Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 5194–5198

Kinase-mediated trapping of bi-functional conjugates of paclitaxel or vinblastine with thymidine in cancer cells

Simon E. Aspland,[a,*] Carlo Ballatore,[a] Rosario Castillo,[a] Joel Desharnais,[a] Trisha Eustaquio,[a] Philip Goelet,[a] Zijian Guo,[a] Qing Li,[a] David Nelson,[a] Chengzao Sun,[a] Angelo J. Castellino[a] and Michael J. Newman[b]

[a]*Acidophil LLC, 2330 W. Joppa Road, Suite 330, Lutherville, MD 21093, USA*
[b]*Dihedron Corporation (Absorbed by Acidophil LLC during 2005), USA*

Received 30 May 2006; revised 3 July 2006; accepted 5 July 2006
Available online 25 July 2006

Abstract—In the present work, we explore the possibility of introducing selectivity to existing chemotherapeutics via the design of non-pro-drug, bi-functional molecules comprising a microtubule-binding agent and a substrate for a disease-associated kinase. The design, synthesis, and in vitro biological evaluation of paclitaxel–thymidine and vinblastine–thymidine bi-functional conjugates are reported here. This work provides the first account of 'kinase-mediated trapping' of cancer therapeutics.

One of the most important problems associated with cancer chemotherapy is the poor selectivity exhibited by the majority of the commonly employed cytotoxic agents, which results in a narrow therapeutic index. Several attempts have been made to improve selectivity of such agents, such as by incorporating a targeting moiety (e.g., Mylotarg),[1] or using pro-drug approaches designed to exploit differences between tumor and normal cells.[2] Over the past several years, advances in elucidating the complex chain of events that take part during the onset and progression of cancer have clearly highlighted the critical role played by protein and small molecule kinases. The level of expression and/or activation of several of these enzymes accounts for some of the most important intracellular differences between normal and malignant cells. Indeed, a number of kinases, including small molecule kinases such as hexokinase 2 (HK2),[3] sphingosine kinase 1 (SK1)[4], and thymidine kinase 1 (TK1),[5] are known to be either over-expressed or aberrantly activated in tumor cells. Interestingly, a growing body of evidence clearly suggests that substrates of HK2,[6] SK1[7], and TK1[8] selectively accumulate in cancer cells, presumably due to the negative charges being added through phosphorylation. This phenomenon, which we refer to as kinase-mediated trapping (KMT), has proven very useful for tumor imaging. For example, TK1-mediated trapping of the thymidine analog 3′-deoxy-3′-[^{18}F]fluorothymidine ([^{18}F]FLT) has been exploited in positron emission topography (PET) for tumor imaging.[9] Based on these observations, we became interested in exploring KMT as a novel mechanism for increasing the selectivity of existing chemotherapeutic agents, by conjugation to substrates of tumor-specific kinases (Fig. 1).

In order to test this approach, several issues need to be considered, namely: (i) the selection of a kinase and its substrate, (ii) the selection of a cytotoxic drug to be paired with this kinase substrate, (iii) the method for linking these components in such a manner that both retain their activities, and (iv) the selection of appropriate assays to evaluate these bi-functional molecules.

We reasoned that TK1 would provide a good trapping mechanism since TK1 has been shown to be over-expressed in ovarian[10] and colorectal[11] carcinomas as well as certain leukemias,[12] and since [^{18}F]FLT has been exploited in tumor imaging by PET. Furthermore, modification of the N3 position of thymidine was explored based on the reports of Tjarks and co-workers, which indicated that relatively large molecules could be attached to this position without drastic reduction in the rate of TK1-mediated phosphorylation.[13] In order to

Keywords: Kinase-mediated trapping; Thymidine kinase 1; Thymidine; Paclitaxel; Vinblastine; Fluorescein.
* Corresponding author. Tel.: +1 443 279 9971; fax: +1 410 494 4247; e-mail: saspland@acidophil.com

0960-894X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2006.07.003

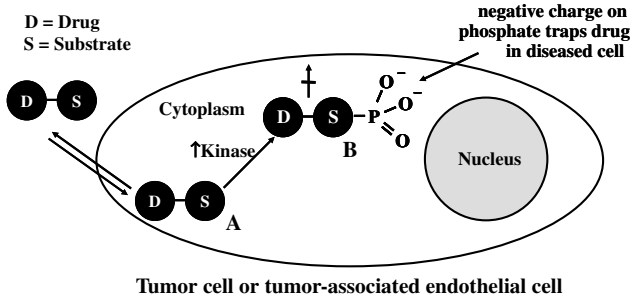


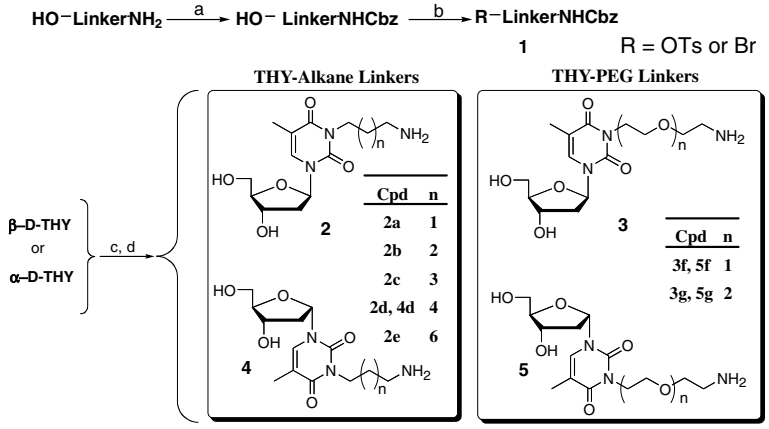
Figure 1. Kinase-mediated trapping of bi-functional molecules: phosphorylation of conjugates by an over-expressed or aberrantly activated kinase selectively traps cytotoxic drugs in tumor cells in preference to normal cells where the same kinase has normal levels of activity. (A) Unphosphorylated conjugate is able to diffuse in and out of all cells; (B) Intracellular phosphorylation of the conjugate mediated by the disease-associated kinase leads to a selective accumulation/retention of the conjugate inside cancer cells.

test TK1 as a drug trapping mechanism we investigated the cellular accumulation of thymidine (THY) conjugated fluorescein (used as a detectable drug surrogate) and the selective cytotoxicity of THY conjugated paclitaxel (PXL) and vinblastine (VBL).

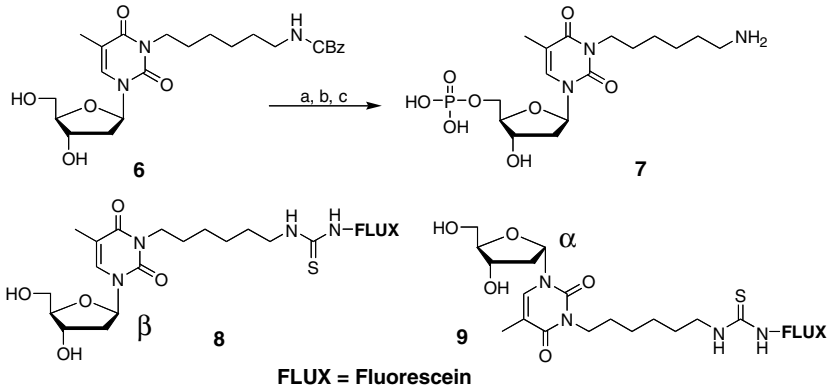
In the present study, we report the design, synthesis and in vitro biological evaluation of bi-functional molecules which comprise either fluorescein or a cytotoxic agent (PXL or VBL) conjugated to THY, the natural substrate for TK1, through hydrolytically and enzymatically stable linkers. The synthetic strategy entailed coupling of THY-linker building blocks with appropriately protected drug derivatives. THY-linkers **2**–**5** (Scheme 1) were prepared via N3-alkylation of α- or β-THY with activated linkers of general structure **1**.

A 5′-phosphorylated THY-linker **7** was also prepared (Scheme 2) for the synthesis of selected phosphorylated cytotoxic-THY conjugates (vide infra, **A9** and **C7**, Table 1). For the fluorescently labeled thymidines, β- and α-THY linkers **2d** and **4d** were reacted with fluorescein–thioisocyanate to give **8** and **9**, respectively (Scheme 2). Interestingly initial studies with these fluorescein–THY conjugates demonstrated that β-D-THY **8** but not its non-phosphorylatable α-anomer **9** leads to differential accumulation of fluorescence in cells as a function of their total TK1 activity level (Fig. 2).

For PXL and VBL conjugates, reported structure–activity relationship studies identified sites expected to be tolerant of modification. Thus, C7 and C10 of PXL[17] as well as C3 of VBL[18] were explored as points of attachment in constructing drug–thymidine bi-functional molecules. Examples of non-phosphorylatable conjugates of PXL or VBL and α-THY were also synthesized for



Scheme 1. Synthesis of thymidine-linker building blocks. Reagents: (a) Cbz-Cl, TEA, MeOH; (b) TsCl, pyridine or PPh_3, CBr_4; (c) **1**, K_2CO_3 (KI), acetone/DMF; (d) H_2, Pd/C.



Scheme 2. Reagents and conditions: (a) $POCl_3$/TEA, TMP −10 °C; (b) ice/water; (c) H_2, Pd/C.

Table 1

Compound	Thy-linker	Yield[a] (%)
A1	**2a**	63
A2	**2b**	69
A3	**2c**	95
A4	**2d**	68
A5	**2e**	40
A6	**3f**	70
A7	**3g**	20
A8	**4d**	40
A9	**7**	10
B1	**2a**	36
B2	**2c**	40
B3	**2d**	10
B4	**3f**	45
B5	**3g**	13
B6	**4d**	65
B7	**5f**	52
B8	**5g**	20
C1	**2a**	8
C2	**2b**	18
C3	**2d**	15
C4	**3f**	39
C6	**4d**	40
C7	**7**	45

[a] Overall yield after HPLC purification.

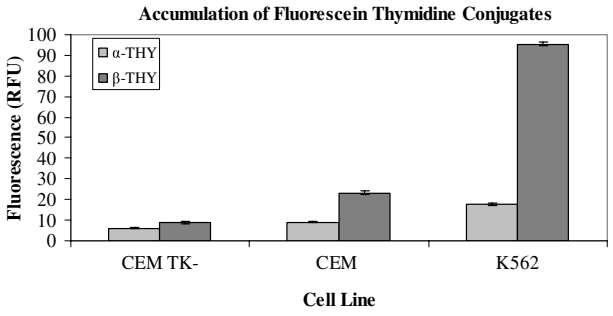


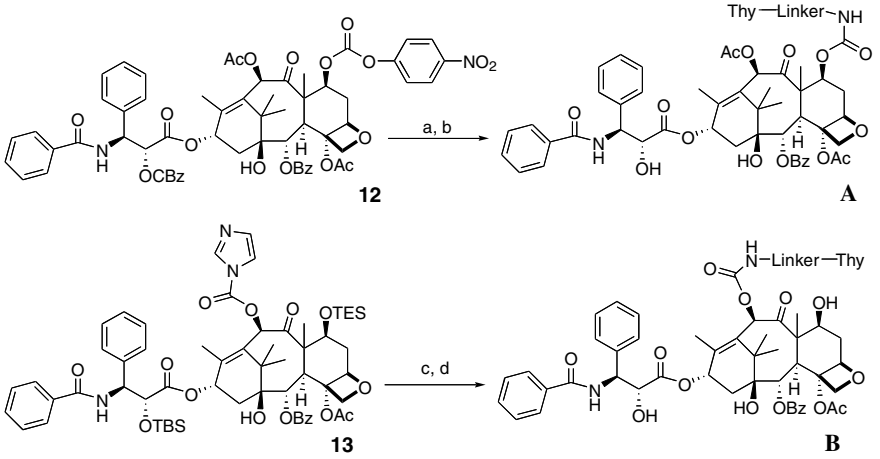
Figure 2. Selective accumulation of fluorescently labeled thymidines in TK1 expressing cells: leukemic cell lines expressing various levels of TK1 (i.e., CEM TK-, (no TK1),[14,15] CEM (medium TK1), and K562 (high TK1)) were incubated with 20 μM of the fluorescein–THY conjugates for 16 h. Cells were then washed and fluorescence was determined by flow cytometry.[16]

comparison. PXL–THY conjugates were obtained by reacting the amino group of the THY-linker inputs **2**–**5**, and **7** with either **12**[19,20] or **13**[21] to give the PXL C-7 or C-10 conjugates of general structures **A** and **B**, respectively (Scheme 3). Likewise VBL–THY conjugates were prepared by reacting the THY-linkers **2**–**4**, and **7** with 4-deacetyl-3-demethoxy-3-azidovinblastine, prepared in situ from **14**[22] (Scheme 4).

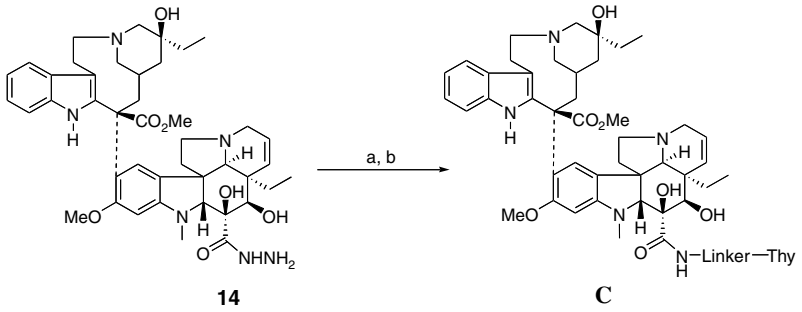
Stability of key conjugates was assessed in tissue culture medium containing 10% fetal calf serum and was found to be comparable or better than the stability of parent drugs (data not shown). All PXL and VBL conjugates were evaluated for their ability to promote or inhibit the polymerization of tubulin in a cell-free system.[23] A TK1 assay was also employed to determine the phosphorylation rates of the conjugates in comparison with THY.[24]

In addition, all but the chemically phosphorylated conjugates were evaluated in a cytotoxicity assay using three tumor cell lines: K562 leukemia, HT29 colorectal carcinoma, and MCF7 mammary carcinoma. A summary of the data obtained from the in vitro assays is shown in Table 2.

Based on the data shown in Table 2 a number of conclusions can be made: (i) With the exception of the non-phosphorylatable analogs, many conjugates were bi-functional in the biochemical assays with phosphorylation rates ranging from 13% to 68% of that of thymidine, and exhibited high levels of drug activity in biochemical assays. Remarkably, several conjugates (e.g., **B1**, **B3**, **B4**, **C2**, and **C4**) maintained microtubule (MT)-binding ability, comparable to the parent drugs (i.e., PXL or VBL). Among PXL conjugates, the C10 series exhibited greater MT-stabilizing ability than the analogous C7 conjugates. (ii) When assayed for their ability to alter the polymerization of tubulin, the chemically phosphorylated conjugates (**A9** and **C7**) exhibited comparable activity to their non-phosphorylated counterparts (**A4** and **C3**), indicating the phosphorylation step does not alter the MT-binding ability of the



Scheme 3. Reagents and conditions: (a) Thy-linker-amine, DIEA, DCM; (b) H_2, Pd/C; (c) Thy-linker-amine, IPA or *t*-BuOH, 82 °C; (d) HF/Py.



Scheme 4. Reagents: (a) $NaNO_2$, HCl, MeOH; (b) Thy-linker-amine, DMF/DCM, DIEA.

Table 2

Compound	TK1 activity[a]	MPA[b]	K562 EC_{50}[c]	HT29EC_{50}[c]	MCF7 EC_{50}[c]	clog P[d]
PXL	N/A	100	0.014	0.006	0.036	4.77
A1	35	66	2.065	3.000	2.030	2.4
A2	68	56	2.875	2.614	1.716	2.94
A3	29	78	1.588	0.875	0.787	3.46
A4	26	83	0.227	0.191	0.096	3.99
A5	13	59	0.207	0.074	0.075	5.05
A6	16	48	4.475	2.000	1.720	1.51
A7	44	35	3.213	3.640	1.600	1.23
A8	ND[e]	74	0.553	1.417	0.296	4.77
A9	N/A	73	—	—	—	—
B1	46	105	0.217	0.114	0.149	1.83
B2	49	86	0.221	0.173	0.107	2.84
B3	45	100	0.091	0.091	0.099	3.42
B4	48	102	0.763	0.145	0.143	1.51
B5	34	91	1.150	1.025	0.265	1.23
B6	ND[e]	92	0.181	0.268	0.114	3.42
B7	ND[e]	87	0.314	0.182	0.112	1.51
B8	ND[e]	89	1.531	1.150	0.390	1.23
VBL	N/A	100	0.001	0.002	0.003	5.23
C1	15	93	0.043	0.106	0.100	1.59
C2	49	107	0.030	0.049	0.151	2.12
C3	27	80	0.010	0.047	0.166	2.82
C4	23	97	0.016	0.021	0.038	1.51
C5	37	84	0.020	0.021	0.076	1.24
C6	ND[e]	93	0.014	—	—	2.82
C7	N/A	97	—	—	—	—

[a] Phosphorylation rates expressed as % of THY.
[b] Ability to promote (PXL) or inhibit (VBL) the polymerization of tubulin, expressed as % of the parent drug.
[c] Concentration of test compound (μM) required to inhibit cell proliferation by 50%, average of at least three assays.
[d] Partition coefficient between water/*n*-octanol.
[e] Not detectable (ND).

conjugates. (iii) Although most conjugates were considerably less potent cytotoxics than the parent drugs, selected compounds (**A4**, **A5**, **B3**, and **C3**) did retain low nanomolar EC_{50} values (10–100 nM). Interestingly, the cell cytotoxicity data show a much broader variability between conjugates (with EC_{50}s ranging from 10 nM to 4 μM) than predicted by the data obtained from the cell-free assays. One possible explanation for the differences between the cell-based and the cell-free results can be found in the different physical–chemical properties of the conjugates (e.g., lipophilicity) that could account for substantial changes in the conjugates' ability to diffuse through cellular membranes. Indeed, the cellular EC_{50} values seem to correlate with the calculated partition coefficients (clog P), whereby the most potent conjugates have a clog P of 3 or greater. (iv) The comparison of EC_{50} values of corresponding pairs of phosphorylatable and non-phosphorylatable analogs (**A4/A8**; **B3/B6**; **B4/B7**; **B5/B8**; and **C3/C6**) does not indicate any overt correlation between potency and TK1-mediated phosphorylation. This could be explained by an insufficient level of intracellular phosphorylation, even though the conjugates were found to be TK1 substrates in the cell-free assay. However, it is also plausible that in vitro cytotoxicity assays may not be the optimal setting for evaluating selectivity enhancement of the conjugates. Therefore, it is possible that only appropriately designed in vivo models will enable the determination of the advantage of KMT of these bi-functional molecules.

In conclusion, a set of conjugates comprising fluorescein, PXL or VBL conjugated to thymidine have been designed and synthesized. Collectively, the data presented here indicate that fluorescent conjugates of thymidine accumulate in cells in a manner that is consistent with KMT. Cytotoxic drug-containing conjugates are bifunctional in the biochemical assays and some of these display acceptable cellular potency. Additional studies will be needed to evaluate a gain in selectivity by KMT.

Acknowledgment

The authors would like to thank Dr. Sydney Brenner for the original idea behind this project.

References and notes

1. Sievers, E. L.; Linenberger, M. *Curr. Opin. Oncol.* **2001**, *13*, 522.
2. Jaracz, S.; Chen, J.; Kuznetsova, L. V.; Ojima, I. *Bioorg. Med. Chem.* **2005**, *13*, 5043.
3. Smith, T. A. *Br. J. Biomed. Sci.* **2000**, *57*, 170.
4. Ogretmen, B.; Hannun, Y. A. *Nat. Rev. Cancer* **2004**, *4*, 604.
5. Hannigan, B. M.; Barnett, Y. A.; Armstrong, D. B.; McKelvey-Martin, V. J.; McKenna, P. G. *Cancer Biother.* **1993**, *8*, 189.
6. Smith, T. A. *Nucl. Med. Commun.* **1998**, *19*, 97.
7. Ettmayer, P.; Billich, A.; Baumruker, T.; Mechtcheriakova, D.; Schmid, H.; Nussbaumer, P. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1555.
8. Buchmann, I.; Neumaier, B.; Schreckenberger, M.; Reske, S. *Cancer Biother. Radiopharm.* **2004**, *19*, 436.
9. Wagner, M.; Seitz, U.; Buck, A.; Neumaier, B.; Schultheiss, S.; Bangerter, M.; Bommer, M.; Leithauser, F.; Wawra, E.; Munzert, G.; Reske, S. N. *Cancer Res.* **2003**, *63*, 2681.
10. Fujiwaki, R.; Hata, K.; Nakayama, K.; Moriyama, M.; Iwanari, O.; Katabuchi, H.; Okamura, H.; Sakai, E.; Miyazaki, K. *Int. J. Cancer* **2002**, *99*, 328.
11. Wu, J.; Mao, Y.; He, L.; Wang, N.; Wu, C.; He, Q.; Skog, S. *Anticancer Res.* **2000**, *20*, 4815.
12. Hengstschlager, M.; Pfeilstocker, M.; Wawra, E. *Adv. Exp. Med. Biol.* **1998**, *431*, 455.
13. Al Madhoun, A. S.; Johnsamuel, J.; Yan, J.; Ji, W.; Wang, J.; Zhuo, J. C.; Lunato, A. J.; Woollard, J. E.; Hawk, A. E.; Cosquer, G. Y.; Blue, T. E.; Eriksson, S.; Tjarks, W. *J. Med. Chem.* **2002**, *45*, 4018.
14. Tornenik, Y.; Ullman, B.; Balzarini, J.; Wahren, B.; Eriksson, S. *Biochem. Pharmacol.* **1995**, *49*, 829.
15. The authors would like to thank Professor Staffan Eriksson for the kind gift of CEM and CEM TK-.
16. They would further like to thank Carina Rumold of the SKCC, La Jolla, for all her help with FACS.
17. Kingston, D. G.; Jagtap, P. G.; Yuan, H.; Samala, L. *Fortschr. Chem. Org. Naturst.* **2002**, *84*, 53.
18. Lavielle, G.; Hautefaye, P.; Schaeffer, C.; Boutin, J. A.; Cudennec, C. A.; Pierre, A. *J. Med. Chem.* **1991**, *34*, 1998.
19. Castellino, A. J.; Ballatore, C.; Aspland, S. E.; Desharnais, J.; Newman, M. J.; Sun, C.; Wirsching, P. U.S. Patent Application 20050148534A1.
20. Newman, M.; Aspland, S.; Ballatore, C.; Castillo, R.; Desharnais, J.; Eustaquio, T.; Guo Z.; Li, Q.; Sun, C.; Castellino, A. 16th EORTC-NCI-AACR Symposium, Abstract #611; *Eur. J. Cancer Sup.* **2004**, *2*, 185.
21. Ballatore, C.; Aspland, S. E.; Castillo, R.; Desharnais, J.; Eustaquio, T.; Sun, C.; Castellino, A. J.; Smith, A. B. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2477.
22. Bhushana Rao, K. S.; Collard, M. P.; Dejonghe, J. P.; Atassi, G.; Hannart, J. A.; Trouet, A. *J. Med. Chem.* **1985**, *28*, 1079.
23. The microtubule polymerization assay was done using a fluorescence-based kit (Cytoskeleton) using the manufacturers' recommended protocol. For VBL-containing conjugates, the assay was done in the presence of 20% glycerol, which induces polymerization of tubulin.
24. C-terminal His-tagged human TK1 fusion protein was produced then purified on a His-bind nickel-affinity column (Novagen). Using this TK1, kinase activity is measured with a coupled enzyme assay that follows the production of ADP, via the conversion of β-NADH to β-NAD^+. Initial velocities for test compounds are divided by velocities for the ATP-negative controls and then compared to the velocity of thymidine.