Bioorganic & Medicinal Chemistry Letters 20 (2010) 2908–2911

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Selective targeting of 2′-deoxy-5-fluorouridine to urokinase positive malignant cells in vitro

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ARTICLE INFO

Article history:
Received 29 January 2010
Revised 4 March 2010
Accepted 5 March 2010
Available online 9 March 2010

Keywords:
2′-Deoxy-5-fluorouridine
PAI-2
Anticancer prodrug
Targeted therapy
Selected delivery

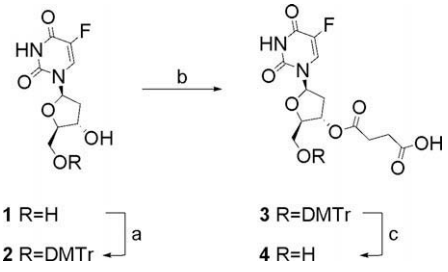
ABSTRACT

A urokinase targeting conjugate of 2′-deoxy-5-fluorouridine (5-FUdr) was synthesized and tested for tumor-cell selective cytotoxicity in vitro. The 5-FUdr prodrug 2′-deoxy-5-fluoro-3′-*O*-(3-carboxypropanoyl)uridine (5-FUdrsuccOH) containing an ester-labile succinate linker was attached to the specific urokinase inhibitor plasminogen activator inhibitor type II (PAI-2) and was found to preferentially kill urokinase-over expressing cancer cells. Up to 7 molecules of 5-FUdr were incorporated per PAI-2 molecule without affecting protein activity. This is the first time a small organic cytotoxin has been conjugated to PAI-2.

The fluoropyrimidine, 5-fluorouracil (5-FU), and its cytotoxic metabolite 2′-deoxy-5-fluorouridine (5-FUdr; **1**) are used in combination with folinic acid as standard treatments in the adjuvant setting for a variety of carcinomas, including those of the stomach, colon and breast.[1–3] However like many cytotoxic chemo-therapeutics, these drugs suffer from a number of disadvantages due to their lack of specificity and, as a result, dose limiting toxicity is often problematical. To overcome this obstacle, efforts have been made to develop a variety of non-toxic prodrugs of 5-FU and 5-FUdr that become activated only at the site of interest. Recently, particular attention has been given to the ligand-directed targeting approach, whereby the targeting of 5-FUdr to tumor cells has been described through the use of rabbit serum albumin,[4] epidermal growth factor,[5] small cyclic peptides and monoclonal antibodies directed against tumor associated antigens.[6–10] This last strategy has demonstrated some therapeutic potential although a number of limitations still persist. These include the inefficient uptake of the antibody conjugates into solid tumors due to poor blood perfusion[11] and large antibody size, loss of antibody activity and specificity after chemical modification,[12] and rapid elimination of the conjugate after initiation of an immune response due to the use of antibodies of non-human origin.[13]

A promising alternative targeting strategy is that based on the urokinase plasminogen activator (uPA) system and its natural inhibitor, plasminogen activator inhibitor type II (PAI-2). PAI-2 has the ability to preferentially target urokinase-positive metastatic breast cancer cells in vitro[14] and tumor xenografts and micrometastates in vivo.[15–17] With these characteristics, together with its relatively small size (47 kDa) and immunoneutrality,[18] it was anticipated that by linking a derivatized version of 5-FUdr to PAI-2, the cytotoxin could be specifically delivered to, and concentrated exclusively within, urokinase expressing tumor cells, thereby reducing collateral damage. Herein we describe the synthesis and characterization of a 2′-deoxy-5-fluorouridine derivative and its conjugation to the targeting protein PAI-2 via a succinate linker. In vitro tumor-cell selective cytotoxicity is also reported.

A series of reactions were performed to prepare 2′-deoxy-5-fluorouridine (5-FUdr, **1**) (Scheme 1) for chemical attachment to PAI-



Scheme 1. Synthesis of 2′-deoxy-5-fluoro-3′-*O*-(3-carboxypropanoyl)-uridine (5-FUdrsuccOH, **4**). Reagents and conditions: (a) 4,4′-dimethoxytriphenylmethyl chloride, DMAP, Et_3N, pyridine, rt, 16 h (61%); (b) succinic anhydride, DMAP, CH_2Cl_2, rt, 24 h (86%); (c) 8:2 AcOH/H_2O, 2 h rt (68%).

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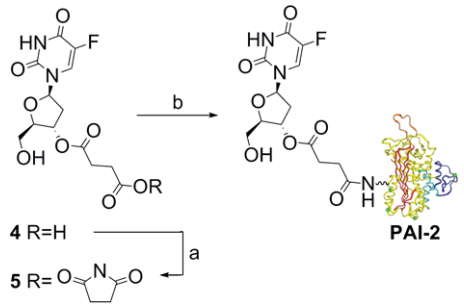
doi:10.1016/j.bmcl.2010.03.029



Scheme 2. Activation of 5-FUdrsuccOH (**4**) to form the active ester **5** and conjugation to PAI-2 to form the uPA directed prodrug 5-FUdrsucc-PAI-2 *via* amide bond formation with free surface lysine residues. Reagents and conditions: (a) *N*-hydroxysuccinimide, dicyclohexylcarbodiimide rt, 3 h; (b) PAI-2 (PBS pH 7.4), rt, 3 h (>85%).

2. This involved the introduction of a free carboxyl group[19] by formation of an ester between the secondary alcohol of a selectively protected 5-FUdr derivative (**2**)[6,20] and succinic acid which proceeded in >65% yield. The carboxyl group of 5-FUdrsuccOH (**4**)[6,21] was then activated by forming the ester **5** with 1.1 equiv of NHS and 6 equiv of DCC in anhydrous DMF (Scheme 2). The molar equivalents employed were based on those described previously for the activation of **4**.[6] However, it was noted that the active ester formed rapidly at RT as evidenced by TLC analysis (R_f 0.55, silica DCM) and longer reaction times, such as those described by Goerlach and co-workers[6] were unnecessary. The active ester **5** was then conjugated without being isolated or further purified to afford 5-FUdrsucc-PAI-2 (Scheme 2).

To optimize coupling, different molar excesses of the activated ester **5** were added to PAI-2 in order to obtain conjugates with the highest possible substitution. The greatest 5-FUdrsucc: PAI-2 ratio was obtained using a 50-fold molar excess of **5**, yielding up to 7 molecules of 5-FUdr for every protein molecule (Fig. 1). This was determined by ESI-MS analysis and corresponds well to previous reports regarding cDTPA incorporation into PAI-2.[15,22] According to the 3D structure of PAI-2 (prepared using the SwissModel® program, ExPaSy), at least 5 free lysine groups are accessible for chemical conjugation at the surface of the protein. However, this was only determined for the amino nitrogens that were >50% accessible and did not include the N-terminal amino acid, suggesting that incorporation of a larger number of cytotoxin molecules is possible. ESI-MS data also demonstrated that the 5-FUdrsucc-PAI-2 preparations contained a mixture of several 5-FUdrsucc-PAI-2 moieties with an average of 3–4 cytotoxin units incorporated per protein molecule. The relative amount of free, unmodified PAI-2 was <10%.

The ability of 5-FUdrsucc-PAI-2 to form stable complexes with uPA was next investigated in order to confirm that the uPA inhibitory activity of PAI-2 was retained after modification. 5-FUdrsucc-PAI-2 was found to form stable complexes of ~75 to 100 kDa with both the B chain (25 kDa) and the A + B chain (50 kDa) of uPA, respectively. This was similar to that observed for unmodified PAI-2, confirming that the modification of lysine functional groups leading to the incorporation of up to 7 molecules of 5-FUdrsucc does not compromise the inhibitory ability of the protein. Furthermore, when a sample of the conjugate was retested after 1 year (after sterile storage at 4 °C), it was found to retain its inhibitory activity as evidenced by the formation of high molecular weight uPA/PAI-2 complexes (Fig. 2).

To assess the targeting ability of the conjugate in vitro, the newly synthesized 5-FUdrsucc-PAI-2 and corresponding free cytotoxins were evaluated for inhibitory activity in two breast adenocarcinoma cell lines (Table 1) varying in their expression levels of uPA. The cytotoxicity of 5-FUdr with the succinate linker attached (5-FUdrsuccOH, **4**) against the metastatic MDA-MB-231 cell line (expressing high levels of uPA and uPAR), was 13-fold lower than that of 5-FUdr (IC_{50} 0.21 μM and IC_{50} 2.85 μM, respectively, Table 1), suggesting that succinylation of the 3′-group of 5-FUdr results in the loss of some activity. Similarly, Goerlach et al. reported a 10-fold decrease in activity for 5-FUdrsuccOH against a murine thymoma cell line in vitro and hypothesized that it may have been due to the inefficient uptake of the modified compound into the cell by the nucleoside transport system.[6] The lowered IC_{50} value of **4** compared to **1** may also be indicative of the relative stability of the succinate linker in culture media.[10] The IC_{50} of the 5-FUdrsucc-PAI-2 conjugate, with an average of 3 mol-

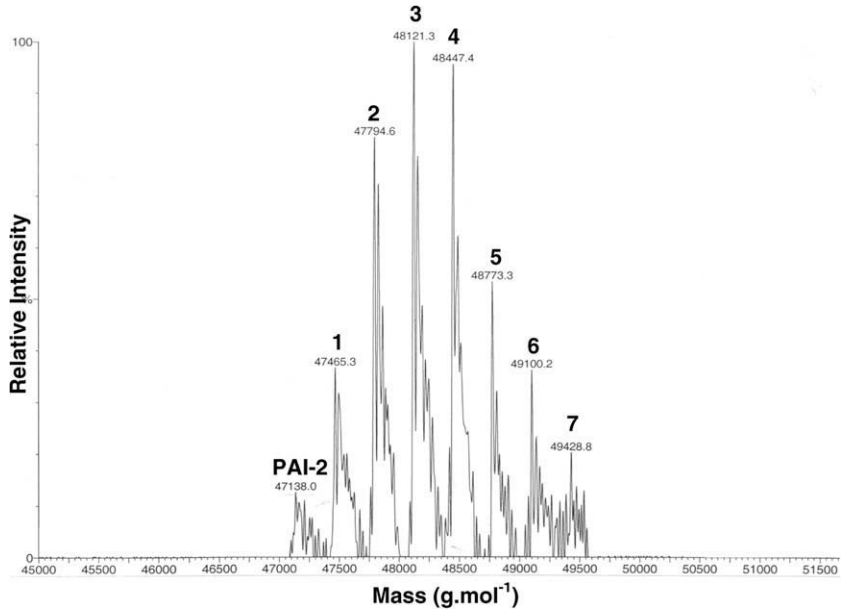


Figure 1. ESI-MS of 5-FUdrsucc-PAI-2. Conjugates were prepared for electrospray ionization mass spectrometry (ESI-MS) in Milli Q water and were made up fresh to a final concentration of 1–10 μM. Numbers displayed on the peaks represent the number of 5-FUdrsucc molecules incorporated into the PAI-2 targeting ligand. The difference in mass for each species represents the exact weight of 5-FUdr, plus the succinate linker (327 g mol^{-1}), giving a correlation coefficient (R^2) = 1.

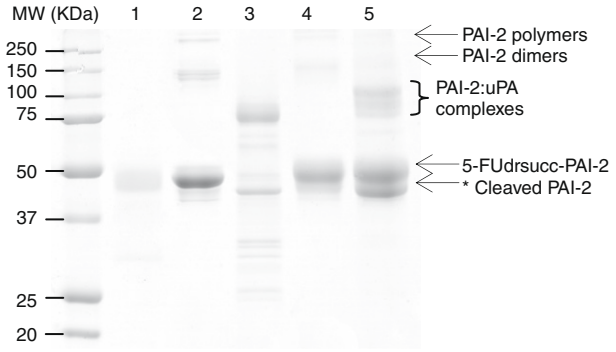


Figure 2. Samples fractioned by SDS PAGE showing the ability of unmodified and modified PAI-2 to form stable complexes with HMW uPA. Briefly, PAI-2 and 5-FUdrsucc-PAI-2 were incubated with uPA (2:1) at 37 °C for 30 min. Samples were then fractionated under non-reducing conditions using 10% acrylamide SDS PAGE and visualized by staining with Coomassie blue. Lane 1: uPA, Lane 2: PAI-2, Lane 3: PAI-2: uPA, Lane 4: 5-FUdrsucc-PAI-2, Lane 5: 5-Fudrsuc-PAI-2: uPA. *Cleaved PAI-2 occurs when a portion of wild-type PAI-2 is cleaved by uPA in the CD-loop.[23]

ecules of 5-FUdr incorporated per protein molecule was calculated to be 0.77 μM against the MDA-MB-231 cell line, which was almost four times lower than 5-FUdrsuccOH. This is a significant finding as potent cytotoxic activity is maintained after chemical modification, a phenomenon which by no means should be assumed. Of importance was that more than four fold[24] greater activity was observed in the high uPA expressing cell line (MDA-MB-231) than the low uPA expressing cell line (MCF-7) after treatment with the cytotoxin conjugate (Table 1, Fig. 3A). This suggests that PAI-2 aids in the cellular uptake of the modified cytotoxin through receptor mediated endocytosis which results in partial hydrolysis of the succinate linker and subsequent release of 5-FUdr. Similarly, Zhang et al. report partial release of 5-fluoro-2′-deoxyuridine from the cyclic peptide CNGRC using the same ester-labile linking system.[10] The specific targeting ability of PAI-2 was further exemplified in the colorectal cell line HCT-116 where a non-specific negative control conjugate (5-FUdrsucc-BSA) was found to be inactive at the highest concentration tested (Fig. 3B), while the PAI-2 targeted conjugate killed 35% of cells at the highest concentration (31 nM equiv protein concn). Furthermore, saturation of the urokinase plasminogen activator receptor (uPAR) on MCF-7 cells by pre-incubation with exogenous uPA, followed by treatment with 3.8 μM 5-FUdrsucc-PAI-2 for 48 h showed a significant increase in cytotoxicity (as measured by the decrease in cell viability ($P = 0.0002$)) compared to MCF-7 cells with endogenous receptor bound uPA only (Fig. 3A, inset). MDA-MB-231 cells with high endogenous uPAR bound uPA were even more sensitive (viability = 6.0% ± 0.29, data not shown), showing a reduction in cell viability of between 3 and 4 times that of MCF-7 cells treated with (+) or without (−) uPA. Considering cell surface uPA is essential for PAI-2:uPA:uPAR endocytosis,[26] this indicates that cytotoxicity is most likely mediated via a uPA-dependent mechanism. This has previously been reported for PAI-2 conjugated to the α-emitting radioisotope ^{213}Bi whereby PAI-2-^{213}Bi was found to be selectively toxic to uPA expressing breast cancer cells in vitro, but not to freshly isolated, normal human leukocytes on which cell-surface localized active uPA was not detectable.[22] Although the full mechanism of action of the 5-FUdrsucc-PAI-2 remains to be elucidated, it is anticipated that the conjugate would be digested in the lysosomes after internalization via receptor mediated endocytosis. Non-specific esterases present in the lysosomes could hydrolyze the ester bond between 5-FUdr and the succinyl linker to liberate free 5-FUdr, which is likely to be metabolized to produce the active compo-

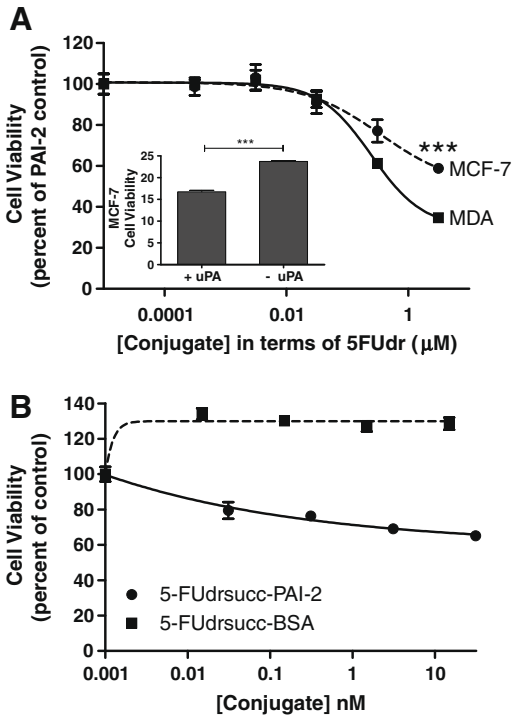


Figure 3. The in vitro cytotoxicity of 5-FUdrsucc-PAI-2 against the high and low uPA and uPAR expressing breast adenocarcinoma cell lines, MDA-MB-231 and MCF-7 and the colorectal cell line HCT-116. (A) Breast cancer cells (1.0×10^4) were cultured for 48 h in the presence of increasing concentrations of the protein cytotoxin conjugate 5-FUdrsucc-PAI-2 and percent viability of the cells determined in reference to PBS vehicle controls. Inset: 5-FUdrsucc-PAI-2 at a single concentration of 3.8 μM was incubated with MCF-7 cells pre-treated with (+) or without (−) exogenous uPA (18 nM) for 48 h. Percent viability was determined in reference to PBS controls. (B) HCT-116 cells (1.0×10^4) were cultured for 24 h in the presence of increasing concentrations of the protein cytotoxin conjugate 5-FUdrsucc-PAI-2 or the negative control conjugate 5-FUdrsucc-BSA and percent viability of the cells determined in reference to PBS vehicle controls (see Supplementary data (Fig. S1) for 72 h results). Data points are the means of triplicate experiments ±SE. *** P <0.001 extremely significant.

Table 1
The effect of 5-FUdrsucc-PAI-2 and unconjugated cytotoxins 5-FUdr (**1**) and 5-FUdrsuccOH (**4**) on two mammary adenocarcinoma cell lines varying in their expression levels of uPA

Cell line	uPA[b]	IC_{50}[a] (μM)			
		5-FUdr (**1**)	5-FUdrsuccOH (**4**)	PAI-2	5-FUdrsucc-PAI-2
MCF-7	Low	NR[c] [≫20.3]	NR [>144]	NA[d]	NR [>3.13]
MDA-MB-231	High	0.21 (±0.003)	2.85 (±0.331)	NA	0.77 (±0.167)

[a] IC_{50} values were calculated based on moles of cytotoxin from sigmoidal dose response curves (variable slope), generated using GraphPad Prism V. 5 (GraphPad Software Inc.). Values are the mean of triplicate experiments ± SEM.
[b] Relative expression levels of uPA.[22,25]
[c] NR: Not reached, even at the highest concentration tested, in brackets.
[d] NA: Not active, even at the highest concentration tested (i.e., 1 μM).

nents required for inhibition of thymidylate synthase and hence DNA synthesis.

In conclusion, the use of the cytotoxic metabolite 5-FUdr in the clinic is currently limited due to its narrow therapeutic window. PAI-2, an irreversible, specific inhibitor of the metastatic marker uPA has the ability to overcome this problem by specifically delivering the potent molecule to urokinase expressing tumor cells, ultimately reducing toxic side effects and overcoming many of the limitations associated with MAb use in targeted therapy. This is the first time a small organic cytotoxin has been conjugated to PAI-2. Further studies assessing the in vivo efficacy of this prodrug are under investigation.

Acknowledgments

This study was supported by the University of Wollongong through the Centre for Medical Bioscience (CMB), the Centre for Medicinal Chemistry and Pharmacology (CMCP), a URC Small Grant, a University Cancer Research Grant and a UPA student scholarship for K. L. Vine. We are also grateful to the Illawarra Cancer Carers Inc., Kiama, Minnamurra and Gerringong Sunrise Rotary, The Robert East Memorial Fund, Professor P. Clingan and other private donors for funding assistance and to Dr. K. Benkendorff for support. Assoc. Professor M. Ranson was a recipient of a Cancer Institute NSW Fellowship award.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.03.029.

References and notes

1. Meyerhardt, J. A.; Mayer, R. J. *N. Eng. J. Med.* **2005**, *352*, 476.
2. Kelder, W.; Hospers, G. A.; Plukker, J. T. *Expert Rev. Anticancer Ther.* **2006**, *6*, 785.
3. van Laar, J. A.; Rustum, Y. M.; Ackland, S. P.; van Groeningen, C. J.; Peters, G. J. *Eur. J. Cancer* **1998**, *34*, 296.
4. Barbanti-Brodano, G.; Fiume, L. *Experientia* **1974**, *30*, 1180.
5. Poznansky, M. J.; Juliano, R. L. *Pharmacol. Rev.* **1984**, *36*, 277.
6. Goerlach, A.; Krauer, K. G.; McKenzie, I. F.; Pietersz, G. A. *Bioconjugate Chem.* **1991**, *2*, 96.
7. Henn, T. F.; Garnett, M. C.; Chhabra, S. R.; Bycroft, B. W.; Baldwin, R. W. *J. Med. Chem.* **1993**, *36*, 1570.
8. Krauer, K. G.; McKenzie, I. F.; Pietersz, G. A. *Cancer Res.* **1992**, *52*, 132.
9. Pietersz, G. A.; Krauer, K. *J. Drug Target* **1994**, *2*, 183.
10. Zhang, Z.; Hatta, H.; Ito, T.; Nishimoto, S. *Org. Biomol. Chem.* **2005**, *3*, 592.
11. Jain, R. K. *J. Natl. Cancer Inst.* **1989**, *81*, 570.
12. Wu, A. M.; Senter, P. D. *Nat. Biotechnol.* **2005**, *23*, 1137.
13. Dillman, R. O. *Antibody Immunocon. Radiopharm.* **1990**, *3*, 1.
14. Ranson, M.; Tian, Z.; Andronicos, N. M.; Rizvi, S.; Allen, B. J. *Breast Cancer Res. Treat.* **2002**, *71*, 149.
15. Allen, B. J.; Tian, Z.; Rizvi, S. M.; Li, Y.; Ranson, M. *Br. J. Cancer* **2003**, *88*, 944.
16. Li, Y.; Rizvi, S. M.; Ranson, M.; Allen, B. J. *Br. J. Cancer* **2002**, *86*, 1197.
17. Stutchbury, T. K.; Al-Ejeh, F.; Stillfried, G. E.; Croucher, D. R.; Andrews, J.; Irving, D.; Links, M.; Ranson, M. *Mol. Cancer Ther.* **2007**, *6*, 203.
18. Croucher, D. R.; Saunders, D. N.; Lobov, S.; Ranson, M. *Nat. Rev. Cancer* **2008**, *8*, 535.
19. *Compound* **3**: ^{1}H NMR ($CDCl_3$) δ 2.34 (ddd, J = 6, 8, 14 Hz, 1H, H2′), 2.58 (dd, J = 6, 14 Hz, 1H, H2′), 2.69 (m, 4H, $COCH_2CH_2CO$), 3.39 (dd, J = 2, 11 Hz, 1H, H5′), 3.52 (dd, J = 3, 11 Hz, 1H, H5′), 3.78 (s, 6H, 2 × OCH_3), 4.19 (d, J = 2 Hz, 1H, H4′), 5.45 (d, J = 6 Hz, 1H, H3′), 6.27 (br t, J = 6 Hz, 1H, H1′), 6.84 (dd, J = 3, 9 Hz, 4H, ArH), 7.22 (t, J = 7 Hz, 1H, phenyl ArH), 7.28–7.31 (overlapping m, 6H, ArH), 7.38 (d, J = 7 Hz, 2H, ArH), 7.83 (d, J_{HF} = 6 Hz, 1H, H6). ^{13}C NMR (CD_3OD) δ 25.1, 24.8, 34.2, 51.2, 59.4, 71.3, 80.1, 81.3, 83.4, 109.3, 109.4, 123.2, 123.9, 124.1, 126.0, 131.0 (d, J_{CF} = 29 Hz, CFCH), 136.9 (d, J_{CF} = 239 Hz, *C*F), 140.0, 145.2, 153.4 (d, J_{CF} = 26 Hz, CFCO), 154.7 (2C), 167.6, 172.4. LRES-MS m/z 646.9 ($[M]^-$).
20. *Compound* **2**: ^{1}H NMR ($CDCl_3$) δ 2.25 (ddd, J = 7, 7, 14 Hz, 1H, H2′), 2.51 (ddd, J = 3, 7, 14 Hz, 1H, H2′), 3.38 (dd, J = 3, 11 Hz, 1H, H5′), 3.42 (dd, J = 3, 11 Hz, 1H, H5′), 3.76 (s, 6H, 2 × OCH_3), 4.10 (d, J = 3 Hz, 1H), 4.56 (t, J = 3 Hz, 1H), 6.31 (t, J = 6 Hz, 1H, H1′), 6.83 (dd, J = 2, 9 Hz, 4H, ArH), 7.20 (t, J = 7 Hz, 1H, ArH), 7.29 (overlapping m, 6H, ArH), 7.40 (d, J = 7 Hz, 2H, ArH), 7.83 (d, J_{HF} = 6 Hz, 1H, H6). ^{13}C NMR ($CDCl_3$) mixture of rotamers δ 41.0, 55.0, 55.1, 55.2, 55.4, 63.3, 71.96, 72.03, 85.5, 85.6, 86.5, 87.1, 113.3, 127.0, 128.0, 129.9, 130.0, 135.2 (d, J_{CF} = 23 Hz, CFCH), 140.6 (d, J_{CF} = 238 Hz, *C*F), 144.2, 149.1, 157.1 (d, J_{CF} = 26 Hz, CFCO), 158.6. LREI-MS m/z 548 ($[M]^+$).
21. *Compound* **4**: ^{1}H NMR (CD_3OD) δ 2.29 (ddd, J = 6, 6, 14 Hz, 1H, H2′), 2.44–2.50 (overlapping m, 3H, CH_2 and H2′), 2.60 (t, J = 7 Hz, 2H, CH_2), 3.81 (d, J = 3 Hz, 2H, H5′), 4.14 (d, J = 2 Hz, 1H, H4′), 5.30 (d, J = 6 Hz, 1H, H3′), 6.25 (t, J = 7 Hz, 1H, H1′), 8.19 (d, J_{HF} = 6 Hz, 1H, H6). ^{13}C NMR (CD_3OD) 31.6, 32.9, 38.4, 62.7, 76.3, 86.5, 86.8, 126.1 (d, J_{CF} = 35 Hz, CFCH), 142.0 (d, J_{CF} = 234 Hz, *C*F), 151.4, 160.3 (d, J_{CF} = 25 Hz, CFCO), 175.3, 181.1. LRES-MS m/z 344.9 ($[M]^-$).
22. Ranson, M.; Tian, Z.; Andronicos, N. M.; Rizvi, S.; Allen, B. J. *Breast Cancer Res. Treat.* **2002**, *71*, 149.
23. Lobov, S.; Wilczynska, M.; Bergstrom, F.; Johansson, L. B.; Ny, T. *J. Mol. Biol.* **2004**, *344*, 1359.
24. This value is likely to be considerably higher. An exact IC_{50} value was not obtained due to limited amounts of material.
25. Andronicos, N. M.; Ranson, M. *Br. J. Cancer* **2001**, *85*, 909.
26. Al-Ejeh, F.; Croucher, D.; Ranson, M. *Exp. Cell Res.* **2004**, *297*, 259.