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Synthesis and Preliminary Analysis of a P-Glycoprotein-Specific [^{3}H]-Benzophenone Photoaffinity Label Based on (−)-Stipiamide

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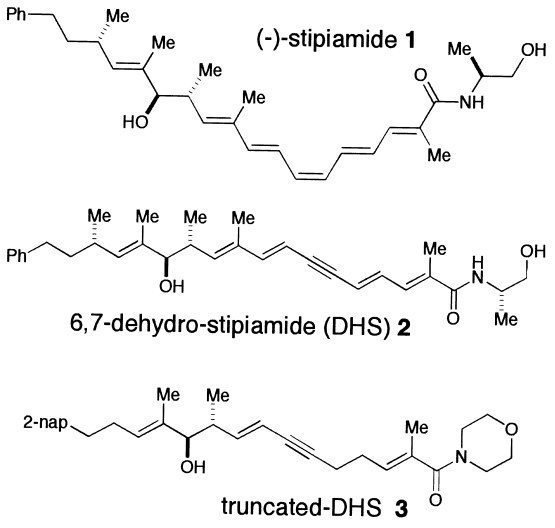
Abstract—A benzophenone photoaffinity label **9** based on the polyene natural product (−)-stipiamide has been constructed using a diaminoethane spacer and the radioactive agent [^{3}H]-BZDC (*N*-succinimidyl *p*-benzoyl-(2,3-^{3}H)-dehydrocinnamate). Photoaffinity experiments show specific binding to human P-glycoprotein (Pgp) in the presence of *cis*-flupentixol but not with cyclosporin A.

P-glycoprotein (Pgp) is an ATP-dependent drug efflux pump whose overexpresion confers multidrug resistance (MDR) to cancer cells. The development of resistance in cancer cells to chemotherapeutic agents has been a major impediment to effective clinical treatment.[1] Recently, we have developed synthetic routes to new MDR reversal agents, (−)-stipiamide **1**, a highly toxic polyene natural product and a designed, more potent, nontoxic compound 6,7-dehydrostipiamide **2** (DHS) (Scheme 1) that restores the cytotoxicity of adriamycin to resistant human breast cancer cells (MCF-7adrR) at low concentration (4 nM).[2] A simplified DHS compound has also been shown, through competitive displacement studies, to bind Pgp and stimulate ATP hydrolysis at the same concentration observed for MDR reversal. In addition, a solution-phase, two-dimensional indexed combinatorial library of DHS compounds was generated and screened.[3] Many potent, non-natural compounds were identified including the truncated 2-naphthyl morpholino amide DHS compound **3** shown. This template is now used as part of an effort to develop more efficient labeling reagents for Pgp.

While various arylazide photoaffinity reagents have been used to map the substrate-binding domains of Pgp,[4] none have identified specific residues within the proposed binding domains. Problems with the availability of large amounts of purified protein and labeling efficiency are the main reasons limiting the process of characterizing Pgp binding residues. Photoaffinity analogues of colchicine, daunomycin, forskolin, and prazosin, interact with Pgp at distinct sites;[5] however, binding to other proteins cannot be ruled out in that Pgp made up only 1–5% of the total protein in the samples used in these assays. Iodoarylazidoprazosin labeled two separate sites on a large segment of mouse Pgp that included TM6 and TM12 using antibody based assays.[6] Low nitrene insertion efficiency (< 2%) and limited amounts of protein precluded the identification of individual



Scheme 1.

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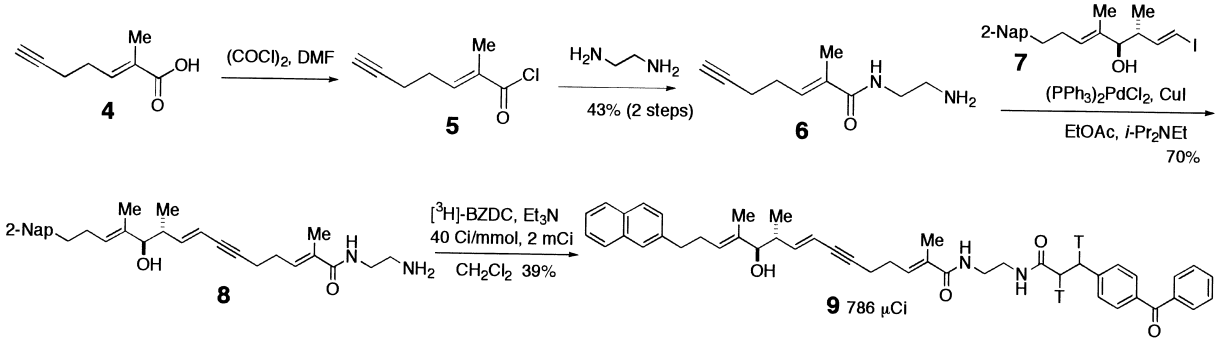
amino acid residues. These regions have also been implicated by azidopine and forskolin affinity reagents.[5] These studies can be used to conclude that residues within TM5-6 and TM11-12 function in a concerted manner for drug binding. The close proximity of the ATP-binding sites supports the hypothesis that ATP hydrolysis induces conformational changes that are conveyed to the TM5-6 and TM11-12 regions causing drug displacement and efflux. The conformational changes in the substrate-binding domain following ATP hydrolysis have been experimentally determined.[7]

Prestwich and others have recently developed successful labeling strategies using benzophenone derivatized substrates for a variety of targets including mouse Pgp.[8] The benzophenone label offers the advantage of being more reactive than the diazo and azide labels with preferential C–H reactivity over X–H bonds. It also is more chemically and room light stable and more lipophilic. Diazo and azide compounds activate irreversibly at 254 nM and are not as stable to room light. Benzophenones, on the other hand, are activated reversibly at 350–365 nM, outside of the protein damaging 250 nM range. Labeling efficiencies, the percentage of target derivatized, are typically 5–10% at best for nitrenes and carbenes, whereas benzophenones appear to be much higher.

The initial stipiamide based labeling agent contains a benzophenone attached on the right hand side via the extended aminoethyl amide (Scheme 2). This approach required the development of an efficient coupling strategy for 1,2-aminoethane and allowed for the use of intermediates previously developed for the non-natural variants of (−)-stipiamide. Enyne acid **4**[2b] was converted to the acid chloride, with oxalyl chloride and DMF, which was then used crude with excess 1,2-diaminoethane.[9] Use of lower equivalents of diamine resulted in the efficient production of the undesired ethyl diamide dimer. With 16 equiv of 1,2-diaminoethane in methylene chloride, aminoethyl amide **6** was obtained in pure form in 43% overall yield for the two steps. Sonogashira coupling using the newly found optimized conditions with **6** and vinyl iodide **7**[3] at −20 °C reacted with palladium chloride and copper iodide catalysts and diisopropylethylamine in ethyl acetate gave enyne **8** in 73% isolated yield.[10] Reaction with the radioactive labeling agent [^{3}H]-BZDC (*N*-succinimidyl *p*-benzoyl-(2,3-^{3}H)-dehydrocinnamate) (40 Ci/mmol) gave the desired ditritiated benzophenone [^{3}H]-**9** (787 μCi).[11] The reaction was also carried out using cold [^{1}H]-BZDC to afford non-tritiated **9** (37%).[12]

The radiolabel **9** was used in Pgp photoaffinity experiments using crude plasma insect cell membranes (lanes 1 and 2) as well as NIH3T3-MDR1 cells expressing human Pgp (lanes 3–5, Fig. 1).[13] Irradiation at 365 nM of the materials in media for 1 h followed by SDS-PAGE incubation and autoradiography exposure.[14] Experiments were also conducted with a compound known to bind and be transported by Pgp, cyclosporin A (K_d 150 nM), and *cis*-flupentixol a modulator of Pgp function with a reversal concentration of approximately 25 μM. With the crude insect cell membranes, only a small fraction of the protein present was labeled by **9** as seen in the control lane 1. With added cyclosporin A, most of the label was effectively displaced leaving an even smaller fraction of labeled Pgp (lane 2). With the NIH3T3-MDR1 cells approximately 50% of the Pgp was labeled by **9** (lane 3). Cyclosporin A, an inhibitor of Pgp function has previously been shown to directly compete with and displace photoaffinity analogues such as ^{125}I labeled iodoarylazidoprazosin (IAAP), an analogue of prazosin, ^{125}I labeled iodomycin, an analogue of daunorubicin, ^{3}H-labeled azidopine, and 6-*O*-[2-[3-(4-azido-3-[^{125}I]iodophenyl)propionamido]ethylcarbamyl] forskolin (AIPPF).[4] The fact that cyclosporin A efficiently displaces **9** in both intact Pgp expressing cells (lane 4) and in crude membrane preparations strongly suggests that the binding of **9** to Pgp is specific. Note that in Figure 1 there is also a strong binding of **9** to another band of < 78 k-Da. However, this binding is not sensitive to cyclosporin A and thus is probably nonspecific. In the presence of *cis*-flupentixol, the Pgp modulator, only about half of the label **9** appears to be removed (lane 5). In this case, the reactivity of the benzophenone label **9** was lower compared to arylazide labels and a longer irradiation time was needed. Both these results made sequence analysis impossible to conduct with this label.

With a route secure for the production of new benzophenone photoaffinity labels, efforts to develop more efficient labels with higher reactivity can now proceed for the identification of specific residues involved in substrate binding by sequence analysis. By placing the benzophenone at different locations along the linear compound various residues of Pgp can be identified.



Scheme 2.

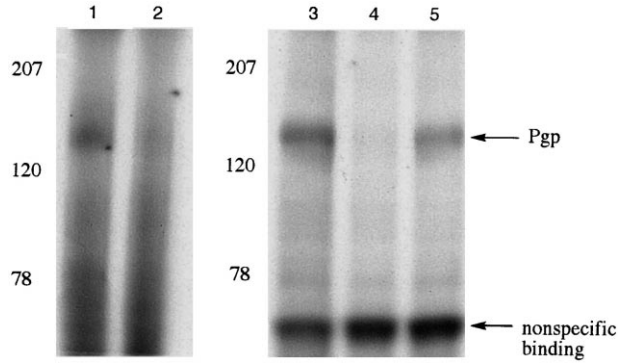


Figure 1. Pgp was photo-crosslinked with 6 μM [^{3}H]-**9** at 365 nm for 1 h at rt, followed by SDS-PAGE. The gels were incubated in Enlightning enhancer (NEN Life Sciences, Boston, MA) for 15 min, dried under vacuum and exposed to Bio-Max MR film at −70 °C for 48 h. The autoradiogram shows: lane 1, untreated crude membranes prepared from High Five insect cells infected with the recombinant baculovirus carrying the human *MDR1* cDNA with a 6 histidine tag at the C-terminal end (BV-MDR1 (H6)); lane 2, crude membranes pretreated with cyclosporin A, 1 μM for 5 min at rt and then treated with [^{3}H]-**9**; lane 3, untreated NIH3T3-MDR1 cells; lane 4, NIH3T3-MDR1 cells pretreated with cyclosporin A, 1 μM for 5 min at rt prior to addition of [^{3}H]-**9**; and lane 5, NIH3T3-MDR1 cells pretreated with *cis*-flupentixol (25 μM) for 5 min at rt prior to addition of [^{3}H]-**9**.

With the benzophenone in the interior, for example, less degrees of freedom will be available leading to more efficient labeling within the binding site. Label **9**, now with its demonstrated specific labeling of Pgp, represents the starting point for future efforts that will be reported in due course.

Acknowledgements

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9. Preparation of **6**: Acid **4** (22 mmol) in CH_2Cl_2 (350 mL) was treated with oxalyl chloride (220 mmol) and one drop of DMF at rt for 4 h. The solution was concentrated to give acid chloride **5**. Acid chloride **5** (22.0 mmol) was redissolved in CH_2Cl_2 (220 mL), cooled to −50 °C and a solution of ethylene diamine (360 mmol) in CH_2Cl_2 (144 mL, precooled to −50 °C) was added via cannula. The mixture was allowed to warm to rt and stirred overnight. The solution was diluted with 100 mL of $NaHCO_3$, extracted with CH_2Cl_2, and concentrated. Flash chromatography (10:1.5:0.2, CH_2Cl_2:MeOH:NH_4Cl) gave 1.69 g (43% yield) of the desired volatile product **6**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.40 (bs, 1H), 6.33 (t, J = 6.9 Hz, 1H), 3.32 (q, J = 5.4 Hz, 2H), 2.85 (t, J = 4.8 Hz, 2H), 2.39–2.24 (m, 4H), 1.96 (t, J = 2.7 Hz, 1H), 1.84 (s, 3H), 1.71 (bs, 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 169.7, 133.5, 132.6, 83.6, 69.2, 42.4, 41.5, 27.5, 18.1, 13.0. Anal. calcd for $C_{10}H_{17}ON_2$: C, 66.64; H, 8.95; found C, 66.41; H, 8.70.
10. Preparation of **8**: A solution of **7** (0.1 mmol) and **6** (0.2 mmol) in EtOAc (3.3 mL) was cooled to −20 °C and $(Ph_3P)_2PdCl_2$ (0.01 mmol), CuI (0.04 mmol), and *i*-Pr_2NH (1.1 mL) were added. The reaction was immediately removed from the cold bath and allowed to warm to rt. The flask was protected from light and allowed to stir for 17 h. Radial chromatography (1:1:0.01, CH_2Cl_2:MeOH:NH_4Cl) gave 0.033 g of product **8** (73% yield): $[\alpha]_D$ −15.1° (*c* 0.94, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.82–7.75 (m, 3H), 7.71 (s, 1H), 7.47–7.39 (m, 2H), 7.34 (d, J = 8.4 Hz, 1H), 6.37 (bt, 2H), 6.00 (dd, J = 15.9, 8.4 Hz, 1H), 5.52 (d, J = 15.9 Hz, 1H), 5.45 (t, J = 6.3 Hz, 1H), 3.62 (d, J = 8.7 Hz, 1H), 3.38 (bd, 2H), 2.95–2.80 (m, 3H), 2.50–2.28 (m, 8H) 2.09 (bs, 3H), 1.88 (s, 3H), 1.57 (s, 3H), 0.78 (d, J = 6.6 Hz, 3H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 169.9, 146.2, 139.6, 136.1, 133.9, 133.8, 132.6, 132.2, 128.2, 128.0, 127.8, 127.6, 127.5, 126.7, 126.1, 125.4, 111.1, 88.5, 81.8, 80.0, 42.1, 41.5, 36.0, 29.5, 27.8, 19.2, 16.9, 13.2, 11.2; HRMS FAB (M+H) calcd for $C_{30}H_{38}O_2N_2Na$, 481.2833, found 481.2835.
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12. Preparation of **9**: **CAUTION** — Reagents were used in a safety hood approved for radioactive material. A solution of [^{3}H]-BZDC (40 Ci/mmol, 0.25 mL, 2 mCi, 50 nmol) in CH_2Cl_2 (1 mL) was charged with **8** (150 nmol), and Et_3N (0.02 mL) and the reaction was stirred overnight at rt. The solution was diluted with 1 mL brine and extracted with $NaHCO_3$, H_2O, and NH_4Cl. The organic layers were dried (Na_2SO_4) and concentrated (N_2) to give [^{3}H]-**9** (786.6 μCi, 39% yield). The reaction using cold BZDC, under similar conditions, afforded non-tritiated **9** (37% yield): $[\alpha]_D$ −0.94° (*c* 0.43, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.80–7.71 (m, 7H), 7.60 (s, 1H), 7.59 (d, J = 6.0 Hz, 1H), 7.50–7.26 (m, 7H), 6.46 (bs, 1H), 6.38 (bs, 1H), 5.98 (dd, J = 15.9, 8.4 Hz, 1H), 5.52 (d, J = 15.6 Hz, 1H), 5.45 (t, 1H), 3.69–3.61 (m, 1H), 3.63 (d, J = 8.4 Hz, 1H), 3.40 (s, 4H), 3.02 (t, J = 7.5 Hz, 2H), 2.88–2.80 (m, 2H), 2.54–2.28 (m, 9H), 1.84 (s, 3H), 1.55 (s, 3H), 0.76 (d, J = 6.9 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.2, 170.6, 146.1, 146.4,

139.6, 137.9, 136.0, 135.9, 134.9, 133.8, 132.6, 132.2, 131.8, 130.7, 130.2, 128.7, 128.6, 128.52, 128.50, 128.4, 128.1, 127.3, 127.6, 127.5, 127.1, 126.7, 126.1, 125.4, 111.3, 88.4, 81.8, 80.0, 41.5, 40.7, 40.6, 37.9, 35.9, 31.7, 29.6, 27.9, 19.1, 16.9, 13.1, 11.2; HRMS FAB (M + Na) calcd for $C_{46}H_{50}O_4N_2Na$ 717.3655, found 717.3642.

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14. Photoaffinity labeling with [^{3}H]-**9**: Crude plasma membranes containing Pgp expressed from High Five insect cells infected with recombinant baculovirus carry the human *MDR1* gene and NIH3T3-MDR1 cells were used for these experiments. The preparation for these membranes is described elsewhere (Ramachandra, M.; Ambudkar, S. V.; Chen, D.; Hrycyna, C. A.; Dey, S.; Gottesman, M. M.; Pastan, I. *Biochemistry* **1998**, *37*, 5010). The crude plasma membrane in Tris–HCl, pH 7.4 was incubated with the MDR modulator (cyclosporin A at 1 μM or *cis*-flupentixol at 25 μM) for 5 min at rt. [^{3}H]-**9** (6 μM) was added in the dark at rt and the mixture was incubated for 5 min. The membranes were exposed to a UV lamp (G.E. no. F15T8-BLB, 365 nM) for 1 h at rt. 5× SDS-PAGE sample buffer was added to each of the samples and these were incubated at rt for 30 min. Samples were then run on an 8% Tris-glycine gel at constant voltage. Gels were dried and exposed to autoradiography film.