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Selective Tumor Cell Targeting by the Disaccharide Moiety of Bleomycin

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Abstract

In a recent study, the well documented tumor targeting properties of the antitumor agent bleomycin (BLM) were studied in cell culture using microbubbles that had been derivatized with multiple copies of BLM. It was shown that BLM selectively targeted MCF-7 human breast carcinoma cells, but not the “normal” breast cell line MCF-10A. Further, the BLM analogue deglycobleomycin, which lacks the disaccharide moiety of BLM, was found not to target either cell line, indicating that the BLM disaccharide moiety is necessary for tumor selectivity. Not resolved in the earlier study was the issue of whether the BLM disaccharide moiety alone is sufficient for tumor cell targeting, as well as the possible cellular uptake of the disaccharide. In the present study, we have conjugated BLM, deglycoBLM and BLM disaccharide to the cyanine dye Cy5[®]. It was found that the BLM and BLM disaccharide conjugates, but not the deglycoBLM conjugate, bound selectively to MCF-7 cells, and were internalized. The same was also true for the prostate cancer cell line DU-145 (but not for normal PZ-HPV-7 prostate cells) and for the pancreas cancer cell line BxPC-3 (but not for normal SVR A221a pancreas cells). The targeting efficiency of the disaccharide was only slightly less than that of BLM in MCF-7 and DU-145 cells, and comparable to BLM in BxPC-3 cells. These results establish that the BLM disaccharide is both necessary and sufficient for tumor cell targeting, a finding with obvious implications for the design of novel tumor imaging and therapeutic agents.

The bleomycins (BLM A₅, Fig. 1) are a family of glycopeptide-derived antitumor antibiotics used clinically for the treatment of squamous cell carcinomas and malignant lymphomas.^{1,2} Their antitumor activity is thought to result from selective oxidative cleavage of 5'-GC-3' and 5'-GT-3' sequences in DNA, and possibly also oxidative degradation of RNA.³ In addition to its anti-tumor activity, bleomycin has been recognized for its ability to target tumors and has been demonstrated to act as a tumor-imaging agent.⁴ Identification of the molecular elements in BLM responsible for tumor cell targeting would not only enable analogues with improved properties to be explored but might also allow for the selective delivery of other drugs to tumor cells.

We have shown previously that BLM A₅ conjugated to microbubbles binds selectively to tumor cells.⁵ Microbubbles, used traditionally as contrast agents for ultrasonography, have recently been modified with ligands that bind to specific receptors on cancer (and other) cell

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Supporting Information

Experimental procedures for the synthesis and characterization of BLM, deglycoBLM and BLM disaccharide dye conjugates. This material is available free of charge via the Internet at <http://pubs.acs.org>.

surfaces in an effort to probe ligand-cell surface interactions.⁶ For this study, the C-terminus of BLM was acylated with biotin and bound to commercially available streptavidin-derivatized microbubbles. It was shown that BLM-derivatized, but not streptavidin-derivatized microbubbles bound to MCF-7 human breast carcinoma cells but not to the “normal” MCF-10A breast cell line.⁵ To define the structural elements in BLM responsible for tumor targeting, the same experiment was performed using deglycoBLM, i.e. lacking the disaccharide moiety. Cellular recognition was not observed either for MCF-7 or MCF-10A cells.⁵ Subsequently, we have carried out analogous experiments using BLM and deglycoBLM conjugated to a cyanine dye (Cy5**). This permitted more facile quantification of the results, as well as investigation of internalization of the conjugates.

Our previous experiments established that the disaccharide moiety of BLM was necessary for tumor cell recognition, but did not address the issue of its possible sufficiency. Thus, the BLM disaccharide, consisting of L-gulose linked to 3-carbamoylmannose, was synthesized utilizing a procedure similar to that described previously.⁷ This disaccharide was coupled to a commercially available linker that had been protected as the benzyloxycarbonyl (CBz) derivative (**1**) to afford **2** in 66% yield (Scheme 1). Deprotection of the primary amine in **2**, followed by deacetylation and subsequent conjugation to the cyanine dye Cy5**^{8,9} via treatment with the *N*-hydroxysuccinimide ester of Cy5** (**3**) provided the BLM disaccharide-Cy5** conjugate (2-*O*-(3-*O*-carbamoyl- α -D-mannopyranosyl)-L-gulopyranose linked to Cy-5**) in 45% overall yield for the last three steps. The Cy-5** conjugates of BLM A₅ and deglycoBLM A₅ were also prepared in analogy with a published procedure (Figure 2).^{5,10}

MCF-7 human breast carcinoma cells and MCF-10A “normal” breast cells were cultured on 16-well glass chamber slides for 48 h, and then treated with 50 μ M BLM-Cy5** at 37 °C for 1 h to allow interaction with the cell surface. The cells were then fixed with 4% paraformaldehyde after washing the cells twice with phosphate buffered saline. Fluorescence microscopy imaging was carried out (Zeiss Axiovert 200M inverted microscope, 40x oil objective). As shown in Figure S2, treatment of MCF-7 cells with BLM-Cy5** resulted in significant cell binding and uptake, while no interaction was apparent for the MCF-10A cells. The cells were also treated with DAPI (2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride) in order to stain the cell nuclei, permitting preliminary evaluation of the localization of the BLM-Cy5** conjugate in the MCF-7 cells relative to the cell nuclei. With these results in hand, an experiment was carried out in which the MCF-7 and MCF-10A cells were treated with 50 μ M BLM-Cy5**, deglycoBLM-Cy5**, or BLM disaccharide-Cy5** and then fixed with paraformaldehyde.

Following 3-second irradiation, the images shown in Figure 3 were recorded. As is clear from the figure, both BLM-Cy5** and BLM disaccharide-Cy5** were associated with the MCF-7 cells, but deglycoBLM-Cy5** was not. None of the conjugates bound to the normal breast (MCF-10A) cells. The dye itself exhibited no affinity for either cell line. Shown in Figure 4 are the results obtained for similar treatment of prostate cancer cell line DU-145, and the normal prostate cell line PZ-HPV-7. The results in these two cell lines are qualitatively identical to those obtained for the MCF-7 and MCF-10A cell lines, although more efficient binding of BLM-Cy5** and BLM disaccharide-Cy5** was observed for DU-145 and PZ-HPV-7 cells. The results shown in Figures 3 and 4 are quantified in Figure 5. The binding/uptake in MCF-7 cells was shown not to vary significantly with the extent of cell confluence (Figure S3).

Also studied was the targeting of BxPC-3 pancreas cancer cells by BLM-Cy5**, deglycoBLM-Cy5** and BLM disaccharide-Cy5**. Unlike the results obtained with MCF-7 and DU-145 cell lines, the binding/uptake of BLM disaccharide-Cy5** and BLM-Cy5**

were essentially equivalent in BxPC-3 cells. As shown in Figure 6, BLM-Cy5** and BLM disaccharide-Cy5** were associated with the BxPC-3 cells, but deglycoBLM-Cy5** was not. None of the dye conjugates exhibited binding/uptake by the SVR A221a normal pancreas cell line. Two additional cancer cell lines have also been observed to exhibit similar selectivities.¹¹ While the scope of tumor cell lines targeted by BLM and its disaccharide are presently the subject of ongoing investigation, the reports of targeting with radionuclides of BLM have included numerous tumor types, suggesting that the phenomenon observed here may prove to be reasonably general.

The process by which the BLM-Cy5** and BLM disaccharide-Cy5** conjugates are internalized presumably involves initial cell surface binding (as demonstrated for BLM in our earlier report involving microbubbles⁵), followed by internalization.¹² The apparent lack of any dye conjugate bound to the cell surface in Figures 3 or 4 presumably reflects facile internalization following cell surface binding. This assumption was tested directly utilizing biotinylated BLM A₅, deglycoBLM A₅ and BLM disaccharide bound to commercially available streptavidin-derivatized microbubbles (Figure 7), as described previously.⁵ As shown in the Figure, the BLM A₅ and BLM disaccharide-derivatized microbubbles bound to MCF-7 cells, but the deglycoBLM A₅-derivatized microbubbles did not. The large size of the microbubbles (~2.5 μm, each estimated to contain ~1.5 × 10⁶ ligands⁵) precluded facile internalization following cell surface binding. As anticipated, under the same conditions, none of the derivatized microbubbles bound to MCF-10A cells (Figure S5).

The mechanism of uptake of the bound BLM disaccharide-Cy5** conjugate was studied by measuring uptake by MCF-7 cells at 4 °C. As shown in Figure 8, the uptake measured at 4 °C after 1 h was less than that observed at 37 °C, suggesting that uptake is ATP dependent.¹³ Thus internalization of the BLM disaccharide-Cy5** conjugate apparently occurs by a specific uptake mechanism, rather than by passive diffusion.

Current cancer therapies are limited by side effects resulting from a lack of tumor cell selectivity. Accordingly, there is great interest in molecules that can distinguish between healthy and cancerous tissue. A few such compounds, typically polypeptides or polysaccharides, have been described;¹⁴ however, size can limit their pharmacologic utility. Prospects for successful utilization in classic therapeutic strategies^{15,16} would increase dramatically if the size of these tumor-targeting molecules could be reduced. For example, smaller compounds tend to be less immunogenic, easier and less expensive to prepare, and more readily amenable to use in prodrug conjugates or as diagnostic agents.

In summary, we have identified an uncomplicated disaccharide that selectively targets a variety of cultured tumor cells.¹⁷ The unique character of this disaccharide at the levels of simplicity and selectivity argue for its potential utility. For example, this finding should facilitate the identification of the cell surface target for bleomycin, and the mechanism(s) of cellular uptake. Whatever the cellular target, the implications of this finding for cancer diagnosis and therapy seem clear. Utilization of this sugar as a tumor-imaging agent can be envisioned with ultrasound imaging or radionuclide imaging. Also, incorporation of this moiety into preexisting or novel cancer therapeutics could in principle increase drug delivery directly to the tumor cells, lowering the necessary dosage and potentially reducing side effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Levi JA, Raghavan D, Harvey V, Thompson D, Sandeman T, Gill G, Stuart-Harris R, Snyder R, Byrne M, Kerestes Z. *J Clin Oncol*. 1993; 11:1300. [PubMed: 7686216]
2. Sikic, BI.; Rozencweig, MSK.; Carter, SK., editors. *Bleomycin Chemotherapy*. Academic Press; Orlando, FL: 1985.
3. (a) Holmes CE, Carter BJ, Hecht SM. *Biochemistry*. 1993; 32:4293. [PubMed: 7682845] (b) Kane SA, Hecht SM. *Prog Nucleic Acid Res Mol Biol*. 1994; 49:313. [PubMed: 7532315] (c) Claussen CA, Long EC. *Chem Rev*. 1999; 99:2797. [PubMed: 11749501] (d) Hecht SM. *J Nat Prod*. 2000; 63:158. [PubMed: 10650103] (e) Abraham AT, Lin JJ, Newton DL, Rybak S, Hecht SM. *Chem Biol*. 2003; 10:45. [PubMed: 12573697] (f) Chen J, Stubbe J. *Nat Rev Cancer*. 2005; 5:102. [PubMed: 15685195] (g) Tao ZF, Konishi K, Keith G, Hecht SM. *J Am Chem Soc*. 2006; 128:14806. [PubMed: 17105281]
4. (a) Jones SE, Lilien DL, O'Mara RE, Durie BG, Salmon SE. *Med Pediatr Oncol*. 1975; 1:11. [PubMed: 60694] (b) Silverstein MJ, Verma RC, Greenfield L, Morton DL. *Cancer*. 1976; 37:36. [PubMed: 55300] (c) Bekerman C, Moran EM, Hoffer PB, Hendrix RW, Gottschalk A. *Radiology*. 1977; 123:687. [PubMed: 860022] (d) Burton IE, Todd JH, Turner RL. *Br J Radiol*. 1977; 50:508. [PubMed: 68793] (e) Goodwin DA, Meares CF, DeRiemer LH, Diamanti CI, Goode RL, Baumert JE Jr, Sartoris DJ, Lantieri RL, Fawcett HD. *J Nucl Med*. 1981; 22:787. [PubMed: 6168750] (f) Stern PH, Helpert SE, Hagan PL, Howell SB, Dabbs JE, Gordon RM. *J Natl Cancer Inst*. 1981; 66:807. [PubMed: 6164811]
5. Chapuis JC, Schmaltz RM, Tsosie KS, Belohlavek M, Hecht SM. *J Am Chem Soc*. 2009; 131:2438. [PubMed: 19187019]
6. (a) Klibanov AL. *Invest Radiol*. 2006; 41:354. [PubMed: 16481920] (b) Willmann JK, Paulmurugan R, Chen K, Gheysens O, Rodriguez-Porcel M, Lutz AM, Chen IY, Chen X, Gambhir SS. *Radiology*. 2008; 246:508. [PubMed: 18180339]
7. Boger DL, Honda T. *J Am Chem Soc*. 1994; 116:5647.
8. PCT WO. 2008/139206 A2.
9. Although the scope of dyes that can support selective tumor cell targeting by BLM or its disaccharide is presently undefined, we have found that conjugates prepared using Atto 647 (Kolmakov K, Belov VN, Wurm CA, Harke B, Leutenegger M, Eggeling C, Hell SW. *Eur J Org Chem*. 2010:3593.) gave results comparable to those obtained with the Cy5** conjugates.
10. Cu(II)•BLM A₅ and Cu(II)•deglycoBLM A₅ were treated with the N-hydroxysuccinimide ester of Cy5**, in analogy with attachment of BLM A₅ to a solid support (Abraham AT, Zhou X, Hecht SM. *J Am Chem Soc*. 2001; 123:5167. [PubMed: 11457377]). The conjugates were purified by C₁₈ reversed phase HPLC (99:1 0.1% aq TFA-CH₃CN → 45:55 0.1% aq TFA-CH₃CN), treated with EDTA to remove Cu²⁺, and then again purified by reversed phase HPLC, using the same method (Figure S1).
11. Selective targeting of the BLM-Cy5** and BLM disaccharide-Cy5** conjugates have also been observed using A498 human kidney cancer cells (but not CCD 1105 KIDTr kidney cells) and A549 lung carcinoma cells (but not WI-38 lung cells) (Figure S4).
12. Alternatively, selectivity may be due to a metabolic process specific to tumor cells.
13. Smalley KSM, Koenig JA, Feniuk W, Humphrey PPABr. *J Pharmcaol*. 2001; 132:1102.
14. Holick, MF.; Ramanathan, H. PCT Int Appl. US20050255038A1. 2003.
15. Avidor Y, Mabeesh NJ, Matzkin H. *Southern Med J*. 2003; 96:1174. [PubMed: 14696868]
16. Eggert US, Superti-Furga G. *Nat Chem Biol*. 2008; 4:7. [PubMed: 18084271]
17. Since observation of tumor cell targeting/uptake is dependent on a conjugated dye, it is not currently possible to exclude the possibility that the dye is essential for these processes. However, the fact that structurally unrelated dyes conjugated to the disaccharide gave the same effect⁹ argues against this possibility, as do the microbubble conjugate data (Figure 7).

Figure 1.
Structure of BLM A₅, with an inset highlighting the BLM disaccharide moiety.

Figure 2.
Structures of BLM-Cy5**, deglycoBLM-Cy5** and BLM disaccharide-Cy5**.

Figure 3.

Uptake of Cy5** conjugates in MCF-7 breast carcinoma cells and MCF-10A normal breast cells. The cells were treated with 50 μ M BLM-Cy5**, deglycoBLM-Cy5**, BLM disaccharide-Cy5** or Cy5** at 37 $^{\circ}$ C for 1 h. After washing with phosphate buffered saline, the cells were fixed with 4% paraformaldehyde. The cell nuclei were stained with DAPI. Fluorescence imaging was carried out after a 3-second exposure.

Figure 4.

Uptake of Cy5** conjugates in DU-145 prostate carcinoma cells and PZ-HPV-7 normal prostate cells. The cells were treated with 25 μ M BLM-Cy5**, deglycoBLM-Cy5**, BLM disaccharide-Cy5** or Cy5** at 37 °C for 1 h. After washing with PBS, the cells were fixed with 4% paraformaldehyde. The cell nuclei were stained with DAPI. Fluorescence imaging was carried out after a 1-sec exposure.

Figure 5.

Quantification of the binding/uptake of BLM-Cy5**, deglycoBLM-Cy5**, BLM disaccharide-Cy5** and Cy5** by MCF-7 and MCF-10A cells (upper panel) or by DU-145 and PZ-HPV-7 cells (lower panel). The MCF-7 and MCF-10A cells were treated with 50 μ M dye conjugates and irradiated for 3 sec prior to fluorescence imaging. The DU-145 and PZ-HPV-7 cells were treated with 25 μ M dye conjugates and irradiated for 1 sec prior to imaging.

Figure 6.

Quantification of the binding/uptake of BLM-Cy5**, deglycoBLM-Cy5**, BLM disaccharide-Cy5** and Cy5** by BxPC-3 and SVR A221a cells. The cells were treated with 25 μ M dye conjugates and irradiated for 1 sec prior to imaging.

bleomycin–microbubble conjugate

BLM disaccharide–microbubble conjugate

deglycoBLM-microbubble conjugate

Figure 7.

Monolayers of cultured MCF-7 breast cancer cells treated with BLM-derivatized microbubbles (top), BLM disaccharide-derivatized microbubbles (center) or deglycoBLM-derivatized microbubbles (bottom).

Figure 8.

The effect of incubation temperature on the internalization of BLM disaccharide-Cy5** conjugate in MCF-7 cells. The cells were treated with 50 μ M BLM disaccharide-Cy5** or Cy5** at 4 °C or 37 °C for 1 h. After washing with phosphate buffered saline, the cells were fixed with 4% paraformaldehyde. Fluorescence imaging was carried out with a 3-sec exposure time.

Scheme 1.

Synthetic Route Employed for the Preparation of BLM Disaccharide-Cy5**