cultures[12] were treated with increasing concentrations of compounds and grown for 48 h. **1** displayed a concentration-dependent cytotoxicity, with an IC_{50} of approx. 1.5 ± 0.28 nM (Table 1, Fig. 1). This is consistent with previous data on the same cell type and demonstrates that neuroblastoma cells are particularly susceptible to this chemotherapeutic insult.[13] When the hybrid molecule **4** was tested, there was an approximately 500-fold loss of toxicity. We then tested whether linking combretastin to the nitrogen mustards via longer chains might have restored cytotoxic activity. Indeed, **5**, where an ether chain was used to bridge the two functional groups, was approximately 3-fold more potent than **4**. Last, an ester linkage was used to join combretastatin to the nitrogen mustard (**6**). Surprisingly, **6** was significantly more potent than **1**, with an IC_{50} value of 0.64 ± 0.11 nM. To establish whether this increase in potency was due to an increased tubulin binding of **6** or was attributable to the presence of two separate functional entities, we incubated neuroblastoma cells with chlorambucil, **1**, or a combination of the two. In SH-SY5Y cells, chlorambucil was a poor cytotoxic agent up to concentrations of 10 μM (Fig. 1B). Nonetheless, when this anticancer agent was added along with com-

Table 1. Cytotoxicity of combretastatin and mustard derivatives on SH-SY5Y neuroblastoma cells

Compound	**1**	**3**	**4**	**5**	**6**
IC_{50} (nM)	1.5 ± 0.28	>10,000	860 ± 220	230 ± 72	0.64 ± 0.11



Scheme 1. Reagents and conditions: (a) $CaCO_3$, 2-chloroethanol, water, reflux; (b) methansulfonyl chloride, TEA, DMF, rt. and then LiCl, 80 °C; (c) tris(2-chloroethyl)amine, potassium *tert*-butylate, DMF, rt; (d) chlorambucil, EDCI, DMAP, CH_2Cl_2, rt.

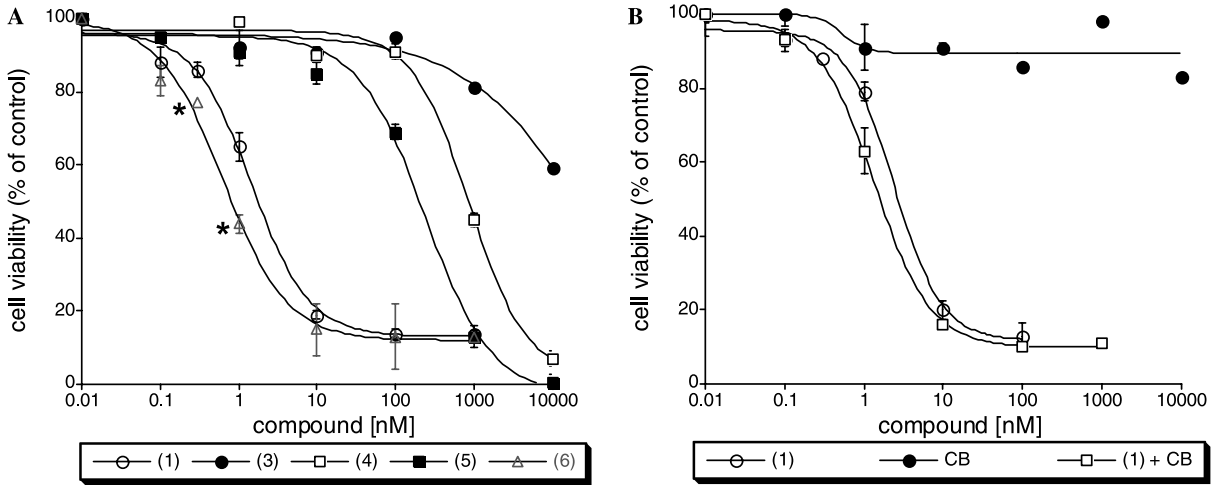


Figure 1. Concentration–response curves of combretastin, chlorambucil, and their hybrids. $n = 12–36$ from at least three separate experiments,

bretastatin, a modest, but not statistically significant, potentiation was observed. To test whether the relative rank order of potency of **1** and **6** was maintained in other cancerous cell lines, we performed concentration–response curves in mesothelioma (REN) and mastocyte (RBL) cell lines.[12] Surprisingly, in these latter cell lines, **1** displayed cytotoxicity higher than that of **6**, although **6** remained relatively potent (IC_{50} below 3 nM in both REN and RBL cells). These data hint at the idea that increased potency observed with **6** in SH-SY5Y cells is cell specific and is attributable to the chlorambucil moiety. Although our data are not conclusive, the difference between the cell lines might reside either in their relative esterase activity or in the ability of **6** to display, alongside tubulin binding, a different mechanism of action.

To establish the mechanism of action of these compounds, we performed a cytofluorimetric analysis using propidium iodide to label DNA. Agents that act on tubulin, such as **1**, are known to induce a selective block in G2/M, most likely due to the disruption of mitotic spindle architecture. Indeed, **1**, **4**, **5**, and **6** all induced a selective increase of the G2/M phase, suggesting that their cytotoxic nature is attributable to tubulin binding (data not shown). Such a cytofluorimetric signature was not observable with chlorambucil, which appears to induce a modest cell cycle arrest in the G1 phase (data not shown).

To establish conclusively whether all these agents act on tubulin, an intact-cell polymerization assay was employed. To measure the degree of tubulin polymerization, we adopted the method described by Minotti et al.[14] as modified by Tron et al.[13] In brief, cells were grown for 24 h in the presence of drugs and were then lysed in the presence of paclitaxel, an agent that prevents further depolymerization. Subsequently, polymerized and unpolymerized tubulin were separated by centrifugation. It has been previously reported that shorter incubation times, but higher concentrations of drugs, are required to observe changes in tubulin polymerization forms compared to the concentrations required in cell viability experiments[15] and we therefore chose concentrations 50-fold higher than the respective IC_{50}s. Indeed, at these concentrations, compounds **1**, **4**, **5**, and **6**, but not chlorambucil, induced a significant shift of tubulin from the polymerized to the unpolymerized form (Fig. 2). Such experiments would suggest that all the synthesized compounds retain the original mechanism of action of combretastatin. Yet, when cells were treated for the same length of time with 5-fold higher concentrations, compound **4** and compound **5** induced tubulin polymerization. In other words, the addition of chlorambucil moiety induced these compounds to change from polymerization inhibitors (colchicine-like drugs) to depolymerization inhibitors (taxol-like drugs). Although we have not investigated further the mechanism by which this shift in biological activity takes place, we propose that compounds **4** and **5** are attracted to

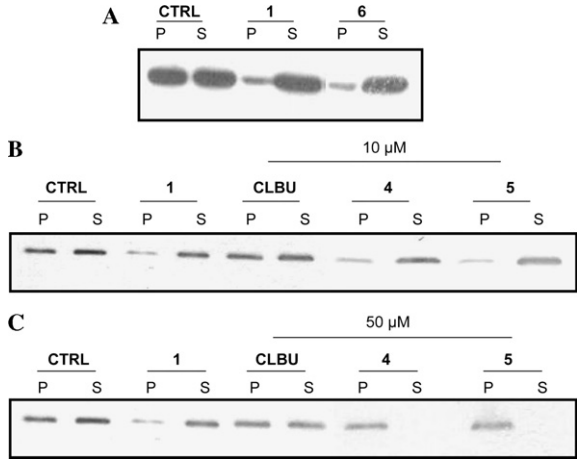


Figure 2. Inhibition of tubulin polymerization or depolymerization by combretastatin, chlorambucil, and their hybrids. P represents the pelletable fraction (polymerized tubulin) and S represents the soluble fraction (unpolymerized tubulin). Western blots are representative of at least three experiments that gave similar results.

tubulin via their active combretastatin group. If high enough concentrations are placed in the vicinity of a microtubule, then the nitrogen mustard will alkylate tubulin or an accessory protein to prevent depolymerization. Indeed, it is surprising that although compounds **4** and **5** are significantly less potent than **1** and **6**, their efficacy appears to be even greater. In cells treated with combretastatin, there appears to be a small, but significant, residual cell viability (approx. 10%), while no cells appear to resist at high concentrations of **4** or **5** (Fig. 1). It is exciting to speculate that capacity of these compounds to act either by increasing or decreasing depolymerization in a concentration-dependent manner may be responsible for this increased efficacy. Indeed, it has been previously suggested that antitumor therapy with a combination of polymerizing and depolymerizing agents provides an added value.[2]

References and notes

1. McGown, A. T.; Fox, B. W. *Cancer Chemother. Pharmacol.* **1990**, *26*, 79.
2. Tozer, G. M.; Kanthou, C.; Parkins, S. A.; Hill, S. A. *Int. J. Exp. Pathol.* **2002**, *83*, 21.
3. Lin, C.; Singh, S. B.; Chu, P. S.; Dempcy, R. O.; Schmidt, J. M.; Pettit, G. R.; Hamel, E. *Mol. Pharmacol.* **1988**, *34*, 200.
4. Dowlati, A.; Robertson, K.; Cooney, M.; Petros, W. P.; Stratford, M.; Jesberger, J.; Rafie, N.; Overmoyer, B.; Makkar, V.; Stambler, B.; Taylor, A.; Waas, J.; Lewin, J. S.; McCrae, K. R.; Remick, S. C. *Cancer Res.* **2002**, *62*, 3408.
5. (a) Pedley, R. B.; Hill, S. A.; Boxer, G. M.; Flynn, A. A.; Boden, R.; Watson, R.; Dearling, J.; Chaplin, D. J.; Begent, R. H. J. *Cancer Res.* **2001**, *61*, 4716; (b) Chaplin, D. J.; Hill, S. A. *Int. J. Radiat. Oncol. Biol. Phys.* **2002**, *54*, 1491.
6. West, C. M. L.; Price, P. *Anti Cancer Drugs* **2004**, *15*, 179.
7. Ohsumi, K.; Nakagawa, R.; Fukuda, Y.; Hatanaka, T.; Morinaga, Y.; Nihei, Y.; Ohishi, K.; Suga, Y.; Akiyama, Y.; Tsuji, Y. *J. Med. Chem.* **1998**, *41*, 3022.
8. Cushman, M.; Nagarathnam, D.; Gopal, D.; Chakraborti, A. K.; Lin, C. M.; Hamel, E. *J. Med. Chem.* **1991**, *34*, 2579.
9. Compound **3**: yellow oil (35%). MS (ESI) *m*/*z*: 404 $(M+H)^+$; ^1H NMR ($CDCl_3$) δ: 7.10 (m, 2H, arom.), 6.82 (d, 1H, arom., $J = 8.8$ Hz), 6.51 (d, 1H, olefinic, $J = 11.5$ Hz), 6.45 (d, 1H, olefinic, $J = 11.5$ Hz), 6.44 (s, 2H, arom.), 3.86 (s, 3H, OMe), 3.82 (s, 3H, OMe), 3.70 (s, 6H, OMe), 3.45 (br t, 2H, $-NCH_2CH_2OH$), 3.08 (br s, 2H, $-NCH_2CH_2OH$). Compound **4**: yellow oil (49%). MS (ESI) *m*/*z*: 440 $(M+H)^+$; ^1H NMR ($CDCl_3$) δ: 6.98 (dd, 1H, arom., $J = 8.5/1.9$ Hz), 6.86 (br s, 1H, arom.), 6.76 (d, 1H, arom., $J = 8.5$ Hz), 6.48 (s, 2H, arom.), 6.44 (s, 2H, olefinic), 3.83 (s, 3H, OMe), 3.82 (s, 3H, OMe), 3.70 (s, 6H, OMe), 3.42 (br s, 8H, $-NCH_2CH_2Cl$); Compound **5**: deep yellow oil (10%). MS (ESI) *m*/*z*: 484 $(M+H)^+$; ^1H NMR ($CDCl_3$) δ: 6.86 (dd, 1H, arom., $J = 8.2/1.9$ Hz), 6.80 (d, 1H, arom., $J = 1.9$ Hz), 6.76 (d, 1H, arom., $J = 8.2$ Hz), 6.51 (s, 2H, arom.), 6.47 (d, 1H, olefinic, $J = 12.1$ Hz), 6.41 (d, 1H, olefinic, $J = 12.1$ Hz), 3.85 (t, $-OCH_2CH_2N$, $J = 5.8$ Hz), 3.83 (br s, 6H, OMe), 3.69 (s, 6H, OMe), 3.51 (t, $-NCH_2CH_2Cl$, $J = 7.1$ Hz), 2.99–2.94 (m, $-OCH_2CH_2N + NCH_2CH_2Cl$); Compound **6**: white solid (89%). mp 118–119.5 °C; MS (ESI) *m*/*z*: 624 $(M+Na)^+$; ^1H NMR ($CDCl_3$) δ: 7.10 (dd, 1H, arom., $J = 8.0/1.9$ Hz), 7.08 (d, 1H, $J = 8.0$ Hz), 6.99 (d, 1H, arom., $J = 1.9$ Hz), 6.84 (d, 2H, arom., $J = 8.2$ Hz), 6.62 (d, 2H, arom., $J = 8.5$ Hz), 6.50 (s, 2H, arom.), 6.44 (s, 2H, olefinic), 3.82 (s, 3H, OMe), 3.80 (s, 3H, OMe), 3.70 (s, 6H, OMe), 3.69–3.60 (m, $-NCH_2CH_2Cl$), 2.62 (t, $COCH_2CH_2CH_2Ph$, $J = 7.4$ Hz), 2.53 (t, $COCH_2CH_2CH_2Ph$, $J = 7.4$ Hz), (q, $COCH_2CH_2CH_2Ph$, $J = 7.7$ Hz). Purity of target compounds was checked by HPLC analysis using a Phenomenex Luna (250×4.6 mm, 5 μm particle size) column on a Shimadzu HPLC system. Solvents for the separation were: solvent A: water; solvent B: acetonitrile at a flow rate of 1 ml/min and a sample injection volume of 210 μl. For compound **6**, eluants A and B were delivered isocratically at a 5:95 ratio. For compounds **4** and **5**, a linear gradient was used from 70:30 to 30:70 in 30 min. All three compounds displayed a purity of at least 95%.
10. Gaukroger, K.; Hadfield, J. A.; Hepworth, L. A.; Lawrence, N. J.; McGown, A. T. *J. Org. Chem.* **2001**, *66*, 8135.
11. Pinney, K. G.; Mejia, M. P.; Villalobos, V. M.; Rosenquist, B. E.; Pettit, G. R.; Verdier-Pinard, P.; Hamel, E. *Biorg. Med. Chem.* **2000**, *8*, 2417.
12. The SH-SY5Y human neuroblastoma cell line and the RBL cell line were obtained from ATCC (LGC Promochem Teddington, UK), The REN human mesothelioma cells[16] were a kind gift of Professor G. Gaudino
13. Tron, G. C.; Pagliai, F.; Del Grosso, E.; Genazzani, A. A.; Sorba, G. *J. Med. Chem.* **2005**, *48*, 3260.
14. Minotti, A. M.; Barlow, S. B.; Cabral, F. *J. Biol. Chem.* **1991**, *266*, 3987.
15. Prinz, H.; Ishii, Y.; Hirano, T.; Stoiber, T.; Camacho, G. J. A.; Schmidt, P.; Düssman, H.; Burger, A. M.; Prehn, J. H. M.; Günther, E. G.; Unger, E.; Umezawa, K. *J. Med. Chem.* **2003**, *46*, 3382.
16. Smythe, W. R.; Kaiser, L. R.; Hwang, H. C.; Amin, K. M.; Pilewski, J. M.; Eck, S. J.; Wilson, J. M.; Albelda, S. M. *Ann. Thorac. Surg.* **1994**, *57*, 1395.