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Synthesis and analysis of polyethylene glycol linked P-glycoprotein-specific homodimers based on (−)-stipiamide

Merritt B. Andrus,[a,*] Timothy M. Turner,[a] Emily P. Updegraff,[a] Zuben E. Sauna[b] and Suresh V. Ambudkar[b]

[a]*Brigham Young University, Department of Chemistry and Biochemistry, C100 BNSN, Provo, UT 84602-5700, USA*
[b]*National Cancer Institute, NIH, Laboratory of Cell Biology, Division of Basic Sciences, Building 37, Room 1B-17 37 Convent Dr., MSC4255 Bethesda, MD 20878-4255, USA*

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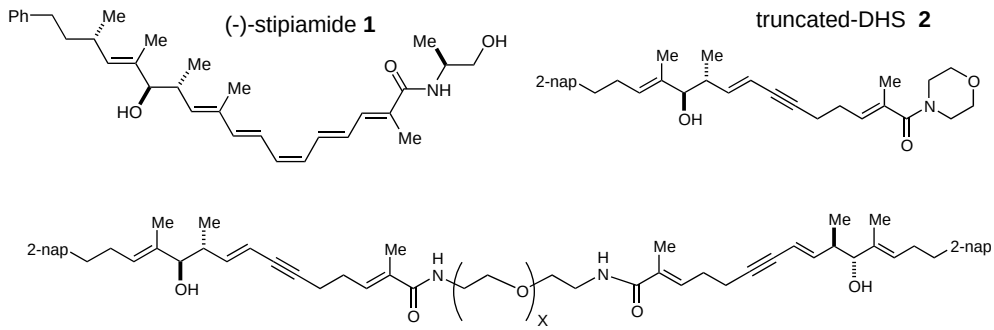
Abstract—A series of five homodimeric polyethylene glycol (PEG) linked homodimers based on the multidrug resistance reversal agent (−)-stipiamide were made and tested for their ability to interact with P-glycoprotein, the protein responsible for multidrug resistance, using ATPase and photoaffinity displacement assays. Key reactions include a new alkoxide-mesylate displacement for the assembly of the PEG linkers and a double Sonogashira coupling reaction. © 2001 Elsevier Science Ltd. All rights reserved.

Polyvalency has become a popular strategy for increasing the binding affinity of ligands to multimeric receptors.[1] A weak binder can be converted into a polyvalent tight binder provided the receptor possesses various binding sites or can be dimerized. The origin of the overall enhanced binding can be attributed to the more favorable second and subsequent binding events where much less entropy is lost due to induced proximity. Among the successful examples are the FK506 and cyclosporin dimers of Schreiber,[2] the vancomycin dimers of Whitesides[3] and Griffin,[4] and the polysaccharides of Kiessling,[5] Whitesides, and Fan.[6] Critical issues include the nature of the linker and the polymeric support, the length and position of attachment of the linker, and compatibility with assays. A particularly challenging target is P-glycoprotein (Pgp) the ATP-dependent drug efflux pump whose overexpression 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 treatments.[7] Recently we reported synthetic routes to the new MDR reversal agents, (−)-stipiamide **1**, a highly toxic polyene and a designed, more potent, nontoxic compound 6,7-dehydrostipiamide (DHS) that restores the cytotoxicity of doxorubicin to resistant human breast cancer cells (MCF-7adrR) at low concentration (Scheme 1).[8] A solution-phase combinatorial library of DHS compounds was generated using a key Sonogashira coupling reaction and was screened.[9]



Scheme 1.

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* Corresponding author.

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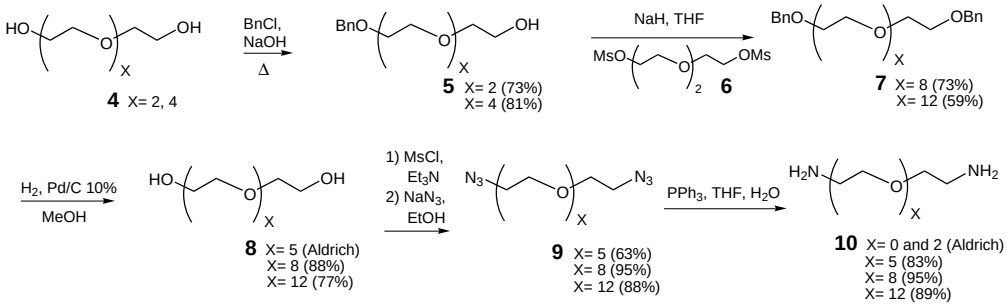
Many potent, non-natural compounds were identified including the 2-naphthyl morpholino amide **2** shown.

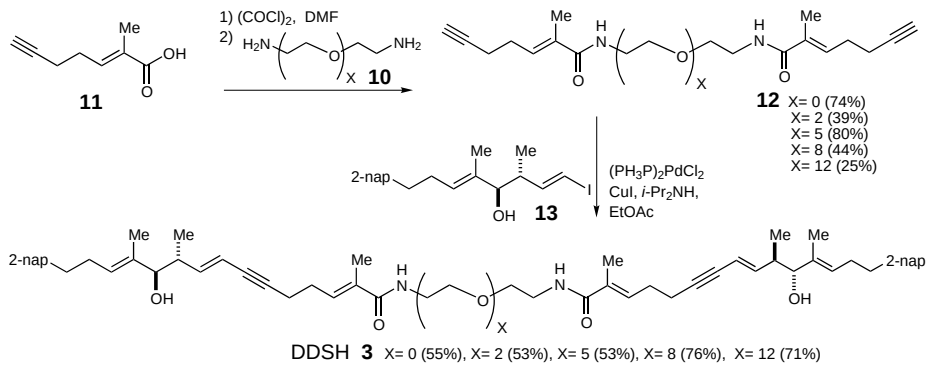
We now report initial efforts to further increase MDR reversal potency by creating bivalent homodimeric polyenes based on stipiamide linked with ethylene glycol spacers **3** (Scheme 1). Photoaffinity labeling has been used to identify two non-identical sites most likely formed by the TM5-6 and TM11-12 regions of Pgp.[10,11] Close proximity of the cytosolic ATP-binding sites supports the notion that ATP hydrolysis induces conformational changes that are conveyed to the TM regions leading to drug displacement and efflux. Conformational changes in the substrate-binding domain following ATP hydrolysis have recently been determined.[11] Additionally, a low resolution (25 Å) electron diffraction structure for Pgp shows the protein to be doughnut shaped with an approximate 50 Å pore opening.[12] The proximity of the TM5-6 and TM11-12 helices and the identity of residues involved in substrate binding remain unknown. Success of the polyvalency approach requires a proper linker length in order to span the binding sites. A new approach involving displacement of a dimesylate was used to access PEG linkers that now provide for a distance range from 3 to 50 Å. Double Sonogashira couplings, using a recently developed set of conditions, were also used to generate the homodimers **3**. Pgp inhibition and binding was determined using ATPase and competitive displacement of photoaffinity analog binding assays.

The key to the synthesis of the homodimers was the development of the general, efficient route to the polyethylene glycols that are not commercially available.[13] Unlike previous reports that have employed chlorides[14] and tosylates[15] as leaving groups, we have found that mesylates function as the most efficient coupling partners. Diols **4** ($x=2$, 4) were first monobenzylated using 50% aqueous hydroxide at reflux to give the protected alcohols **5** (Scheme 2). The key step that allowed for reproducible glycol production employed sodium hydride with alcohol **5** followed by dropwise addition of dimesylate **6**[16] and reflux to provide **7**.[17] Hydrogenation (200 psi) with palladium on carbon (10%) in methanol was used to give diols **8**. Dimesylate formation and sodium azide displacement provided diazides **9**. Commercially available hexaethylene glycol **8** ($x=5$) was used for conversion to **9** ($x=5$). Reduction with triphenylphosphine was used to generate the key diamines **10**. Completing the series are **10** ($x=0$ and 2) which were purchased.

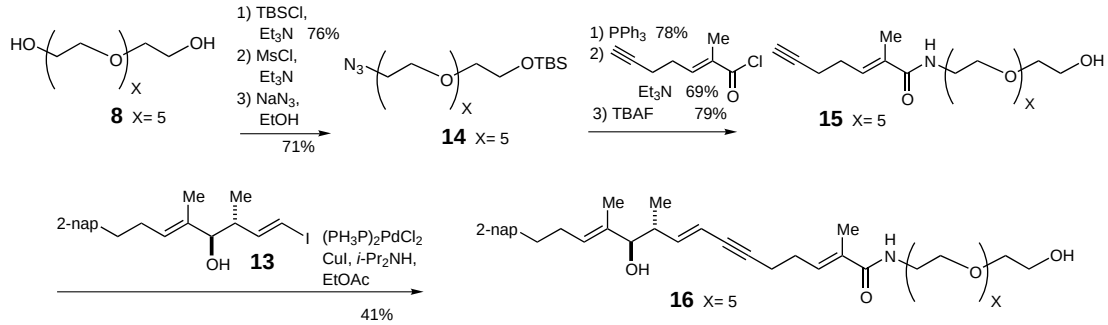
Treatment of the diamines **10** (1.2 equiv.) at –40°C with the known acid (1 equiv.) generated from carboxylic acid **11**[9b] led to the formation of the diamides **12** (Scheme 3). Difficulties with low yields in some cases ($x=8$, 12) may be attributed to the very hydroscopic nature of the diamine glycols. Double Sonogashira couplings were then performed with the bis-amide alkynes **12** reacted with the previously reported vinyl iodide **13**[9b] (3 equiv.) under palladium chloride, copper iodide catalysis. These optimized conditions include diisopropylamine together with the moderately polar ethyl acetate as solvent and a starting temperature of –20°C followed by immediate warming to room tempera-



Scheme 2.



Scheme 3.



Scheme 4.

ture.[8b] The yields for this double process were moderate (50%) for the shorted linked amides **3** ($x=0$, 2, 5) and good (70%) for the longer products **3** ($x=8$, 12).[18] These conditions were developed previously for the synthesis of the stipiamide variants and were also used in the solution-phase library.[9] More polar solvents DMF and NMP, and less polar solvents THF and benzene, gave much lower yields at near 1:1 stoichiometry. Beginning the reaction at rt also lowered product yields. Previous Sonogashira coupling examples have shown that amides containing acetylenes are very poor coupling partners.[19] Recently this new Sonogashira protocol has been employed as the key step in the synthesis of callipeltoside.[20]

A monomeric ethylene glycol-amide reversal agent was also made for control purposes in the MDR and Pgp assays (Scheme 4). Monosilyl ether protection, mesylation and azide displacement were uneventful with hexaethylene glycol **8** ($x=5$) to give **14**. Phosphine reduction, acid chloride coupling, and TBAF deportation generated amide **15**. Coupling with **13** then gave the desired control substrate **16**.

Preliminary activity of the dimers along with the monomeric control agents **2** and **16** were investigated using Pgp ATPase activity and photoaffinity displacement assays (Table 1). Stimulation of Pgp ATPase activity was determined along with displacement of the prazosin photoaffinity label, iodoarylazidoprazosin (^{125}IAAP) following the known protocol.[21] The dimer effect on both ATPase activity and IAAP binding to Pgp appear to be significant. ATPase stimulation reaches a maximum at low concentration (1 μM) and steadily drops off as the concentration is increased (not shown). In contrast **3** ($x=5$) slowly achieves maximum stimulation up to 50 μM and this level is maintained as the concentration increases. The other dimers are similar to other known monomeric MDR reversal compounds where maximum stimulation is achieved and rapidly drops off as concentration increases. Most remarkable is the dimer **3** ($x=12$) where near complete ATPase inhibition was found. Potent interaction with Pgp is also seen in the photoaffinity displacement results where **3** ($x=12$) binds most tightly at 1.7 μM. The other dimers were less effective at ^{125}IAAP displacement with K_is in the 10–20 μM range. These results clearly indicate an interaction with Pgp for the dimers that varies greatly with tether length. As the tether length increases, ATPase activity goes from being stimulatory to strongly inhibitory. Control **16**, containing only a PEG tail also inhibits ATPase activity, but not to the same degree as **3** ($x=12$). Also the K_i of **16** is less potent at 7.6 μM. This suggests that there may be potential to reverse MDR using a dimer strategy. Assays using drug resistant MCF7adrR and NIH-3T3 cells, to be reported elsewhere, will be used to establish reversal concentrations for the dimers along with more detailed Pgp binding studies to further correlate tether length. These methods and results will be used to develop other polyvalent agents to achieve stronger interaction with Pgp and more effective reversal of MDR.

Table 1. ATPase activity, and inhibition of IAAP binding by homodimers **3** and control amide **16**

Cmpd	x	d (Å)[a]	ATPase activity[b]	K_i[c]
2			3.20	2.7
16			0.34	7.6
3	0	3	3.30	19.1
3	2	11	1.90	9.7
3	5	22	1.90	15.1
3	8	35	0.70	10.1
3	12	50	0.00	1.7

[a] Approximate distance between amide nitrogens.
[b] ATP-hydrolysis, fraction of control, values >1 represent stimulation, values <1 inhibition.
[c] K_i (mM) inhibition of ^{125}IAAP binding to Pgp.

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16. All intermediates were characterized by ^{1}H and ^{13}C NMR, and HRMS. Dimesylate **6** was produced from triethylene glycol, mesyl chloride (2.1 equiv.), and triethylamide (2.4 equiv.) in methylene chloride (0.2 M). Sodium bicarbonate work-up and silica gel chromatography were used to isolate the product (97%).
17. Preparation of **7** ($x=8$): 95% NaH (0.526 g, 20 mmol) was added to 20 mL THF followed by tri(ethylene glycol) benzylether **5** ($x=2$) (5.0 g, 20 mmol in 12 mL THF) and the mixture was stirred for 1 h. Tri(ethylene glycol) dimesylate **6** (3.18 g, 10 mmol in 7.5 mL THF) was added dropwise over 30 min and the solution was refluxed for 20 h. Upon cooling, the solution was diluted with $NaHCO_3$ (satd) (250 mL) and extracted with CH_2Cl_2. The combined organic layers were dried ($MgSO_4$), filtered, and concentrated to give a yellow oil. Flash chromatography (100% EtOAc to 10% MeOH/EtOAc) gave the desired product as an oil: 4.51 g (73% yield). $R_f=0.34$ (10% MeOH/EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34–7.26 (m, 10H), 4.56 (s, 4H), 3.69–3.61 (m, 36H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.4, 128.5, 127.9, 127.8, 73.4, 70.8, 70.7, 69.6; HRMS FAB (M+Na) calcd for $C_{32}H_{50}O_{10}Na$ 617.3287, found 617.3272.
Preparation of **7** ($x=12$): Same conditions as above except that **5** ($x=4$) was used (59% yield). $R_f=0.21$ (10% MeOH/EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35–7.27 (m, 10H), 4.57 (s, 4H), 3.69–3.60 (m, 52H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.4, 128.5, 127.9, 127.8, 73.4, 70.8, 70.7, 69.6; HRMS FAB (M+Na) calcd for $C_{40}H_{66}O_{14}Na$ 793.4362, found 793.4374.
18. Preparation of **3**: To a flask containing dialkynylamide **12** ($x=0$) (11.5 mg, 0.038 mmol) was added **13** (47 mg, 0.115 mmol) and EtOAc (3.9 mL). The reaction was cooled to −20°C and $(Ph_3P)_2PdCl_2$ (4.1 mg, 0.006 mmol), CuI (3.8 mg, 0.02 mmol), and i-Pr_2NH (0.58 mL, 0.2 M) were added. The flask was removed from the cold bath and allowed to warm to rt. The solution was filtered through a silica gel plug using 30% MeOH/EtOAc and concentrated. Purification via radial chromatography (100% EtOAc) gave 18.0 mg (55% yield) of **3** ($x=0$) as an oil. $R_f=0.5$ (2% MeOH/EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.81–7.67 (m, 6H), 7.60 (s, 2H), 7.47–7.40 (m, 4H), 7.34 (dd, $J=8.4$, 1.5 Hz, 2H), 6.61 (bs, 2H), 6.38 (t, $J=5.7$ Hz, 2H), 5.98 (dd, $J=15.9$, 8.4 Hz, 2H), 5.52 (dd, $J=15.9$, 0.9 Hz, 2H), 5.45 (t, $J=6.6$ Hz, 2H), 3.63 (d, $J=8.7$ Hz, 2H), 3.48–3.46 (m, 4H), 2.87–2.80 (m, 4H), 2.49–2.29 (m, 14H), 1.85 (s, 2H), 1.84 (s, 6H), 1.56 (s, 6H), 0.77 (d, $J=6.6$ Hz, 6H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 170.6, 145.9, 139.6, 135.9, 134.6, 133.7, 132.1, 131.9, 128.3, 128.0, 127.8, 127.6, 127.5, 126.6, 126.1, 125.3, 111.3, 88.4, 81.8, 79.9, 41.5, 40.7, 35.9, 29.6, 28.0, 19.1, 16.9, 13.1, 11.2; HRMS FAB (M+Na) calcd for $C_{58}H_{68}O_4N_2Na$ 879.5071, found 879.5065. Compounds **3** ($x=2$, 5, 8, 12) were formed followed the same procedure with yields indicated (Scheme 3).
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