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Bombesin marine toxin conjugates inhibit the growth of lung cancer cells

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Abstract

Hemiasterlin (Hem) and dolastatin (Dol) are marine natural products which are cytotoxic for cancer cells. Hem, a tripeptide, and Dol, a hexapeptide, were conjugated with linkers (L) to the universal BB agonist DPhe-Gln-Trp-Ala-Val-βAla-His-Phe-Nle-NH_2(BA1) and the effects of the Hem-BB and Dol-BB conjugates investigated on NCI-H1299 lung cancer cells. Hem-LA-BA1 and Hem-LB-BA1 inhibited specific (^{125}I-Tyr^4) BB binding to NCI-H1299 cells, which have BB_2 receptors (R), with IC_{50} values of 15 and 25 nM, respectively. Addition of Hem-LA-BA1 and Hem-LB-BA1 to Fura-2 AM loaded cells containing BB_2R, caused elevated cytosolic Ca^{2+}. In a growth assay, Hem-LA-BA1 and Hem-LB-BA1 inhibited the proliferation of NCI-H1299 cells. Dol-succinamide (Dols)-LD-BA1 and Dols-LE-BA1 bound with high affinity to NCI-H1299 cells and elevated cytosolic Ca^{2+}, but did not inhibit the proliferation of NCI-H1299 cells. Also, Hem-LA-BA1 inhibited ^{125}I-DTyr-Gln-Trp-Ala-Val-βAla-His-Phe-Nle-NH_2 (BA2) binding to Balb/3T3 cells transfected with BB_1R or BB_2R as well as with BRS-3 with IC_{50} values of 130, 8, and 540 nM, respectively. These results show that Hem-BB conjugates are cytotoxic for cancer cells containing BB_2R.
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Keywords: Bombesin; Dolastatin; Hemiasterlin; Lung cancer

Introduction

Marine natural products have been used for the treatment of diseases such as acquired immuno-deficiency disease (Rawat et al., 2006). Marine natural products have been isolated from sponges, moluscs, bryozoans as well as tunicates and range in structure from linear peptides to complex macrocyclic polyethers. Two such peptides include hemiasterlin (Hem), which is isolated from the South African sponge (Talpir et al., 1994) and dolastatin (Dol), which is isolated from the Indian Ocean mollusk (Pettit et al, 1989). Hem and its analog HTI-286 inhibit the proliferation of tumor cells during mitosis by interaction with tubulin (Krisnamurthy et al. 2003). Similarly, Dol and its analog TZT-1027 interact with tubulin and are cytotoxic for cancer cells (Kobayashi et al., 1997). Dol induces G2/M cell cycle arrest and causes apoptosis of cancer cells via mitochondrial and Fas mediated pathways (Sato et al., 2007). Both Hem and Dol are being evaluated in phase I and phase II clinical trials using cancer patients. In some clinical trials, however, Dol 10 had toxic side effects including peripheral neuropathy (Pitot et al., 1999). This toxicity might be decreased if marine toxins can be delivered using molecular targets associated with the cancer cells.

Bombesin/gastrin releasing peptide (BB/GRP) is an autocrine growth factor for some small cell lung cancer cells (SCLC) (Moody et al., 1981; Wood et al., 1981; Cuttitta et al., 1985). Lung cancer, which kills over 160,000 U.S. citizens annually, is traditionally treated with chemo- and/or radiation therapy (Sekido et al., 2005), however, relapse frequently occurs and the median survival time is under 1 year. BB binds with high affinity to a G-protein coupled receptor, the BB_2 receptor (Battey et al., 1991; Spindel et al., 1990). Two other receptors of this family include the neuromedin B receptor, the BB_1 receptor (Wada et al., 1991), and BB receptor subtype-3 (BRS-3) (Fathi et al., 1993). BB receptors are present in over 50% of the tumors

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derived from lung cancer, breast cancer, prostate cancer, head/neck squamous cell cancer, glioblastoma and neuroblastoma (Jensen and Moody, 2006). These BB receptors may serve as molecular targets to develop new drugs for treatment of lung cancer patients.

Prodrugs decrease drug associated side effects and enhance tumor cytotoxicity (deGroot et al., 2001). One group of prodrugs uses ligands for peptide growth factor receptors that are overexpressed in cancer cells. BB, luteinizing hormone-releasing hormone, somatostatin and vasoactive intestinal peptide analogs have been coupled to cytotoxic agents and have potent antitumor effects (Buchholz et al., 2006; Nagy and Schally, 2005). These peptide-chemotherapeutic conjugates are delivered to the cancer cell by receptor-mediated endocytosis and the cytotoxic agent released into the cancer cell after enzymatic catalysis. Previously, we developed BB–camptothecin (CPT) conjugates which bound with high affinity to BB_1R and BB_2R as well as BRS-3 (Moody et al., 2004). BB receptors are present in many lung cancer tumors (Reubi et al., 2002). The BB–CPT conjugates were readily internalized by lung cancer cells containing BB_1R, BB_2R as well as BRS-3 (Moody et al., 2006). The BB–CPT conjugates were metabolized by P450 enzymes releasing the cytotoxic CPT, which is a topoisomerase 1 inhibitor, leading to cytotoxicity of the lung cancer cells (Patterson et al., 1999).

In this communication Hem or Dol were coupled to universal agonist DPhe-Gln-Trp-Ala-Val-βAla-His-Phe-Nle-NH_2 (BA1), which binds with high affinity to all classes of mammalian BB receptors (Mantey et al., 1997; Pradhan et al., 1998), and their effects tested in NCI-H1299 lung cancer cells. Hem-BB and Dol-BB conjugates bound with high affinity to NCI-H1299 lung cancer cells which have BB_2R (IC_{50} values 15–150 nM). Also, the Hem-BB and Dol-BB conjugates bound with high affinity to Balb 3T3 cells transfected with BB_2R and caused elevation of cytosolic Ca^{2+}. The Hem-BB, but not Dol-BB conjugates, inhibited the growth of NCI-H1299 cells. These results indicate that Hem-BB conjugates are cytotoxic for lung cancer cells which have BB receptors.

Materials and methods

Cell culture

NCI-H1299 cells were cultured in RPMI-1640 medium containing 10% heat inactivated fetal bovine serum (FBS). NCI-H1299 cells were split weekly 1/20 with trypsin-EDTA. Balb 3T3 cells stably expressing human BRS-3, BB_1R or BB_2R (Benya et al., 1995) were grown in DMEM supplemented with 300 mg/l G418 sulfate. Cells were mycoplasm free and were

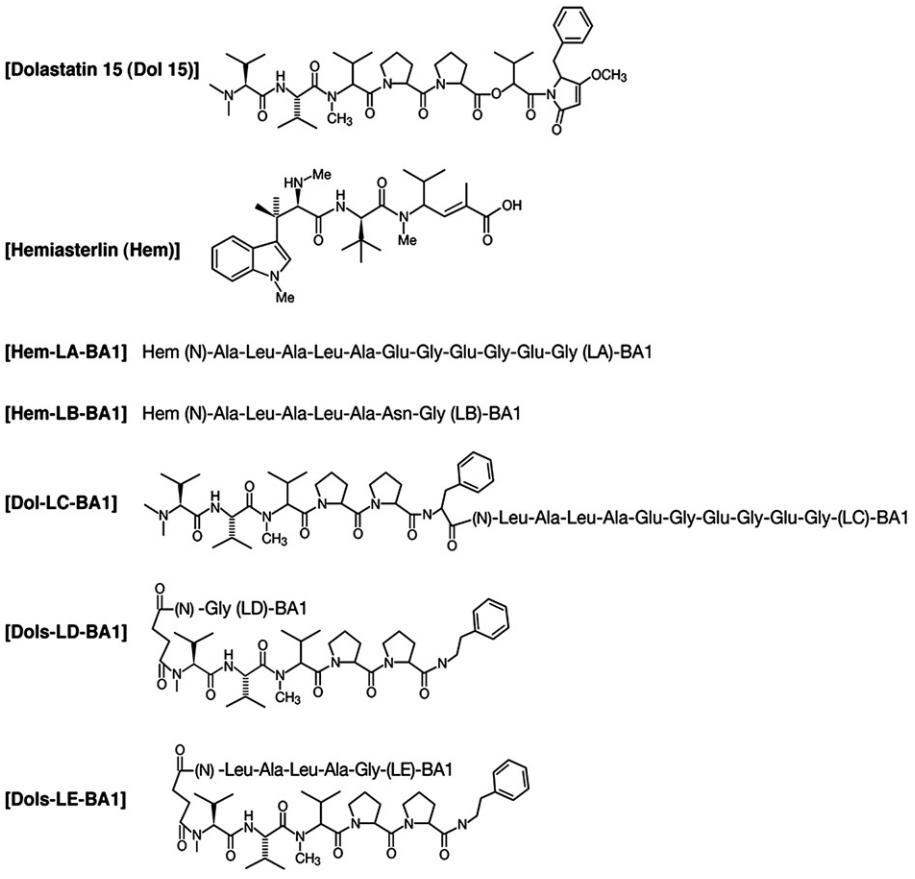

Fig. 1. Structures of peptides. The chemical structures of hemiasterlin, dolastatin-15, Dol and Dols are shown. The peptide structures of LA, LB, LC, LD and LE are shown. (N) represents the N-terminal.

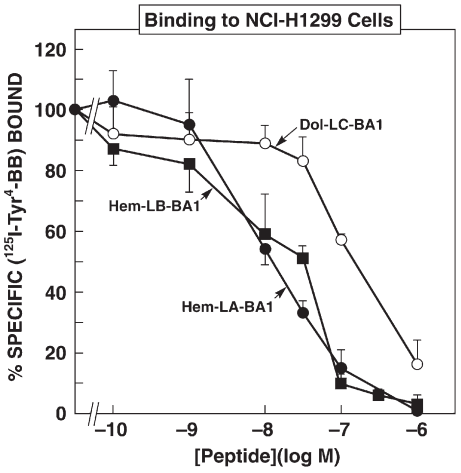

Fig. 2. Binding to NCI-H1299 cells. The ability of varying concentrations of Hem-LA-BA1 (●), Hem-LB-BA1 (■) and Dol-LC-BA1 (○) to inhibit specific (^{125}I-Tyr^4) BB binding to NCI-H1299 cells was investigated. The mean value±S.D. of 4 determinations is indicated. This experiment is representative of 3 others.

used when they were in exponential growth phase after incubation at 37 °C in 5% CO_2 and 95% air.

Receptor binding

(^{125}I-Tyr^4)BB and ^{125}I-DTyr-Gln-Trp-Ala-Val-βAla-His-Phe-Nle-NH_2 (^{125}I-BA2) with specific activities of 2200 Ci/mmol were prepared as described (Moody et al., 2004). The radiolabeled peptides were separated using a C18 Sep-Pak (Waters Associates, Milford, MA) and further purified by reverse-phase high pressure liquid chromatography on a C18 column. The fractions with the highest radioactivity were pooled, neutralized with 0.2 N Tris buffer, pH 9.5, and stored with 0.5% (w/v) bovine serum albumin (BSA) at −20 °C. Binding was performed using human lung cancer NCI-H1299 cells or Balb 3T3 cells stably expressing BB_2R (0.3×10^6/cell), BB_1R (0.03×10^6/cell) or BRS-3 (0.3×10^6/cell). A monolayer of cells in 24 well plates was incubated with 0.05 nM ^{125}I-BA2 in PBS containing 0.25% BSA and 250 μg/ml bacitracin at 37 °C for 20 min or 25 °C for 60 min. The nonsaturable binding was the amount of radioactivity associated with cells in incubations containing 0.05 nM radioligand (2200 Ci/mmol) and 1000 nM unlabeled BA2. Nonsaturable binding was <10% of total binding in all the experiments. Inhibition constants (K_i) were determined using a least-square, curve-fitting program (KaleidaGraph) and the Cheng–Prusoff equation (Cheng and Prusoff, 1973).

Peptide synthesis

Linker(L)-BA1 peptides were synthesized using solid phase techniques. Hem and Dol peptides were synthesized with a Fmoc N-terminal and free carboxyl group. The C-terminal of Hem and Dol were then coupled to the N-terminal of L-BA1 using carbodiimide. The N-terminal of the products was deprotected and Hem-LA-BA1, Hem-LB-BA1 and Dol-LC-BA1 cleaved from the resin (Fig. 1). In addition, solid phase L-BA1 peptides were coupled to Dol in which the N-terminal was succinnylated (Dols). Dols-LD-BA1 and Dols-LE-BA1 were cleaved from the resin (Fig. 1). The molecular weights of Hem-LA-BA1, Hem-LB-BA1, Dol-LC-BA1, Dols-LD-BA1 and Dols-LE-BA1 were 2570, 2183, 2725, 1894 and 2320 Da, respectively. Peptide purity was greater than 98% based on HPLC, amino acid and mass spectroscopy analysis.

Cytosolic calcium $[Ca^{2+}]_i$

The ability of the Dol-BB and Hem-BB conjugates to alter cytosolic $[Ca^{2+}]_i$ was investigated as described previously (Moody et al., 1987). NCI-H1299 cells and Balb/3T3 cells containing BB_2R were harvested (2.5×10^6/ml) and incubated with 5 μM Fura 2 AM at 37 °C for 30 min. The cells, which contained loaded Fura 2, were centrifuged at 1500 ×g for 10 min and resuspended at the same concentration in SIT medium (RPMI-1640 containing 30 nM sodium selenite, 5 μg/ml bovine insulin and 10 μg/ml transferrin). The cells were placed in a cuvette and analyzed using a Delta PTI Scan 1 spectrofluorometer (Photon Technology International, South Brunswick, NJ) equipped with a magnetic stirring mechanism and temperature-regulated cuvette holder. The fluorescence intensity was continuously monitored at dual excitation wavelengths of 340 nm and 380 nm, using an emission wavelength of 510 nm prior to and after the addition of BB-like peptides.

Table 1
Binding to cells containing BB receptors

	NCI-H1299	Balb/3T3 with BB_2R	Balb/3T3 with BB_1R	Balb/3T3 with BRS-3
Peptide	IC_{50}, nM			
BA2	6±1	0.6±0.1	12±1	14±2
Dol-LC-BA1	150±18	21±2	270±30	370±60
Dols-LD-BA1	20±2	4±1	190±30	380±40
Dols-LE-BA1	15±1	18±2	1120±90	1420±130
Hem-LA-BA1	15±2	8±1	130±10	540±50
Hem-LB-BA1	25±3	5±1	69±9	160±30

The mean value±S.D. of 3 determinations each repeated in quadruplicate is indicated. (^{125}I-Tyr^4)BB was bound to NCI-H1299 cells for 20 min at 37 °C. ^{125}I-BA2 was bound to Balb/3T3 cells for 60 min at 25 °C. The structures of the peptides are shown below:
BA1 DPhe-Gln-Trp-Ala-Val-βAla-His-Phe-Nle-NH_2.
BA2 DTyr-Gln-Trp-Ala-Val-βAla-His-Phe-Nle-NH_2.

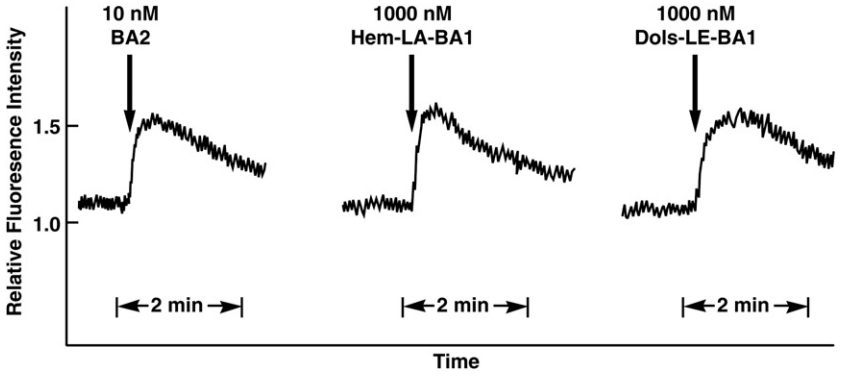

Fig. 3. Cytosolic calcium. The ability of (left) 10 nM BA2, (middle) 1000 nM Hem-LA-BA1 or (right) 1000 nM Dols-LE-BA1 to elevate Ca^{2+} in Fura-2AM loaded Balb 3T3 cells containing BB_2-R is indicated. This experiment is representative of 2 others.

Proliferation assays

Growth studies *in vitro* were conducted using the [3-(4,5 dimethylthiazol-2-yl)-2.5-diphenyl-2H-tetrazolium bromide] (MTT) (Sigma Chemical Co., St. Louis, MO) colorimetric assays. NCI-H1299 cells were placed in 100 µl of SIT medium and various concentrations of Hem-BB or Dol-BB conjugates added. After 4 days, 15 µl (1 mg/ml) of MTT was added and after another 4 h, 150 µl of DMSO was added. After 16 h, the optical density at 570 nm was determined using an ELISA reader.

Results

Hem-BB and Dol-BB conjugates bind with high affinity to cells containing BB_2R

Fig. 2 shows that specific (^{125}I-Tyr^4)BB binding to NCI-H1299 cells was inhibited in a dose-dependent manner by Hem-BB and Dol-BB conjugates. Hem-LA-BA1, Hem-LB-BA1 and Dol-LC-BA1 half maximally inhibited specific (^{125}I-Tyr^4)BB binding with IC_{50} values of 15, 25 and 150 nM, respectively. These results indicate that when the C-terminal of Hem was coupled to the N-terminal of BA1, the resulting conjugate bound with high affinity to lung cancer cells using either linker LA or LB. Table 1 shows that specific (^{125}I-Tyr^4)BB binding to NCI-H1299 cells was inhibited with high affinity by BA2, Dol-LC-BA1, Dols-LD-BA1 and Dols-LE-BA1 with IC_{50} values of 6, 150, 20 and 15 nM, respectively. These results indicate that when the C-terminal of Dol or Dols was linked to the N-terminal of BA1, the resulting conjugates bound with high affinity to cells containing BB_2R. The ability of Hem-BB and Dol-BB conjugates to bind to Balb 3T3 cells stably transfected with human BB receptors was investigated. Table 1 shows that using Balb 3T3 cells transfected with BB_2R, Hem-LA-BA1, Hem-LB-BA1 and Dol-LC-BA1 inhibited specific ^{125}I-BA2 binding with IC_{50} values of 8, 5 and 21 nM, respectively. These results indicate that Hem-BB and Dol-BB conjugates bound with similar affinity to Balb 3T3 cells transfected with BB_2R and human lung cancer cells. Hem-LA-BA1, Hem-LB-BA1 and Dol-LC-BA1 inhibited specific ^{125}I-BA2 binding to Balb 3T3 cells transfected with human BB_1R with IC_{50} values of 130, 69 and 270 nM, respectively. Hem-LA-BA1, Hem-LB-BA1 and Dol-LC-BA1 inhibited specific ^{125}I-BA2 binding to Balb/3T3 cells transfected with human BRS-3 with IC_{50} values of 540, 160 and 370 nM, respectively. These results indicate that Hem-BB and Dol-BB conjugates bind with 1–2 orders of magnitude lower affinity the Balb 3T3 cells transfected with BB_1R or BRS-3 relative to BB_2R.

Hem-BB and Dol-BB conjugates elevate cytosolic Ca^{2+}

The effects of Hem-BB and Dol-BB conjugates were investigated on Fura-2/AM loaded NCI-H1299 or Balb 3T3 cells containing BB_2R. Fig. 3 shows that BA2, Hem-LA-BA1 and Dols-LE-BA1 caused elevated cytosolic Ca^{2+} using BB_2R containing cells. Using 1000 nM Hem-LA1-BA1 or Dols-LE-BAI, the fluorescence intensity strongly increased within seconds after addition to cells containing BB_2R. The response was maximal after 0.2 min then slowly declined over a 2 min period. Similarly, 10 nM BA2 caused a strong Ca^{2+} response in 3T3

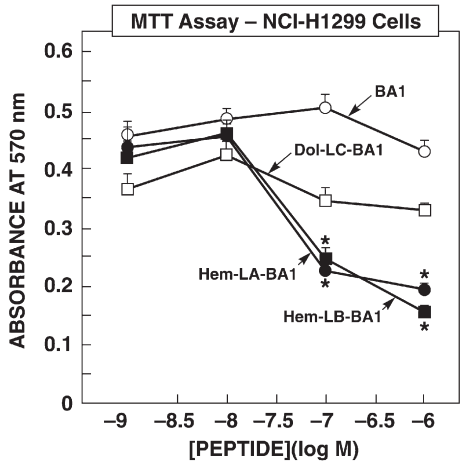

Fig. 4. MTT assay. The ability of varying concentrations of Hem-LA-BA1 (●), Hem-LB-BA1 (■), Dol-LC-BA1 (□) and BA1 (○) to inhibit NCI-H1299 growth was investigated. The mean value ± S.D. of 8 determinations is indicated; $p<0.05$, * using Student's *t*-test. This experiment is representative of 3 others.

cells containing BB_2R or NCI-H1299 cells as did 1000 nM Hem-LB-BA1, 1000 nM Dol-LC-BA1 and 1000 nM Dols-LD-BA1 (data not shown). The results indicate that Hem-BB and Dol-BB conjugates function as BB_2R agonists.

Hem-BB but not Dol-BB conjugates are cytotoxic for NCI-H1299 cells

The ability of Hem-BB and Dol-BB conjugates to alter the proliferation of lung cancer cells was investigated. Fig. 4 shows that Hem-BB but not Dol-BB conjugates or BA1 inhibited the proliferation of NCI-H1299 cells in a concentration dependent manner. Hem-LA-BA1 or Hem-LB-BA1 did not alter NCI-H1299 proliferation at 1 or 10 nM but significantly inhibited proliferation at 100 or 1000 nM. In contrast, Dol-LC-BA1 or BA1 did not significantly alter NCI-H1299 proliferation at any dose tested. Similarly, Dols-LD-BA1 or Dols-LE-BA1 had little effect on NCI-H1299 proliferation (data not shown). These results suggest that Hem-BB conjugates but not Dol-BB conjugates are cytotoxic for NCI-H1299 cells.

Discussion

BB receptors are present in numerous human cancers (Reubi et al., 2002). Using *in vitro* autoradiographic techniques, the BB_2R is present in 100% of prostate cancers, 72% of breast cancers and 33% of SCLC. The BB_1R is present in 46% of intestinal carcinoids. BRS-3 is present in 35% of bronchial carcinoids. In addition, BB_1R and BB_2R mRNA was detected in 100% and 64% respectively of the NSCLC cell lines examined (Siegfried et al., 1999). Agonists, but not antagonists, for each of the three BB receptor subclasses, are rapidly internalized (Slice et al., 1998). Radiolabeled synthetic analogues of BB-related peptides demonstrate these can be used to target tumors/tissues containing BB receptors and these analogues are internalized in high amounts by these tumors (Hoffman et al., 2003). Recently, an ^{111}In derivative of BB was utilized to image prostate cancer tumors (deVisser et al., 2007). It remains to be determined if BB-like peptides can be utilized for early detection and/or treatment of cancer. Peptide hormones which are coupled to cytotoxic agents have been shown to be effective at delivering targeted drugs (Breeman et al., 2007). We have shown that BB–CPT conjugates are cytotoxic for lung cancer cells *in vitro* and *in vivo* (Moody et al., 2006). CPT can be conjugated with the universal BB agonist BA2 with retention of high affinity binding and biological activity (Moody et al., 2004). The BB–CPT conjugate bound with higher affinity to BB_1R, BB_2R or BRS-3 containing cells with higher affinity than did BA2, possibly because CPT increased hydrophobic interactions with BB receptors. The BB–CPT conjugate was internalized by and cytotoxic for cells containing BB_1 or BB_2 receptors as well as BRS-3. The CPT–BB conjugate inhibited NCI-H1299 xenograft growth in nude mice (Moody et al., 2006). Furthermore the BB–CPT conjugate inhibited the growth of 13 additional human cancer cell lines tested as well as 3 tumors. The results demonstrated that BB–CPT conjugates can be utilized in tumors which have BB_2R, BB_1R and/or BRS-3 and that BA2 is an excellent ligand to couple cytotoxic agents to allow BB receptor targeted delivery.

In this communication, BA1 was coupled to the marine natural products Hem or Dol. Hem-LA-BA1 bound with high affinity to cells containing BB_2 receptors (IC_{50}=8 nM) and lower affinity to cells containing BB_1R or BRS-3 (IC_{50}=130 and 540 nM, respectively). Hem-LB-BA1, which has a neutral linker of Ala-Leu-Ala-Leu-Ala, bound with similar affinity to NCI-H1299 cells as did Hem-LA-BA1, which has an acidic linker of Ala-Leu-Ala-Leu-Glu-Gly-Glu-Gly-Glu-Gly. These results suggest Hem-BB conjugates bind with high affinity to cells containing BB_2R regardless of the linker used. In addition Dols-LD-BA1 bound with high affinity to cells containing BB_2R (IC_{50}=4 nM) and lower affinity to cells containing BB_1R or BRS-3 (IC_{50}=190 and 380 nM, respectively). Hem is a tripeptide composed of trimethyltryptophan, tert-leucine and N-methylhomovinylvaline (Fig. 1). Dol is a hexapeptide which has 5 of the same 6 amino acids as does Dol-15. Dol and Dol-15 have the same N-terminal dolavaline which is essential for interaction with cancer cell tubulin and the resulting cytotoxicity (Zask et al., 2005). Dol-15 interacts with tubulin binding sites similar to those of the traditional chemotherapeutic agents vincristine, vinblastin or vinorelbine (Bai et al., 1999).

The Hem-BB and Dol-BB conjugates functioned as agonists. They increased the cytosolic Ca^{2+} in cells containing BB_2R. Previously, we showed that BB–CPT conjugates functioned as agonists in that they increased phosphatidylinositol turnover in cells containing BB_2R, BB_1R or BRS-3 (Moody et al., 2004). Also, the BB–CPT conjugate, which contains a Tyr, was iodinated and found to be internalized by NCI-H1299 cells. Because the Hem-BB or Dol-BB conjugates lack Tyr, it was not possible to iodinate them and determine if they were internalized. Previously, we found that all BB agonists examined which bound with high affinity to the BB_2R, were internalized at 37 °C but not 4 °C (Benya et al., 1995; Mantey et al., 1997; Moody et al., 2004; Moody et al., 2006).

The Hem-BB but not Dol-BB conjugates were cytotoxic for NCI-H1299 cells. The IC_{50} values for Hem-LA-BA1 and Hem-LB-BA1 were 50 and 60 nM, respectively. In contrast, the Dol-BB conjugates or BA1 had no effect on NCI-H1299 cellular toxicity. It is possible that the Dol used, which had a C-terminal Phe, is not as biologically active as is Dol-15, which has a C-terminal 2-hydroxy-isovaline-dola-pyrrolidone. Dol-15 causes apoptosis of A549 lung cancer cells by causing a G2-M cell cycle arrest (Catassi et al., 2006). Dol-15 caused BAD dissociation from 14-3-3 followed by association with BCL-XL, cytochrome *c* release, caspase-3 activation and cleavage of vimentin. The Hem-BB conjugates (Hem interacts with tubulin) are more cytotoxic for NCI-H1299 cells than BB–CPT conjugates (CPT inhibits topoisomerase 1) which had an IC_{50} of 190 nM in the MTT assay.

The Hem and Dol conjugates are linked to BA1 through amide bonds. After undergoing receptor-mediated endocytosis, Hem can be released intracellularly from BA1 by endopeptidase metabolism of the linker. In contrast, the BB–CPT conjugates are linked via a carbamate bond and are metabolized by P450 enzymes. These results suggest that the cell surface BB_2R

receptor can serve as a molecular target to deliver marine toxins into cancer cells. In contrast, normal cells which lack the BB_2R should not be affected by the Hem-BB conjugates. Because there is no receptor for Hem or Dol, the marine toxins may diffuse into plasma membranes and kill both normal and cancer cells, leading to toxic side effects. The Hem-BB conjugates will be cytotoxic for cancer cells enriched in BBR, endopeptidases and tubulin, and should have little toxicity for cells lacking BB receptors. Hem-LA-BA1 will have greater selectivity for cancer cells containing BB_2R than will Hem-LB-BA1. While it will be easier to synthesize Hem-LB-BA1 than Hem-LA-BA1 due to the smaller linker size, Hem-LB-BA1 will likely be cytotoxic for cells containing BB_1R, BB_2R and/or BRS-3. It remains to be determined if Hem will be preferentially released by cancer cell enzymes using Hem-LA-BA1 or Hem-LB-BA1.

Conclusions

Hem-LA-BA1 binds with high affinity cells containing BB_2R. Because the Hem-LA-BA1 functions as an agonist, it may be internalized by lung cancer cells. It remains to be determined if Hem-LA-BA1 may be a useful conjugate for treatment of cancer patients whose tumors are enriched in BB_2R.

Acknowledgments

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