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Comparative subcellular distribution of the copper complexes of bleomycin-A_2 and deglycobleomycin-A_2

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We have compared the cellular uptake and subcellular localization of ^{14}C-labeled bleomycin-A_2 (BLM) and deglycobleomycin-A_2 in living KB_3 cells. Both drugs exhibit poor internalization into cells but reveal significantly different intracellular distribution, with low and high accumulation into the cell nuclei for BLM and deglyco-BLM, respectively. The results indicate that the carbohydrate chain does not constitute a limiting factor for BLM permeation and is directly implicated in the intracellular distribution of the drug into cells.

Key words: Bleomycin, cancer, cellular distribution, cell permeation.

The bleomycins (BLMs) are a family of glycopeptide antibiotics used in the treatment of selected cancers. Their therapeutic activity is attributed to their ability to bind and cleave DNA.[1] The bithiazole moiety of the drug can selectively recognize certain sequences in DNA[2] and contributes to the efficiency of DNA degradation.[3] DNA cleavage by BLM is oxygen-dependent and requires the participation of a redox-active metal ion.[1] BLM-A_2, which bears a terminal sulfonium, group is the most active congener and the major component of the clinically used formulation. BLM rapidly forms a copper complex in blood which is thought to be the form by which the drug enters into cells. BLM has very low cellular uptake capacities,[4] approximately 0.1% of the total drug in the medium associates with HeLa cells.[5] Little is known concerning the mechanisms of BLM transport into cells and nuclear translocation. The functional state of the cell membrane would be critical for determining BLM cytotoxicities. Membrane effecting agents like ethanol,[6] local anesthetics[7] as well as hyperthermia and electric pulses[8] exert a synergistic effect on BLM cytotoxicity. The BLM–Fe complex also induces transient plasma membrane perturbations.[9] Although the BLM–metal complex is slightly more efficient to penetrate into cells than demetallo–BLM,[10] it seems that neither the metal complexing part nor the terminal amine of the drug are responsible for the low efficiency of BLM entering into cells.[11] It is thought that the carbohydrate chain would be involved in the penetration of BLM into cells but its exact role is still a matter of debate. It has been well established that deglyco-BLMs are somewhat less efficient in effecting DNA degradation than the respective BLMs.[12] The glycannic moiety contributes to the activation of molecular oxygen by the BLM–Fe complex.[13] Much less clear is the structure–activity relationship between transport or cellular drug content and the carbohydrate chain of BLM. Information on the precise role of the gulose–mannose sugar residues at the membrane level is meager and this is partly because of the difficulty to synthesize deglyco-BLM or to remove this group without degradating the antibiotic. An efficient procedure using HF solvolysis to specifically cleave the gulose–mannose moiety of BLM-A_2 with high reproducibility was developed in our laboratory.[14] The availability of deglyco-BLM combined with the use of described methods to radiolabeled BLM allowed us to examine the influence of the glycannic group on BLM association with cells *in vitro*. We report here our initial studies in which we have compared the cellular uptake and subcellular distribution of ^{14}C-labeled BLM-A_2 and deglyco-BLM-A_2 in KB_3 cells. This is the first report showing that the removal of the disaccharide moiety has little effect on BLM cellular uptake but fundamentaly affects the intracellular distribution pattern of the drug.

BLM-A_2 was demethylated by pyrolysis[15] and deglycosylated following the procedure based on solvolysis with fluorohydric acid previously described.[14] Desmethyl-BLM-A_2 and deglyco-desmethyl-BLM-A_2 were then methylated with [^{14}C]ICH_3.[16]

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Then 15 ml of KB_3 cells at 5×10^5 cells/ml were incubated with 150 µl of either [^{14}C]BLM-A_2–Cu(II) (0.64 µCi/mg; 15 ng/ml) or [^{14}C]deglyco-BLM]A_2–Cu(II) (0.68 µCi/mg; 11 ng/ml). After 15 min the steady state level of accumulation is reached.[4,11] Cells were collected by centrifugation, washed twice with 0.9% NaCl and then allowed to swell in ice in 10 mM Tris–HCl hypotonic buffer, pH 8.1, containing 50 mM NaCl and 1 mM EDTA. Cell disruption was achieved with a Dounce homogenizer. Although unlikely, we cannot exclude the possibility that a re-distribution of the drug occurs at this stage. The resulting homogenate was layered over a discontinuous 40–10% sucrose gradient. After 1 h centrifugation (95 000 *g* at 4°C) the nuclei sedimented; the white band of membranes is located between the two sucrose layers and the supernatant is referred to as the cytoplasmic fraction. The radioactivity associated with each fraction was counted. The purity of the fractions was assessed by both morphological examination under an electron microscope and by appropriate marker enzyme activities: 5′-nucleotidase, glucose-6-phosphatase and catalase markers for the plasma membrane, endoplasmic reticulum and peroxisomes-lysosomes.[9]

The concentration of either drug in the cell cultures was 10 nM and, as indicated in Table 1, only a very small fraction of labeled drug added to the culture is taken up into cells. Cellular uptake by deglyco-BLM is very weak. Clearly the carbohydrate chain has little effect on the efficiency of BLM penetration into KB_3 cells. Cells treated with BLM and its deglycosylated congener were then subjected to subcellular fractionation based on differential centrifugation. Three fractions were collected. The nuclear fraction contains almost exclusively intact nuclei (and a few intact cells). The fraction referred to as the membrane fraction is morphologically homogeneous and consists principally of plasma membranes with little contamination from endopasmic membranes and ribosomes. The third fraction groups the cytosol and some of its constituents (e.g. some lysosomes and mitochondria). The percentages of distribution of both BLM-A_2 and deglyco-BLM-A_2 in each fraction are reported in Table 1. The large majority of cellular [^{14}C]BLM-A_2 is recovered in the cytoplasmic fraction and only one-fifth of the total intacellular drug molecules is found associated with nuclei. Interestingly, the pattern of distribution of deglyco-BLM-A_2 is fundamentaly different since the carbohydrate-free drug is essentially recovered in the nuclei rather than in the cytoplasm. If one considers the respective quantities of drugs incorporated into KB_3 cells, it appears that the total association on membranes is almost identical for both antibiotics; in the same way, absolute cytoplasmic values are not very different whereas a very large difference can be seen in the nuclear association. Nuclei of KB_3 cells treated with deglyco-BLM-A_2 contain about 4-fold more drugs than cells treated with BLM-A_2. Clearly, the removal of the glycannic moiety facilitates the translocation of BLM-A_2 from the cytoplasm to the nuclei. In other words, the gulose–mannose part of BLM-A_2 hinders the accumulation of the antibiotic in the nuclei. Therefore, it may be envisaged to design BLM analogs with modified sugar residues with the expectation of targeting the drug more efficiently to the cells nuclei where the main target is located.

Table 1. Cellular uptake and subcellular distribution of BLM-A_2 and deglyco-BLM-A_2 in KB_3 cells

	BLM-A_2		Deglyco-BLM-A_2	
Cells	0.02[a] (±0.004)		0.03 (±0.005)	
Membranes	9[b]	(0.018)[c]	5	(0.015)
Nuclei	20	(0.040)	60	(0.18)
Cytoplasm	71	(0.142)	35	(0.105)

[a] Percent of radioactivity recovered after centrifugation and washing of the cells (7.5×10^6 cells), relative to the total amount of labeled drugs added to the cultures (10 nM).
[b] Percent of total cellular radioactivity.
[c] Absolute values of association (in ‰), i.e. amount of drug recovered in the subcellular fraction related to the total cellular association (see footnote 'a'). Mean of three separate experiments.

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