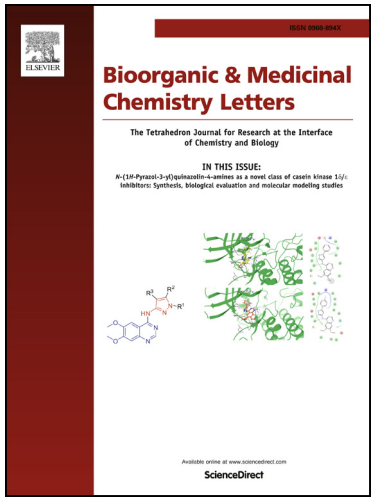
Accepted Manuscript



Rational design, synthesis and preliminary antitumor activity evaluation of a chlorambucil derivative with potent DNA/HDAC dual-targeting inhibitory activity

Rui Xie, Yan Li, Pingwah Tang, Qipeng Yuan

PII: S0960-894X(17)30801-6
DOI: http://dx.doi.org/10.1016/j.bmcl.2017.08.011
Reference: BMCL 25205

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 2 July 2017
Revised Date: 6 August 2017
Accepted Date: 7 August 2017

Please cite this article as: Xie, R., Li, Y., Tang, P., Yuan, Q., Rational design, synthesis and preliminary antitumor activity evaluation of a chlorambucil derivative with potent DNA/HDAC dual-targeting inhibitory activity, *Bioorganic & Medicinal Chemistry Letters* (2017), doi: http://dx.doi.org/10.1016/j.bmcl.2017.08.011

Rational design, synthesis and preliminary antitumor activity evaluation of a chlorambucil derivative with potent DNA/HDAC dual-targeting inhibitory activity

Rui Xie, Yan Li, Pingwah Tang and Qipeng Yuan*

Beijing Laboratory of Biomedical Materials, College of Life Science and Technology, Beijing University of Chemical Technology, 15 Beisanhuan East Road, Beijing 100029, China

*Corresponding author, E-mail addresses: yuanqp@mail.buct.edu.cn

ABSTRACT:

Histone deacetylases (HDACs) play a pivotal role not only in gene expression but also in DNA repair. Herein, we report the successful design, synthesis and evaluation of a chlorambucil derivative named vorambucil with a hydroxamic acid tail as a DNA/HDAC dual-targeting inhibitor. Vorambucil obtained both potent DNA and HDACs inhibitory activities. Molecular docking results supported the initial pharmacophoric hypothesis and rationalized the potent inhibitory activity of vorambucil against HDAC1, HDAC2 and HDAC6. Vorambucil showed potent antiproliferative activity against all the test four cancer cell lines with IC_{50} values of as low as 3.2~6.2 μM and exhibited 5.0~18.3 fold enhanced antiproliferative activity than chlorambucil. Vorambucil also significantly inhibits colony formation of A375 cancer cells. Further investigation showed that vorambucil remarkably induced apoptosis and arrested the cell cycle of A375 cells at G2/M phase. Vorambucil could be a promising candidate and a useful tool to elucidate the role of those DNA/HDAC dual-targeting inhibitors for cancer therapy.

Keywords: Chlorambucil; Nitrogen mustard; Hydroxamic acid; Anticancer

Genotoxic anticancer drugs play a key role in cancer treatment for many years. Among them, the nitrogen mustards represent a major class of genotoxic anticancer drugs for the treatment of various cancers. Chlorambucil as one of the best tolerated nitrogen mustards is in worldwide clinical use (Fig. 1A). The mechanism of action of chlorambucil is based on attacking cellular DNA and causing DNA damage.[1-3] However, the DNA damage caused by genotoxic drugs can be alleviated by cellular DNA repair machinery, so that some cancer cells can survive and eventually lead to treatment failure.[4-6] As a result, nitrogen mustards exhibited poor potency and limited treatment success. Inhibition of DNA repair machinery may be an effective strategy to enhance the efficacy of nitrogen mustards.

Histone deacetylases (HDACs) are a family of epigenetic enzymes with critical roles in chromatin condensation and gene expression by catalyzing the removal of acetyl groups from histones.[7-11] It has been shown that acetylation of the core histones weakens the histone-DNA interactions, thereby increasing DNA accessibility.[12] Recent evidence demonstrates that the structural alterations in chromatin induced by HDAC inhibitors could expose DNA to DNA-damaging agents such as ultraviolet rays, x-ray and genotoxic drugs, eventually leading to double strand breaks in DNA.[13-15] Inhibition of HDAC could down-regulate the DNA repair machinery.[16] The critical role

of HDACs in DNA repair has provide new opportunities for improving traditional genotoxic drugs.[17] Therefore, we devised a strategy to design a novel chlorambucil derivative with the ability to not only bind DNA in much the same way as classical nitrogen mustards drugs but also inhibit HDACs.

In general, the pharmacophore model for HDAC inhibitors is composed of three regions: a zinc binding group (ZBG), a linker and a cap group. The zinc binding group chelates the zinc ion in the active pocket of HDAC. The cap group interacts with the entrance surface of HDAC active pocket. The linker accommodates the tubular access of the active pocket.[18-20] The common ZBG of HDAC inhibitors is hydroxamic acid. There are many HDAC inhibitors with hydroxamic acid as ZBG, such as SAHA and LBH589 (Fig. 1B), which represents the most studied and potent HDAC inhibitor with a hydroxamic acid ZBG. Liu et al. synthesized a bendamustine derivative CY190602 as DNA/HDAC dual-targeting drug.[21] However, CY190602 contains a nitrogen heterocycle (1-methyl-1H-benzimidazole) which is difficult to synthesize. Moreover the nitrogen heterocyclic structure may not be a necessary group to obtain potent antitumor drug. Herein, we report our effort in developing and characterizing a chlorambucil derivative vorambucil (Fig. 1C) with a hydroxamate moiety displaying potent HDAC inhibitory activity and antitumor activity. Vorambucil possesses simple structure, potent antitumor activity and special HDAC isoform inhibitory activity, which may be a promising candidate for cancer therapy.

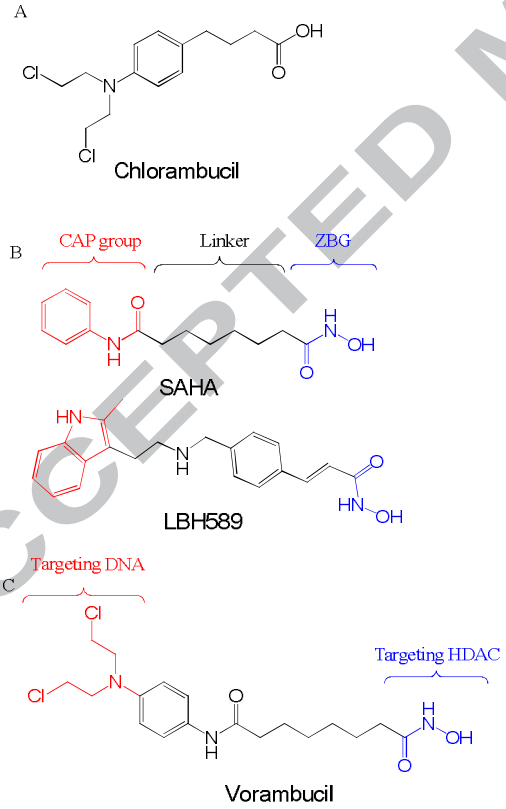
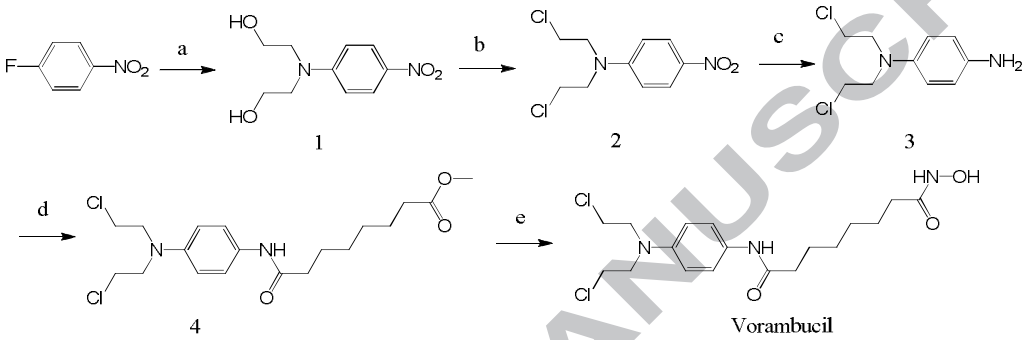


Fig. 1. （A）Structure of chlorambucil;（B）Structure of SAHA and LBH589 ; (C) Design of vorambucil as DNA/HDAC dual-targeting inhibitor.

The preparation of vorambucil was depicted in Scheme 1. Firstly, commercially available 1-fluoro-4-nitrobenzene was treated with 2,2'-iminodiethanol to provide 2,2'-[(4-nitrophenyl)imino]diethanol (intermediate 1). Intermediate 1 was treated with $SOCl_2$ to obtain *N*,*N*-bis(2-chloroethyl)-4-nitroaniline (Intermediate 2). Intermediate 2 was reduced with zinc and hydrochloric acid to obtain *N*,*N*-bis(2-chloroethyl)benzene-1,4-diamine (Intermediate 3). Then, intermediate 3 coupled with 8-methoxy-8-oxooctanoic acid to obtain intermediate 4. Finally, conversion of methyl ester (intermediate 4) to hydroxamic acid using freshly prepared hydroxylamine furnished vorambucil.



Scheme 1. Synthesis of vorambucil. Reagents and conditions: (a) 2,2'-iminodiethanol, 118 ℃, reflux, 87%; (b) $SOCl_2$, THF, 0 ℃ to r.t., 91%; (c) Zn, HCl, CH_3OH, 78%; (d) 8-methoxy-8-oxooctanoic acid, ethanedioyl dichloride, DMF, triethylamine, THF, 0℃to r.t., 64%; (e) KOH, $NH_2OH.HCl$/MeOH, r.t.,57%.

The HDACs family are classified in four classes according to their homology to yeast proteins and catalytic mechanism: class Ⅰ (HDAC 1, 2, 3, and 8), class Ⅱ (HDAC 4, 5, 6, 7, 9 and 10), class Ⅳ (HDAC 11) and class Ⅲ (sirtuin 1-7).[22, 23] It has been shown that the dysregulation of class Ⅰ and class Ⅱ HDACs, especially class Ⅰ isozymes, has been associated with the process of tumor cells proliferation and DNA repair.[24-27] We firstly tested the total HDACs inhibitory activity of vorambucil using a HDACs Assay kit (BML-AK530, Enzo® Life Sciences) to investigate whether vorambucil obtained HDACs inhibitory activity or not. As expected pharmacophoric hypothesis, vorambucil achieve potent HDACs inhibitory activity (Fig. 2). Since class Ⅰ and class Ⅱ HDACs were most closely related to tumor cells proliferation and DNA repair, vorambucil was then assessed for inhibitory activity against HDAC isoforms 1 and 2 (class Ⅰ), and 6 (class Ⅱ). As shown in Table 1, vorambucil displayed potent HDAC1 and HDAC2 inhibitory activity, which was comparable to SAHA. Interestingly, vorambucil showed approximately 20-fold weaker inhibitory activity against HDAC6 than SAHA. The results of HDAC isoforms inhibitory activity demonstrated that vorambucil showed moderately more potent inhibitory activity against HDAC1 and HDAC2 over HDAC6.

In order to understand the potent HDACs inhibitory activity of vorambucil, we carried out docking study of vorambucil to the active pockets of HDAC1 (PDB code: 4BKX), HDAC2 (PDB code: 4LXZ) and HDAC6 (PDB code: 5EDU) using Surflex-dock. As

shown in Fig. 3, Vorambucil could form five hydrogen bonds with HIS145, HIS146, ASP104 and TYR308 in the active site of HDAC2. The binding affinity of vorambucil for HDAC2 was -8.2 kcal/mol. Vorambucil could also form five hydrogen bonds with ARG34, TRP135, ALA136 and GLY137 in the active site of HDAC1. The binding affinity of vorambucil for HDAC1 was -5.5 kcal/mol. Vorambucil could only form three hydrogen bonds with HIS610, GLY619 and SER568 in the active site of HDAC6 and the binding affinity was only -4.5 kcal/mol. The docking results could well explain the potent HDACs inhibitory activity and moderate selectivity of vorambucil against HDAC1 and HDAC2 over HDAC6.

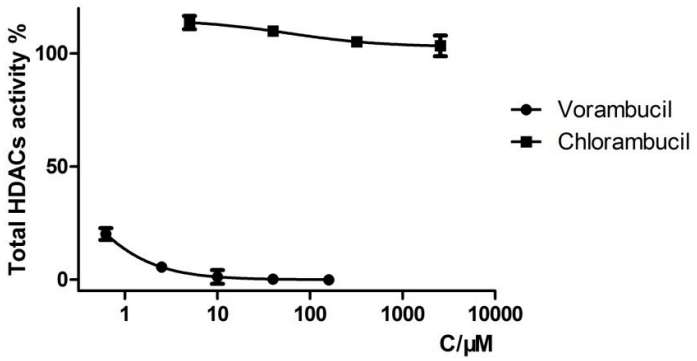


Fig. 2. Total HDACs inhibitory activity of vorambucil and chlorambucil

Table 1 Inhibition of individual HDAC isoforms, IC_{50} (μM).

Compd	IC_{50} (μM) [a]			Isoform selectivity	
	class Ⅰ		class Ⅱ		
	HDAC1	HDAC2	HDAC6	HDAC6/HDAC1	HDAC6/HDAC2
vorambucil	0.27±0.04	0.24±0.05	0.62±0.11	2.3	2.6
SAHA	0.19±0.02	0.18±0.03	0.03±0.004	0.16	0.17

[a] Values are the mean of at least two separate determinations.

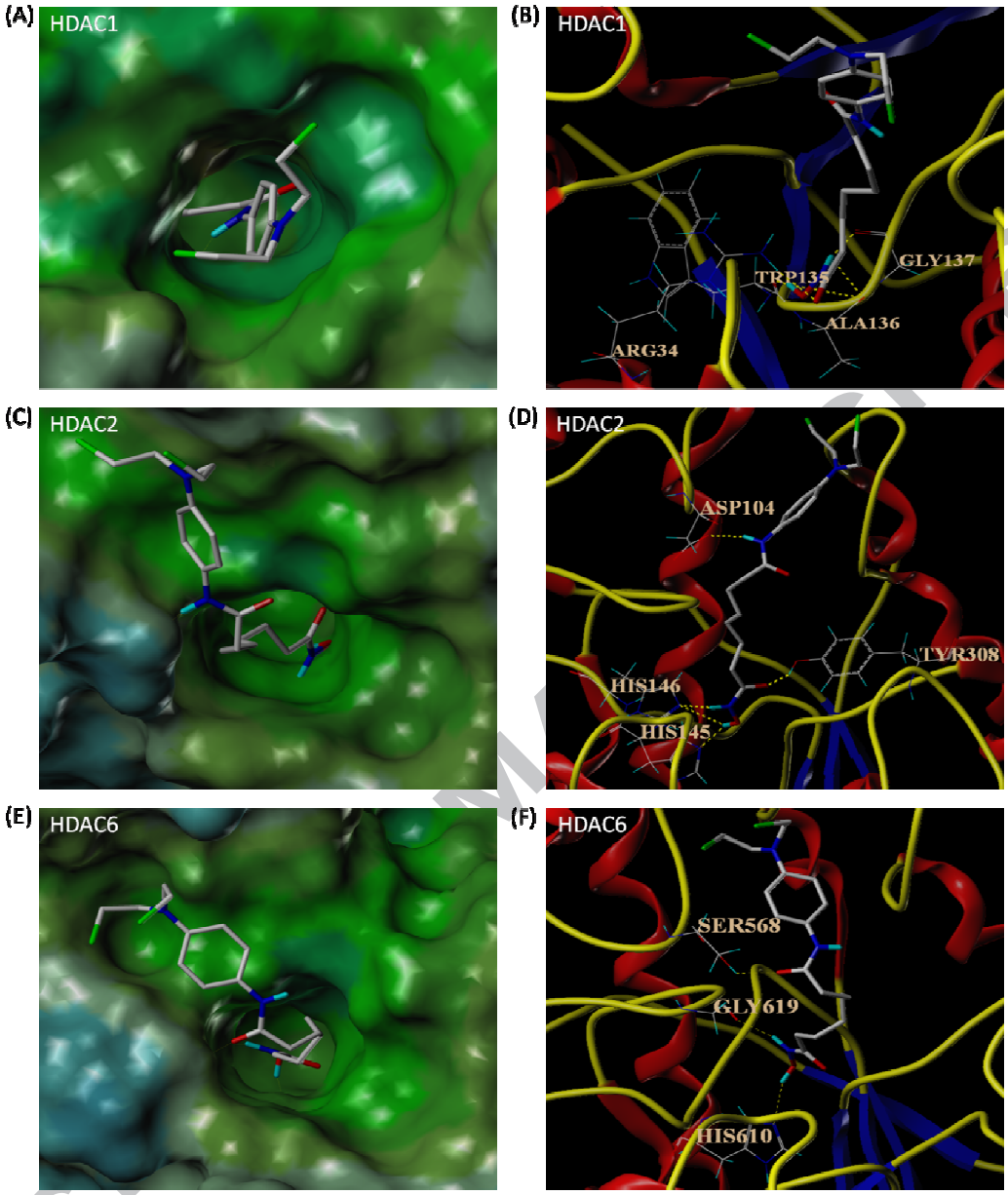


Fig. 3. Predicted binding modes of vorambucil on HDAC1 (PDB code: 4BKX) , HDAC2 (PDB code: 4LXZ) and HDAC6 (PDB code: 5EDU). Interactions between the protein and the ligand are shown as yellow dotted lines. (A) Molecular surface of the HDAC1 binding pocket (B) vorambucil interacted with the actives of HDAC1. (C) Molecular surface of the HDAC2 binding pocket (D) vorambucil interacted with the actives of HDAC2. (E) Molecular surface of the HDAC6 binding pocket (F) vorambucil interacted with the actives of HDAC6.

To examine whether vorambucil triggers DNA damage in cancer cells, we performed DNA damage assay of vorambucil against A375 cancer cells by a DNA damage assay kit (Epigentek, NY, USA). The kit is able to determine the phosphorylation of histone H2AX (γ H2AX), which has been regarded as a marker for DNA-double strand breaks. As shown in Fig. 4, after 48 h exposure, vorambucil

significantly induced more DNA damage (224.1%±2.4% at 16 μM) compared with chlorambucil (128.6%±24.3% at 16 μM). Taken together, those results indicated that vorambucil obtained both enhanced DNA-damaging activity and potent HDAC inhibitory activity.

Does the enhanced DNA-damaging activity and potent HDAC inhibitory activity of vorambucil bring enhanced anticancer activities or not? The antiproliferative activities of vorambucil against four cancer cell lines were evaluated by CCK-8-based assay. As shown in Table 2, vorambucil exhibited significantly enhanced anticancer potency with IC_{50} values (concentration required to reduce viability to 50%) of as low as 3.2~6.2 μM against four human cancer cell lines, more than 5.0~18.3 fold lower than chlorambucil. The dose-dependent antiproliferative activities of vorambucil on cancer cell lines were displayed in Fig. 5, which revealed that the antiproliferative activities of vorambucil showed obvious dose-dependent manner.

To further explore the antitumor ability of vorambucil, we performed colony formation assay of vorambucil against A375 cells with chlorambucil as positive control. Results were summarized in Fig. 6. Vorambucil inhibited the colony formation of A375 cancer cells in a dose dependent manner, and remarkably more potent than chlorambucil. To further investigated whether the potent antiproliferative activities of vorambucil was related to enhanced apoptosis of cancer cells, we carried out flow cytometry assay on A375 cancer cell line and the percentages of apoptotic cells were determined. As shown in Fig. 7, vorambucil significantly induced apoptosis of A375 cancer cells in a concentration-dependent manner. vorambucil induced 15.64%, 49.24% and 69.58% apoptosis of A375 cancer cells at 2 μM, 8 μM and 16 μM respectively. However, chlorambucil only induced 9.93%, 12.85% and 28.86% apoptotic cells at the same concentration. Since genotoxic anticancer drugs block the DNA synthesis, the effect of vorambucil on A375 cell cycle progression was determined. As showed in Fig. 8, vorambucil remarkably induced the accumulation of A375 cells at the G2/M phase (66.5% at 8 μM, Fig. 8), which were obviously more potent than chlorambucil (47.6% at 8 μM, Fig. 8).

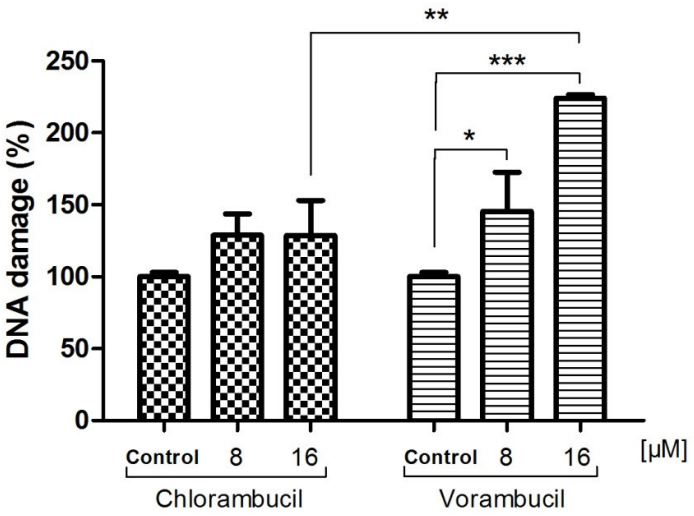


Fig. 4. Effects of chlorambucil and vorambucil on DNA damage in A375 cells. A375 cells were treated with vorambucil or chlorambucil at 8 μM and 16 μM for 48 hours. The DNA damage was detected using the EpiQuik In Situ DNA Damage Assay kit (Epigentek, NY, USA) according to the manufacturer's instructions. Data were expressed as the percent of the control (no drug treatment), as mean±SD (n=3). *P*-values were determined using a *t*-test (* indicates $p<0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$).

Table 2 Antiproliferative activities data (IC_{50}, μM) of vorambucil and chlorambucil against four cancer cell lines

Compd	HepG2	A549	HCT116	A375
Chlorambucil	111.8±17.0	22.2±3.7	57.6±11.0	69.2±9.9
Vorambucil	6.1±1.5	4.4±0.6	3.2±0.4	6.2±0.6

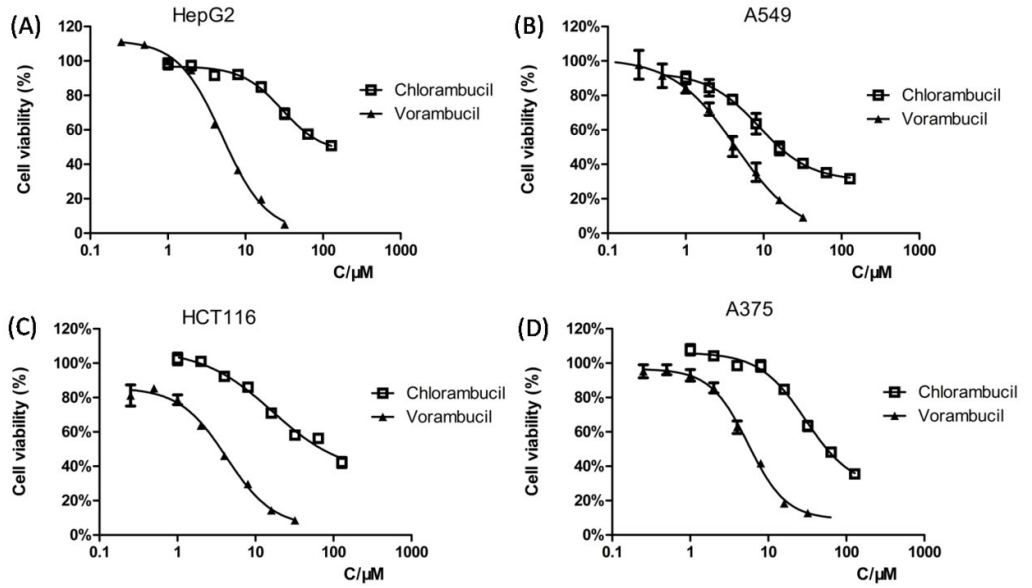


Fig. 5. Dose-dependent antiproliferative activities of vorambucil against four cancer cell lines. The values represent the means of three experiments. (A) treatment in HepG2 cells. (B) treatment in A549 cells. (C) treatment in HCT116 cells. (D) treatment in A375 cells.

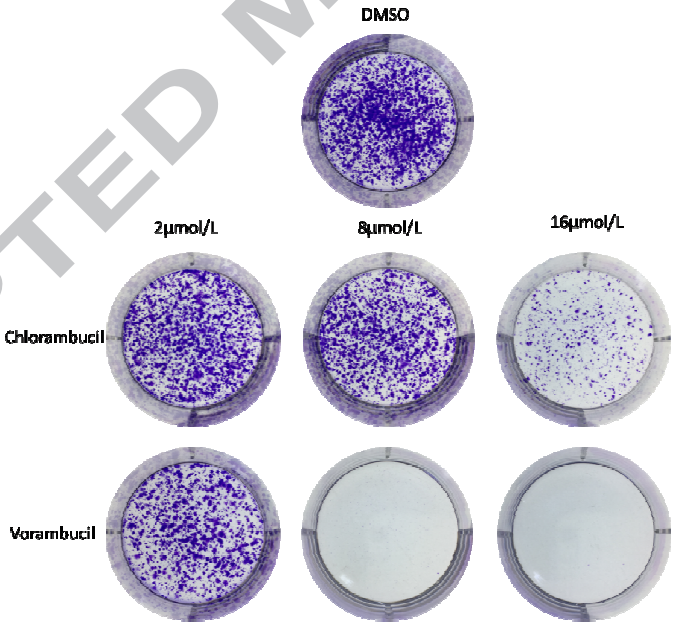


Fig. 6. Vorambucil and chlorambucil inhibit A375 cancer cell colony formation.

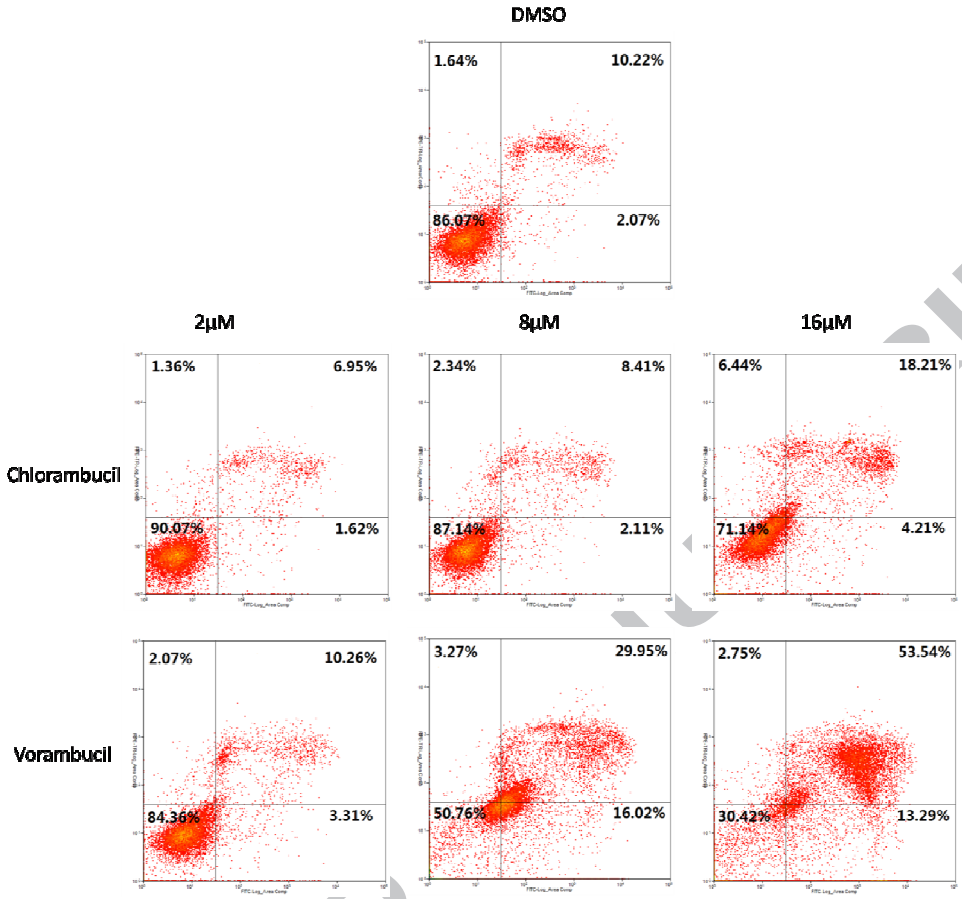


Fig. 7. Induction of apoptosis by vorambucil and chlorambucil in different concentrations in A375 cancer cells. The values represent the means of three separate experiments with SD less than 10%.

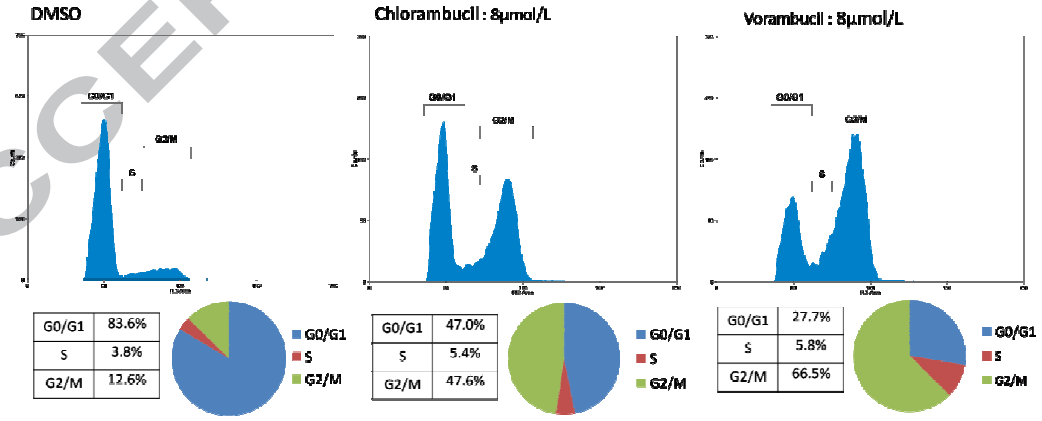


Fig. 8. Effect of vorambucil and chlorambucil on A375 cell cycle progression. The values represent the means of three separate experiments with SD less than 10%.

In conclusion, using structure-based design approach, we have successfully generated a novel chlorambucil derivative named vorambucil with a hydroxamate acid tail as DNA/HDAC dual inhibitor. As expected pharmacophoric hypothesis, vorambucil displayed potent inhibitory activity against both DNA and HDACs. Notably, vorambucil showed slightly selective inhibition against HDAC1 and HDAC2 over HDAC6. Molecular docking results could well support the potent HDACs inhibitory activity and moderate selectivity of vorambucil. Vorambucil caused increased DNA damage than chlorambucil. In vitro cell growth inhibition assays indicated that vorambucil exhibited significantly enhanced anticancer potency than chlorambucil against all the tested cancer cell line with IC_{50} of 3.2-6.2 μM. Moreover, vorambucil was able to inhibit colony formation of A375 cells. Furthermore, vorambucil was found to potently induce A375 cells apoptosis and arrest cell cycle at G2/M phase. Together, vorambucil possessed potent antitumor activity and would be a useful tool to elucidate the role of those DNA/HDAC dual-targeting inhibitors in cancer therapy. More detailed antitumor mechanisms and in vivo activity evaluation of vorambucil are underway in our lab.

Acknowledgements

This work was supported by the National Science Foundation of China (Grant No. 21176018).

Supplementary data

Supplementary data associated with this article can be found in the online version

References and notes

1. Poklar, N.; Pilch, D. S.; Lippard, S. J.; Redding, E. A.; Dunham, S. U.; Breslauer, K. J. *Proc. Natl. Acad. Sci. U. S. A.* **1996,** *93*, 7606.
2. Begleiter, A.; Mowat, M.; Israels, L. G.; Johnston, J. B. *Leuk. Lymphoma* **1996,** *23*, 187.
3. Di Antonio, M.; McLuckie, K. I.; Balasubramanian, S. *J. Am. Chem. Soc.* **2014,** *136*, 5860.
4. Jeggo, P. A.; Pearl, L. H.; Carr, A. M. *Nat. Rev. Cancer* **2016,** *16*, 35.
5. Bouwman, P.; Jonkers, J. *Nat. Rev. Cancer* **2012,** *12*, 587.
6. Lord, C. J.; Ashworth, A. *Nature* **2012,** *481*, 287.
7. Dawson, M. A.; Kouzarides, T. *Cell* **2012,** *150*, 12.
8. Haberland, M.; Montgomery, R. L.; Olson, E. N. *Nat. Rev. Genet.* **2009,** *10*, 32.
9. Biel, M.; Wascholowski, V.; Giannis, A. *Angew. Chem. Int. Ed. Engl.* **2005,** *44*, 3186.
10. Lane, A. A.; Chabner, B. A. *J. Clin. Oncol.* **2009,** *27*, 5459.
11. Kouzarides, T. *Cell* **2007,** *128*, 693.
12. Kim, M. S.; Blake, M.; Baek, J. H.; Kohlhagen, G.; Pommier, Y.; Carrier, F. *Cancer Res.* **2003,** *63*, 7291.
13. Venugopal, B.; Evans, T. *Curr. Med. Chem.* **2011,** *18*, 1658.
14. Stengel, K. R.; Hiebert, S. W. *Antioxid. Redox Signal.* **2015,** *23*, 51.
15. Lee, J.-H.; Choy, M.; Ngo, L.; Foster, S.; Marks, P. A. *Proc. Natl. Acad. Sci. U. S. A.* **2010,** *107*, 14639.
16. Johnstone, R. W. *Nat. Rev. Drug Discov.* **2002,** *1*, 287.
17. Eot-Houllier, G.; Fulcrand, G.; Magnaghi-Jaulin, L.; Jaulin, C. *Cancer Lett.* **2009,** *274*, 169.

18. Xie, R.; Yao, Y.; Tang, P.; Chen, G.; Liu, X.; Yun, F.; Chen, C.; Wu, X.; Yuan, Q. *Eur. J. Med. Chem.* **2017,** *134*, 1.

19. Xie, R.; Shi, J. H.; Qu, Y.; Tang, P. W.; Wu, X. Y.; Yang, M.; Yuan, Q. P. *Med. Chem.* **2015,** *11*, 636.

20. Miller, T. A.; Witter, D. J.; Belvedere, S. *J. Med. Chem.* **2003,** *46*, 5097.

21. Liu, C.; Ding, H.; Li, X.; Pallasch, C. P.; Hong, L.; Guo, D.; Chen, Y.; Wang, D.; Wang, W.; Wang, Y. *EMBO Mol. Med.* **2015,** *7*, 438.

22. Bolden, J. E.; Peart, M. J.; Johnstone, R. W. *Nat. Rev. Drug Discov.* **2006,** *5*, 769.

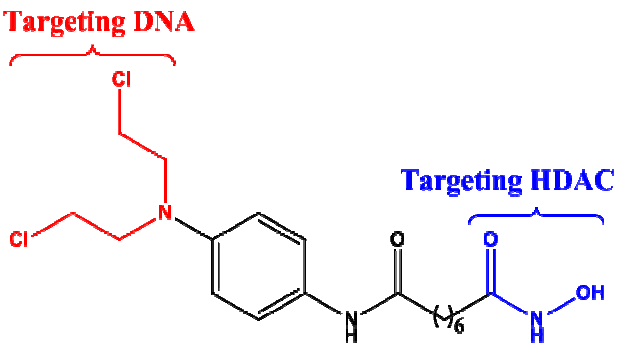
23. Yang, X. J.; Seto, E. *Nat. Rev. Mol. Cell Biol.* **2008,** *9*, 206.

24. Weichert, W.; Roske, A.; Gekeler, V.; Beckers, T.; Ebert, M. P.; Pross, M.; Dietel, M.; Denkert, C.; Rocken, C. *Lancet Oncol.* **2008,** *9*, 139.

25. Weichert, W.; Röske, A.; Niesporek, S.; Noske, A.; Buckendahl, A.-C.; Dietel, M.; Gekeler, V.; Boehm, M.; Beckers, T.; Denkert, C. *Clin. Cancer Res.* **2008,** *14*, 1669.

26. Fukuda, T.; Wu, W.; Okada, M.; Maeda, I.; Kojima, Y.; Hayami, R.; Miyoshi, Y.; Tsugawa, K. i.; Ohta, T. *Cancer Sci.* **2015,** *106*, 1050.

27. Bhaskara, S.; Chyla, B. J.; Amann, J. M.; Knutson, S. K.; Cortez, D.; Sun, Z.-W.; Hiebert, S. W. *Mol. Cell* **2008,** *30*, 61.



HDAC inhibitory activity:

IC_{50} 0.27 μ M against HDAC1

IC_{50} 0.24 μ M against HDAC2

IC_{50} 0.62 μ M against HDAC6

Antiproliferative activity:

IC_{50} 6.1 μ M against HepG2 cell;

IC_{50} 4.4 μ M against A549 cell;

IC_{50} 3.2 μ M against HCT116 cell;

IC_{50} 6.2 μ M against A375 cell;

1. A chlorambucil derivative named vorambucil with a hydroxamic acid tail was synthesized.
2. Vorambucil obtained both potent DNA damage and HDACs inhibitory activities.
3. Vorambucil displayed potent antiproliferative activity with IC_{50} values of as low as 3.2~6.2 μM against 4 kinds of cancer cells.
4. Vorambucil was able to induce apoptosis and arrest cell cycle at G2/M phase of A375 cancer cells.
5. Vorambucil would be an important lead compound to guide the design of novel DNA/HDAC dual-targeting inhibitors.