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SYNTHESIS OF SAMARIUM BINDING BLEOMYCIN - A POSSIBLE NCT RADIOSENSITIZER

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ABSTRACT

Bleomycin (BLM) is a drug that has attractive features for the development of a new radiopharmaceutical, particularly with regard to neutron capture therapy (NCT) sensitized by Sm-149. It has the ability to chelate many metal ions. In vitro studies have shown that up to 78% of BLM present in a cell is accumulated inside the nucleus or in the nuclear membrane. In addition, this drug has higher affinity for tumor tissues than for normal tissues. Radioactive isotopes carried by this antibiotic would be taken preferentially to one important cellular targets DNA. Besides, BLM displays intrinsic anti-tumor activity - it is a chemotherapic antibiotic clinically used against some cancers. This study aimed to obtain bleomycin molecules bound to samarium (BLM-Sm) for NCT studies *in vitro* and *in vivo*. The binding technique employed in this work has great simplicity and low cost. Thin layer chromatography (TLC), high performance liquid chromatography (HPLC), fast protein liquid chromatography (FPLC) and analysis by ICP-AES were applied to verify the binding molecule. ICP-AES results showed the presence of samarium in the sample peaks related to BLM-Sm. However, efficiency and stability of this bond needs to be investigated.

1. INTRODUCTION

Bleomycin (BLM) is a water soluble natural antibiotic isolated from the fungus *Streptomyces verticillus* [1, 2, 3]. It is a well studied glycopeptide, both toxic to mammalian and bacterial cells. Bleomycin toxicity is attributed mainly due to its ability to cleave one or two strands of DNA molecules [1, 2, 4]. Initially no effect on RNA was found. However, cleavage of this type of nucleic acid with consequent protein synthesis inhibition has been reported recently [5]. The ability to oxidize cell membranes has also been described [6, 7]. Bleomycin is most effective in G_2 phase of the cell cycle, but is also active against cells that are not dividing (G_0). Bleomycin causes little myelosuppression and immunosuppression, one contrast with many of anticancer drugs [8, 9].

Bleomycin was first isolated in 1966 by Umezawa. Since then, different applications using this molecule have been developed. One year after its discovery in 1967, Umezawa proposed the use of BLM as chemotherapeutic agent for cancer treatment due to it'sits ability to kill cells in high division rate. Currently bleomycin is clinically used in malignant neoplasias treatment such as: squamous cell carcinoma (head and neck, cervix and vulva); Hodgkin's

and non-Hodgkin's lymphoma; testicular carcinoma; and Kaposi's sarcomas [7, 10, 11]. Bleomycin may be applied alone or as one of a drug cocktail. Cases in which up to five drugs are employed simultaneously can be found [12]. BLM can also be useful as adjuvant to radiation therapy, providing a small increase in control and cure rates of head and neck cancer [13, 14].

The BLM is a molecule of 1400 to 1500 Daltons approximately and presents four different functional groups or domains. The disaccharide group, derived from mannose and galactose, is generally considered responsible for selective uptake, membrane recognition and, possibly, has influence in interaction with metals. There is also the group responsible for chelating capacity and cleavage activation. The bithiazole group, located near the C-terminal, is essential for bleomycin-DNA binding. The threonine-methyl-valerate domain serves as a linker region, providing correct spacing among other groups [7, 15]. Bleomycin's structural formula may show small variations, especially at the C-terminal end. Figure 01 shows a BLM A_2 molecule.

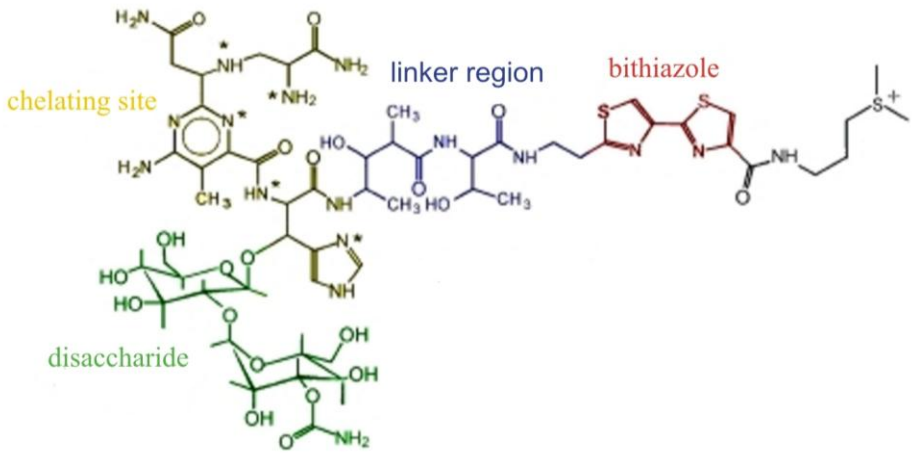

Figure 01 - Bleomycin A_2 molecule and its functional groups: bithiazole - red; linker region - blue; chelating site - yellow; and disaccharide group - green.

Bleomycin sulfate is commercialized under different names: Blenoxane, Lundbeck and Bleocin [16, 17]. These compounds are a mixture of different isomers of bleomycin with minor modifications in some radicals. The BLM A_2 (60%) and BLM B_2 (25%) are predominant isomers [09, 17, 20, 21].

The Samarium-149 is one metal isotope that presents a high cross section for neutrons, thus can provide excellent performance in neutron capture therapy. Bleomycin has a great ability to chelate metal ions. Wide variety of mono, di and trivalent cations has been employed in bleomycin labeling. Divalent cations (Cu-II, Ni-II, Zn-II and Co-II) have shown great affinity. On the other hand, trivalent cations often present binding difficulties. The probability of formation of stable compounds between the BLM and the alkaline earth metals is greatly reduced [1]. The binding of BLM with different metal ions witch are not Fe-II can alter the DNA preferential cleavage sites [4]. However, these changes were not related to decreased

drug effectiveness. Compounds of bleomycin bound to ^{51}Cr and ^{57}Co are clinically applied. The latter showed 98 % specificity in staging lung cancer staging [1].

Bleomycin's most serious side effect is lung toxicity. The most common consequences are: decreased total lung volume, decreased vital capacity and, rarely, pulmonary fibrosis (doses above 300 mg/m^2) [9]. This response is difficult to identify and there are many studies to find early markers that indicate its occurrence. Doses above 400 units are not recommended. This effect occurs in approximately 10% of patients and is fatal in 1% [8]

The analysis of the data presented so far suggests that ^{149}Sm labeled bleomycin can presents attractive characteristics for the development of a drug for neutron capture therapy. BLM has the capacity to chelate a large number of metal ions. It presents intrinsic antitumor activity. Myelosuppression is low - a characteristic shared pratically with no chemotherapeutical agent. It is a clinically used drug and has a greater affinity to tumor than to normal tissue. In addition, studies *in vitro* shown that up to 78% of BLM present in a cell accumulates in the nucleus and in nuclear membrane [1]. Thus, it can be expected that the isotopes carried by this antibiotic would be taken directly to their targets (DNA).

This study aimed to obtain bleomycin molecules bound to samarium (BLM-Sm) for NCT studies *in vitro* and *in vivo*. Thin layer chromatography (TLC), high performance liquid chromatography (HPLC), fast protein liquid chromatography (FPLC) and analysis by ICP-AES were applied to verify de binding.

2. MATHERIALS AND METHODS

2.1. BLM-Sm Synthesis

Initially, was prepared an aqueous bleomycin stock solution in a 2 mg/ml concentration (ie 1.4 μM).

Three solutions of BLM-Sm were prepared from the stock solution for TLC, HPLC and FPLC assays: (1) BLM-Sm solution at the ratio of one molecule of bleomycin to eight molecules of samarium (1:8 M/M); (2) BLM-Sm solution (1:1 - M/M); and (3) BLM-Sm solution (2:1 - M/M).

Solutions (1), (2) and (3) were placed in eppendorf tubes and shaked at vortex for 30 seconds. After mixing, the solutions were left at room temperature for 12 hours. The conservation of the prepared solutions was made at temperatures from 2 to 8 °C.

2.2. TLC Assays

Thin layer chromatographies were performed with SiO_2 plates, in supersaturated chamber. The protocol was adapted from Stahl, 1969 and Braga & Collins, 1988 [18, 19].

The mobile phase employed was (0.50: 0.45: 0.05, v/v) methanol: ammonium acetate 10% - aqueous solution: 10% ammonium hydroxide - aqueous solution. This solvent combination resulted in higher R_f values and good separation of BLM A_2 and BLM B_2 isomers.

Potassium permanganate, a stain for sugars and polyalcohols, was utilized. The disaccharide group derived from galactose and mannose was the main target on bleomycin molecule.

At least three runs of the following bleomycin solutions were carried out: BLM in aqueous solution [2mg/ml] (control), BLM-Sm (1), (2) and (3), and saline BLM. The latter one was used to determine if the presence of chloride anions in the aqueous solution could cause changes in R_f of A2 and B2 isomers.

2.3. HPLC Assays

BLM-Sm samples with samarium excess (solution 1) were chosen for the HPLC assays.

A C_{18} column (4.6 x 150 mm) was employed. The mobile phase was: (A) 0.1% TFA (trifluoroacetic acid) v/v in water and (B) 0.1% TFA v/v in acetonitrile.

The gradient was: 0% to 30% of (B) in 30 minutes, 30% to 100% of (B) in 30 minutes, and 100% of (B) maintained for 10 minutes.

UV detection (280 nm) was utilized.

2.4. FPLC Assays

BLM-Sm samples with samarium excess (solution 1) were chosen for the FPLC assays.

A cation-exchange column Mono S HR 5 / 5 (5 x 50 mm) has been used. Mobile phase was: (A) - 0.05 M ammonium formate and (B) - 1.00 M ammonium formate.

The gradient was: 0% to 5% of (B) in 30 minutes; 5% to 25% of (B) in 20 minutes; 25% to 100% of (B) in 10 minutes; and 100% of (B) maintained for 10 minutes [20].

UV detection (280 nm) was utilized.

2.5. ICP-AES Analysis

ICP analyses were performed in four samples at the Centre National de la Recherche Scientifique (CNRS) of France. Four tubes containing elution fractions from FPLC assays derived from unbound BLM and BLM-Sm were chosen. These tubes correspond to: (A) - A_2 isomer of BLM-Sm; (B) - A_2 Isomer of unbound BLM; (C) – B_2 isomer of BLM-Sm; and (D) – elution fraction of BLM-Sm without bleomycin isomers.

3. RESULTS

3.1. TLC Assays

Figure 02 shows the photograph of a TLC assay performed to identify the binding between bleomycin and samarium. Differences between the spots produced by the BLM and BLM-Sm

could be observed. Bleomycin in aqueous solution (unbound) showed larger R_fs values than the BLM-Sm ones.

The BLM A2 isomer apparently was subdivided into two spots called BLM A_2 and BLM A_2'. This was not observed in TLC assay with the (1:1 v/v) methanol: ammonium acetate 10% - (aqueous solution) solvent used in pilot-testing. The presence of a tenuous spot ahead of BLM A_2' should indicate another bleomycin isomer.

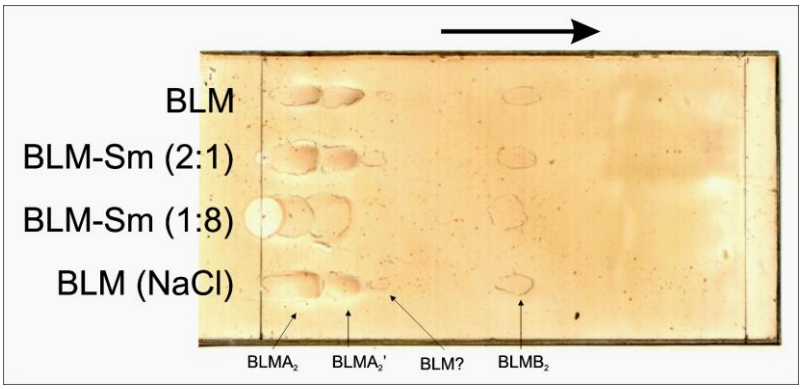

Figure 02 - TLC assay with SiO_2 plate in supersaturated chamber. Mobile phase was (0.50: 0.45: 0.05, v/v) methanol: ammonium acetate 10% - aqueous solution: 10% ammonium hydroxide - aqueous solution. The horizontal arrow indicates the solvent run direction.

It was also observed the presence of a white halo at the location where the samples containing samarium were applied (in the origin). This halo is probably due to the presence of free Sm. The following evidences support this hypothesis: i) bigger halo in sample with samarium excess; ii) smaller halo in sample with bleomycin excess; and iii) no white halo in aqueous BLM solution and BLM saline solution samples. In addition, the literature describes that the solvent employed is not capable to carry metal ions (like free samarium) [18].

Table 01 – Mean R_f values and standard deviation for BLM isomers of bound and unbound solutions.

Isomer	R_f: BLM Aqueous Solution		R_f: BLM-Sm Solution (1)	
	Mean	SD	Mean	SD
$BLMA_2$	0,08	0,01	0,06	0,01
$BLMA_2$'	0,16	0,01	0,14	0,02
$BLMB_2$	0,53	0,01	0,49	0,05

The Table 01 presents mean R_f values and standard deviation obtained considering all TLC assays accomplished for Control Solution and for Solution (1) of BLM-Sm (1:8). Statistically significant differences between the migration patterns of the two solutions were not observed.

3.2. HPLC Assays.

Figure 03 shows two HPLC chromatograms performed to check the Bleomycin-samarium binding. The absorbance (UV - 280 nm) is represented by the red line. The blue line represents the concentration gradient of solvent B (TFA 0.1% v / v in acetonitrile). Figure 03A corresponds to the chromatogram of a sample of bleomycin without samarium, while Figure 03B corresponds to the BLM-Sm - Solution (1).

Both chromatograms show five peaks. The first (and biggest) of them corresponds to the A_2 isomer. The second peak represents the B_2 isomer. The others represent minority isomers, contaminants or BLM degradation products.

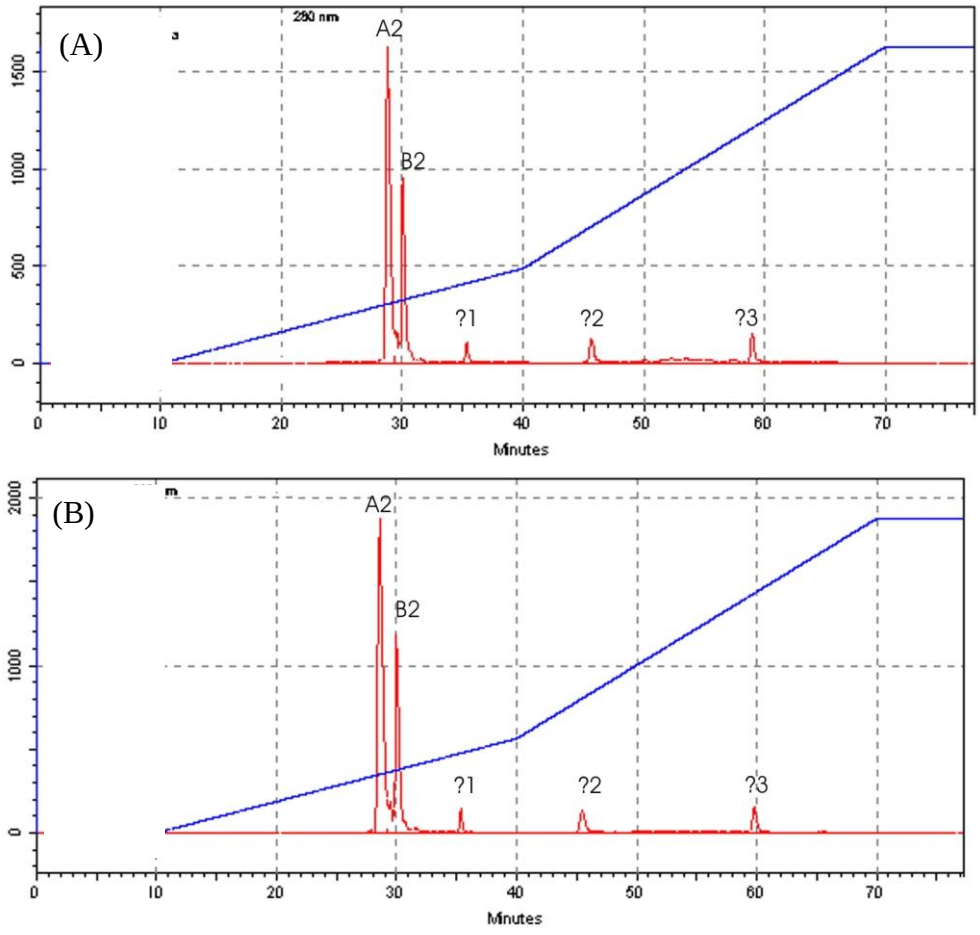

Figure 03 – HPLC chromatograms. (A) - BLM unbound (bleomycin in aqueous solution). (B) – BLM-Sm, solution (1). Were applied 100 µg of bleomycin.

The retention times and each isomer proportion (based on absorbance) to aqueous BLM (unbound) and BLM-Sm (bound) are found in Table 02. For both samples, retention times (RT) were practically identical considering each isomer. This information can also be seen in Figure 03.

Table 02 – Retention times and the proportions of each isomer (based on absorbance) for BLM and BLM-Sm.

Isomer	BLM (Aqueous Solution)		BLM-Sm (Solution (1))	
	RT (min)	Proportion (%)	RT (min)	Proportion (%)
BLM-A_2	18.5	55.17	18.5	53.52
BLM-B_2	20.0	32.76	20.0	33.80
BLM-?$_1$	25.5	3.45	25.5	4.23
BLM-?$_2$	35.5	3.45	35.5	4.23
BLM-?$_3$	49.0	5.17	50.0	4.23

3.3. FPLC Assays.

The FPLC assay results for unbound BLM and BLM-Sm (solution 1) are shown in Figure 04. Both samples present retention times (RT) for A_2 and B_2 isomers practically identical. BLM B_2 peaks showed low resolution. The normalized absorbance indicates 54-62% of A_2 isomer and 22-26% of B_2 isomer considering both samples. Samarium chloride sample don't show any peak. The UV detector could not detect the $SmCl_3$.

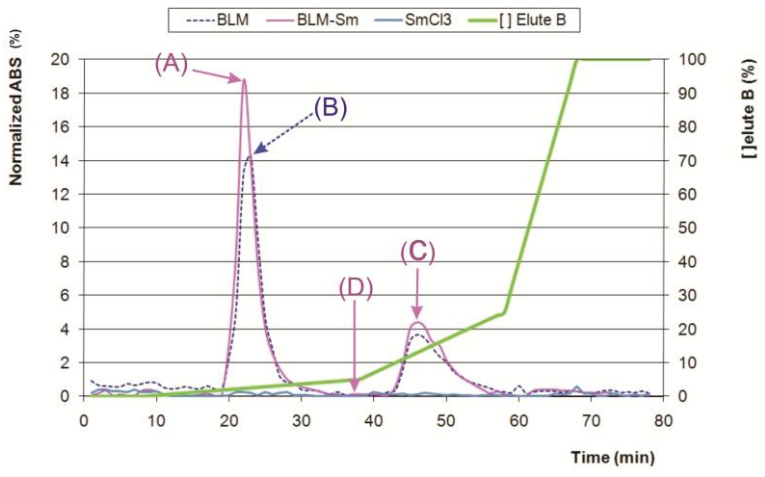

Figure 04 – FPLC Chromatogram for unbound BLM sample (dotted) and BLM-Sm sample (magenta). The green line indicates the concentration gradient of solvent B. The light blue full line represents the $SmCl_3$ sample. The points (A), (B), (C) and (D) indicate the elution fractions that were sent to the CNRS.

The tubes containing the elution fractions corresponding to points (A), (B), (C) and (D) presented in Figure 04 were sent to the Centre National de la Recherche Scientifique (CNRS) in France to determine samarium concentration by ICP-AES. The point (A) refers to the elution fraction containing the isomer A_2 from the samarium containing solution (BLM-Sm). Point (B) represents the elution fraction containing the A_2 bleomycin isomer from unbound BLM sample. Point (C) refers to the elution fraction containing the B_2 isomer from BLM-Sm solution. The point (D) represents an elution fraction from BLM-Sm sample that didn't show any isomer of bleomycin. This last point serves as a negative control, because in the absence of bleomycin isomers is not expected that there are samarium atoms.

3.4. ICP-AES Analysis.

ICP analyses were performed for the elution fraction tubes corresponding to points A, B, C and D (Fig. 04). The results are shown in Table 03.

The result referring to point (B) indicated the absence of samarium atoms. As expected the BLM A_2 isomer from the unbound-BLM sample don't show any trace of samarium. On the other hand, the presence of different isotopes of natural samarium in the A and C samples related to BLM-Sm solution indicates that there was binding of BLM A_2 isomers to the Samarium atoms. The result for sample D, related to BLM-Sm solution, showed that samarium was not detected in the elution fraction in which BLM isomers were not expected to be present.

Table 03 – ICP-AES analysis referring to samples A, B, C and D, presented in Figure 04.

Isotope / Natural Abundance	**Samarium Concentration (ppb)**			
	A **(BLM-Sm A_2)**	**B** **(BLM A_2)**	**C** **(BLM-Sm B_2)**	**D** **(BLM-Sm no isomer)**
Sm-147 / 15.0%	3.9	0.1	0.5	-0.4
Sm-149 / 13.8%	3.7	-0.3	0.3	-0.7
Sm-152 / 26.7%	4.0	-0.2	0.7	-0.6
Sm-154 / 22.7%	3.7	0.1	0.0	-0.2

4. DISCUSSION

The TLC results show that the solvent employed allowed good separation of bleomycin isomers. However, it was not possible to identify the binding of BLM with the samarium atoms. The sample of BLM-Sm (2:1) showed a small white halo at the origin (where the sample was applied). This fact seems to indicate the presence of samarium atoms not bound even with the excess of bleomycin molecules.

Regarding to HPLCs tests, analysis of Table 02 confirms that the proportions found for A_2 (~60%) and B_2 (~25%) bleomycin isomers are very similar to those mentioned in literature as

well as presence of minority isomers [09, 17, 20]. The number of peaks and the retention times (RT) found for BLM and BLM-Sm were practically the same. This could indicate that the BLM didn't chelate the samarium. However, is noted that there is the possibility that the binding could not change the BLM affinity to the column. Thus, the retention time would be the same for BLM and BLM-Sm. It is also possible that bleomycin have been bound to samarium, but with low yield. In this case, the amount of labeled molecules may have been below the device detection limit.

The analysis of Figure 04, related to FPLC assays, allows confirmation that the technique used enables a good separation between the A_2 and B_2 isomers. The value found to BLM A_2 RT was identical to that reported in the literature for this type of chromatography [20]. The B_2 isomer RT value was slightly smaller (38 - 40 min), however, as mentioned before, this peak showed low resolution which may be the cause of this difference. As in the HPLC assays, was not possible to observe changes in isomers retention time of unbound BLM and BLM-Sm samples.

The ICP-AES results (Table 03) of samples from tubes (elution fraction) corresponding to points A, B, C and D (Fig. 04) shed some light on the effectiveness of the binding reaction. It was verified that bleomycin bind to samarium atoms. Furthermore, it can be stated that bleomycin bound to samarium showed the same retention time as the non-bound one, considering the chromatographic system utilized. The low concentration of samarium observed (of the order of ppb) seems to indicate a low yield binding. Confirmed this in samples of A and C must be found labeled and unlabeled BLM isomers.

5. CONCLUSIONS

Bleomycin is a drug that has great potential to carry ^{149}Sm as a NCT radiosensitizer. The binding technique employed in this work has great simplicity and low cost. Data obtained from FPLC assays and ICP analyses lead us to conclude that the Bleomycin binds to samarium element. However, efficiency and stability of this bond needs to be investigated.

New FPLC assays combined with ICP-AES analysis, as well as the synthesis of BLM-Sm with radioactive samarium, may further clarify binding efficiency of the technique. TLC and HPLC techniques, properly adapted may also be useful in future experiments.

Other binding forms of samarium and bleomycin should be explored as well as the possibility of binding this chemotherapeutic antibiotic to other NCT sensitizers as ^{10}B and ^{157}Gd.

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