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STABLE [^{57}Co]-BLEOMYCIN COMPLEX WITH A VERY HIGH SPECIFIC RADIOACTIVITY FOR USE AT VERY LOW CONCENTRATIONS

Bruno PODDEVIN, Jean BELEHRADEK Jr., and Lluis M.MIR*

Laboratoire de Pharmacologie Moléculaire
U 140 INSERM, URA 147 CNRS, Institut Gustave Roussy
39, rue Camille Desmoulins F-94805 Villejuif, France

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A method for the preparation of ^{57}Co-labelled bleomycin (BLM) possessing very high specific radioactivity and suitable for use at the nanomolar concentration range is described, and validated using a biological assay. Chelation of BLM with Co(II) results in a very stable complex. However, association does not occur below the micromolar concentration range. A nanomolar [^{57}Co]BLM solution with maximal specific radioactivity can be easily prepared, without handling unreasonable amounts of radioactivity, provided that : equimolar solutions of BLM and [^{57}Co]Cl_2 are first mixed at the micromolar concentration range and that the mixture is then diluted a thousand times to reach the nanomolar concentration range.

Bleomycin (BLM) (1) is a glycopeptidic, hydrosoluble antibiotic endowed with cytotoxic properties against eukaryotic cells (2) and used as a human antitumor agent (3). BLM displays characteristic properties which are still under investigation : metal chelation (4), interactions with DNA (5,6), and association with cells (6,7). Studies have been generally performed at BLM concentrations in the micromolar range, i.e. at the level at which the drug begins to be cytotoxic (2,7). However, after cell electropermeabilization, cytotoxicity is detected at the nanomolar concentration range (8). For experiments at such low concentrations, we determined conditions for a stable and sensitive labelling of BLM using ^{57}Co, which we present in this report.

MATERIAL AND METHODS

Preparation of cobalt-bleomycin complex (CoBLM) : 15mg of lyophilized BLM (Roger Bellon, France) were dissolved in isoosmotic PBS (Phosphate Buffered Saline) at pH=7, diluted with the same buffer to the desired concentration and stored at -20°C. [^{57}Co]Cl_2 (440 Ci/mmol) (New England Nuclear) in a 50 mM HCl solution was diluted to the desired specific radioactivity in a 50 mM HCl solution of cold $CoCl_2$. The final pH of the labelled $CoCl_2$ solution was adjusted to 7 with 0.375 M Na_2CO_3. Just before use, BLM and $CoCl_2$ were mixed at two different molar ratios BLM/$CoCl_2$: 3/1 (gel permeation

*To whom correspondence should be addressed.

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chromatographies), 1/1.1 (cell viability measurements) and incubated for 1 hr at room temperature. The exact specific radioactivity (CPM/μl) of each [^{57}Co]BLM preparation was controlled on an Automatic Gamma Counting System MR 252 (Kontron).

Gel Permeation Chromatography : G-10 Sephadex (Pharmacia) or P2-Biogel (Biorad) was pre-swollen in the eluant [5 mM CH_3COOH/500 mM NaCl in distilled water adjusted to pH=7 with 8 N NaOH] and degassed. Five to 7 ml of these preparations were poured in columns of 6 mm inside diameter (gel height : 20 to 27 cm). The packing of each column was checked and the void volume measured by the filtration of a sample of blue dextran 2000 (Pharmacia) at a concentration of 2 mg/ml. Void volumes were between 2 and 3 ml for an average total volume of 6 ml. The flow rate of the eluant was 2.5 min/ml for the G-10 Sephadex columns and 7 min/ml for the P2-Biogel columns.

The volume of the samples deposited at the top of the columns was 100 μl. At the bottom, 500 μl fractions were manually collected and their radioactivity was counted. At each filtration, the activity of the initial 100 μl sample was compared with the sum of the activities of the final 500 μl fractions in order to check that no radioactive molecules had been retained in the column. Control preparations, where BLM was replaced by PBS, were analysed in the same manner.

Cells and cell treatments : DC-3F cells, a Chinese hamster lung fibroblast line (9), were maintained in previously described culture conditions (8). Cell electropermeabilization was performed using a square waved pulse generator, the "electropulsator" PS 15, commercially available from JOUAN (Saint-Herblain, France). The output voltage from this generator is between 0 and 1500 volts and can be maintained for periods of time ranging from 5 μsec to 24 msec. This square wave signal can be delivered either ad libitum or at a preprogrammed frequency (ranging from 0.1 Hz to 10 Hz). The efficiency of the impulses was checked through a digital storage oscilloscope VC-6025 (Hitachi), connected to the generator. Treatments with BLM or CoBLM were performed 24 hrs after the plating of 10^7 cells per T75 culture flask. After trypsinization of exponentially growing cells and inactivation of trypsin by complete medium, cells were washed three times in 0.5 mM Ca^{2+} supplemented S-MEM (without serum) (Gibco). Cells were then resuspended in the same ice-cooled medium at a density of 2.2×10^7 cells/ml. Aliquots of 90 μl of the monodispersed cell suspension were mixed with 10 μl of drug solutions 10 fold in concentration. Fifty μl of the mixture were immediately deposited between the two electrodes (2 mm apart) and exposed to electric treatment (8 pulses of 100 μs and 1500 V/cm at a frequency of 1 Hz). After the shock, the cells were maintained for 5 min at 24°C, then diluted about 10^4 times and seeded in triplicate in complete culture medium (500 cells per cell culture dish 60 mm in diameter) for colony inhibition assay. Colonies were counted after 5 days. Results are expressed as the percentage of the number of colonies obtained with control cells treated in the absence of drug. Absolute cloning efficiency of the controls is usually about 60%.

RESULTS

The separation of free [^{57}Co] from [^{57}Co]BLM was performed using gel permeation chromatography (Fig.1 and 2). In these figures, the ratio between the radioactivity of each fraction collected and the total radioactivity eluted from the column was expressed as a function of the elution volume.

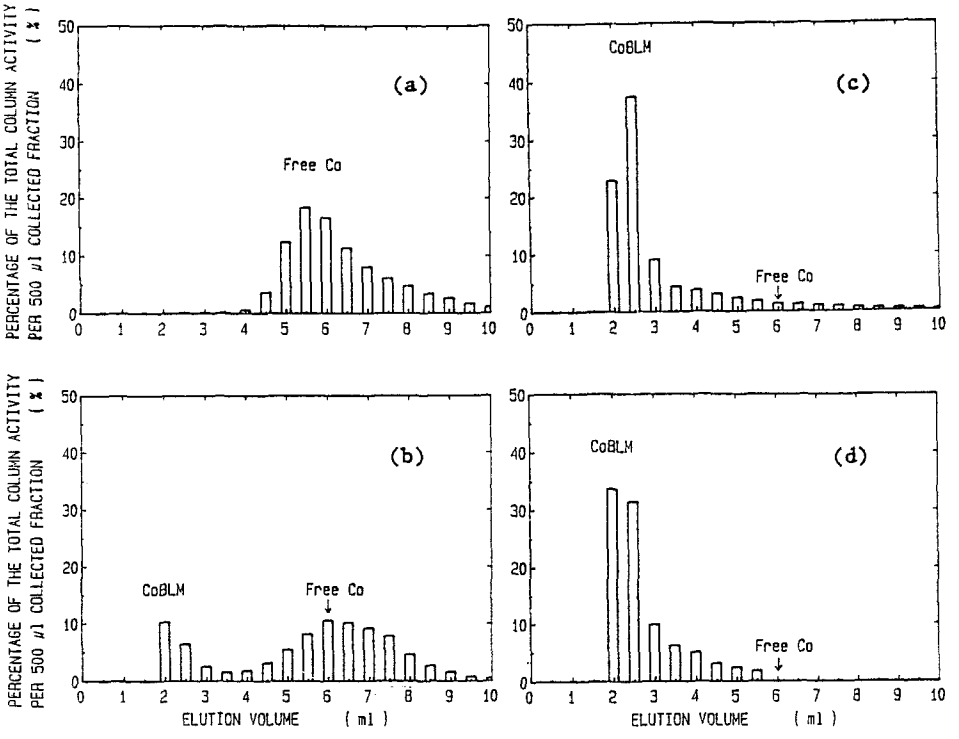

Figure 1 . Gel filtration on G-10 Sephadex of free ^{57}Co (a), of a mixture containing 1µM BLM/ 0.3µM $CoCl_2$ (b), and of a mixture containing 10µM BLM/ 3µM $CoCl_2$ (c). The mixture containing 10µM BLM/ 3µM $CoCl_2$ was diluted ten thousand times with PBS and filtrated on G-10 Sephadex (d).

Under optimal conditions (pH, ionic strength, column package), the passage of different compounds through a dextran gel such as Sephadex G-10 depends upon the molecular weight of the molecules. With an eluant of low ionic strength, Sephadex G-10 columns retained Co. However, complete elution of Co was achieved using eluants of high ionic strength (data not shown). Under these conditions, after appropriate calibration procedures (filtration of blue dextran), the [^{57}Co] elution volume was similar to the

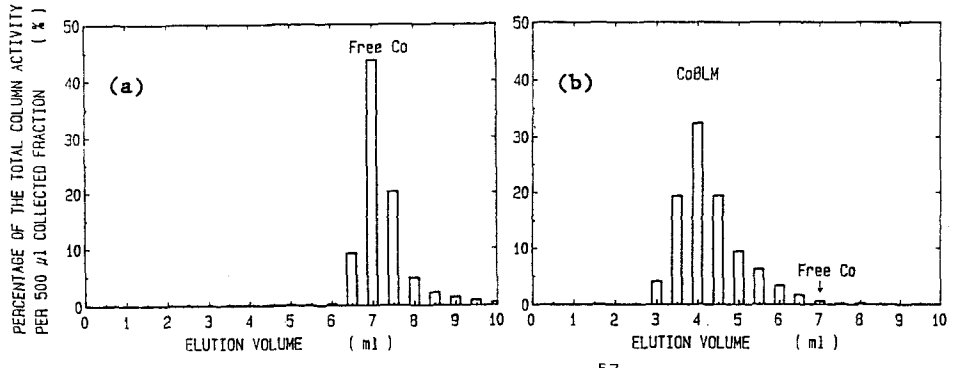

Figure 2 . Gel filtration on P2-Biogel of free ^{57}Co (a), and of a thousand times diluted mixture containing initially 10µM BLM/ 3µM $CoCl_2$ (b).

total column volume (Fig.1a) whereas the [^{57}Co]BLM elution volume was identical to the void volume (Fig.1c).

Fig.1b shows that only a partial formation of the CoBLM complex was obtained in a solution containing 1 μM BLM and 0.3 μM $CoCl_2$; a large fraction of cobalt remained unbound to the drug. However, when a solution ten times the initial concentration was used (Fig.1c), the association was almost complete. In addition, Fig.1d shows that this more concentrated mixture can be diluted ten thousand times without any detectable decrease in association.

Using P2-Biogel we observed that peaks were sharper and higher than those obtained using Sephadex G-10. More quantitative analyses were possible : in a solution containing 10 μM BLM and 3 μM $CoCl_2$, less than 2% of cobalt remained free, even after the mixture had been diluted a thousand times (Fig.2).

DC-3F cells were electropermeabilized in the presence of either various concentrations of free BLM or various dilutions of a mixture of 10 mM BLM and 11mM $CoCl_2$. The survival plots of treated cells, shown in Fig.3, reveal that at the same BLM concentrations, CoBLM solutions, obtained as previously described, are at least 350 times less cytotoxic than free BLM solutions. Given that CoBLM is known to be non cytotoxic (7) this result suggests that less than 0.3% BLM remained unbound in the different CoBLM solutions.

DISCUSSION

Our objective was to obtain labelled BLM displaying very high specific radioactivity suitable for use at the nanomolar concentration range. Many forms of radiolabelled BLM exist. Nevertheless the use of [^{3}H]BLM or [^{14}C]BLM is restricted at low concentrations because of the specific

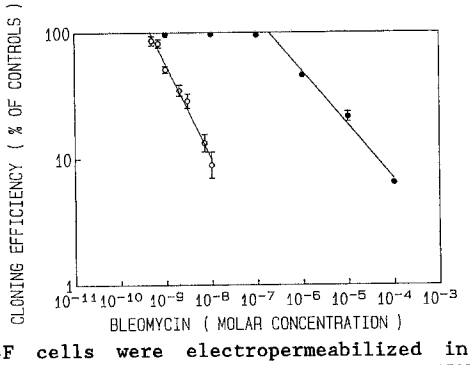

Figure 3 . DC-3F cells were electropermeabilized in the presence of different concentrations of BLM (open symbols), or CoBLM (closed symbols). CoBLM solutions were prepared by dilution in PBS of the most concentrated one. After electrical procedure, relative cloning efficiencies of treated cells were determined. Logarithm of cell survival was reported as a function of the logarithm of BLM concentration in the shock medium.

activity of these compounds ([^{14}C]BLM : 40 Ci/mmol (10); [^{3}H]BLM : 90 to 2500 mCi/mmol (6,7); Cu(II)[dimethylsulfonium-^{3}H]BLMA$_2$: 43 Ci/mmol (11)). BLM can also be labelled by chelation with metallic radionuclides : ^{111}In, ^{67}Ga or ^{59}Fe can bind BLM (12), but the most stable complexes are those formed with ^{57}Co. The theoretical limit of the specific activity of BLM labelled using a pure ^{57}Co radioisotope is 440 Ci/mmol, i.e. several ten or hundred times higher than the specific activity of [^{3}H]BLM or [^{14}C]BLM. Thus [^{57}Co]BLM seemed to be suitable for use at the nanomolar concentration range provided its stability was adequate at these concentrations.

It is usually agreed that, at 20°C, in isoosmotic buffers, at neutral pH, cobalt binds to BLM instantaneously (13) to form a Co(II)BLM complex which spontaneously oxidizes to the extremely stable Co(III)BLM species (4). Many authors have taken advantage of the very rapid and easy association of Co(II)BLM in studies concerning the binding of metallo-bleomycins to cells (6,7) and BLM-DNA interactions (5).[^{57}Co]BLM has already been used as a tumor scanning agent (14). However, none of these applications needed both very diluted and highly radioactive CoBLM.

The highest radioactive [^{57}Co]BLM solution is obtained when every molecule of BLM is bound to a ^{57}Co atom. The simplest way to prepare such a solution is to mix together equimolar solutions of pure radioactive cobalt and bleomycin, in conditions and at a concentration level where total association occurs between the two species. We performed gel permeation experiments with a BLM/$CoCl_2$ molar ratio of 3/1 in order to ensure that no cobalt atom remained free because of an excess of cobalt. Our first results (Fig.1b,c) suggested that under our preparation conditions, the apparent constant of the BLM/CoBLM equilibrium was in the range of 1 μM. Thus no association is to be expected with the equimolar mixing of nanomolar solutions. However, once almost complete association at adequately chosen concentrations is obtained, the CoBLM solution can be diluted down to the nanomolar range without significant dissociation of the complex (Fig.1d and 2). As previously mentioned, this property probably results from the rapid post-association oxidation of Co(II).

Thus a nanomolar [^{57}Co]BLM solution with maximal specific radioactivity can be easily prepared, without handling unreasonable amounts of radioactivity, provided that : first, equimolar solutions of BLM and [^{57}Co]Cl_2 are mixed at the micromolar range of concentrations; the mixture is subsequently diluted a thousand times to reach the nanomolar concentration range.

This experimental procedure was checked using a biological assay. It is known that (i) CoBLM is not cytotoxic (7), and that (ii) in vitro cytotoxicity of BLM is highly potentiated by cell

electropermeabilization.(8). Fig.3 shows that cytotoxicity of free BLM is detectable on electropermeabilized cells for a concentration below 10^{-9}M. Thus the cytotoxicity of a mixture of BLM and $CoCl_2$, revealed by electropermeabilization, allows the detection of even very low concentrations of free BLM, and, consequently the determination of the level of the CoBLM complex formation in the mixture. Using this biological assay, the presence of free BLM in amounts greater than 0.3% was not detected in the CoBLM mixtures tested. Thus, this biological assay confirms the chromatographic observations and further validates the method proposed by us for [^{57}Co]BLM preparation.

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REFERENCES

1. Umezawa, H., Maeda, K. and Takeuchi, T. (1966) J. Antibiot. 19, 200-209.
2. Terasima, T., Takabe, Y., Katsumata, T., Watanabe, M. and Umezawa, H. (1972) J. Natl. Cancer Inst. 49, 1093-1100.
3. Droz, J. P., Cvitkovic, E., Armand, J. P. and Khoury, S. (1988) Handbook of chemotherapy in clinical oncology F.I.I.S., Paris.
4. Stubbe, J. and Kozarich, J. W. (1987) Chem. Rev. 87, 1107-1136.
5. Byrnes, R. W., Templin, J., Sem, D., Lyman, S. and Petering, D. H. (1990) Cancer Res. 50, 5275-5286.
6. Roy, N. and Horwitz, S. B. (1984) Cancer Res. 44, 1541-1546.
7. Lyman, S., Ujjani, B., Renner, K., Antholine, W., Petering, D. H., Whetstone, J. W. and Knight, J. M. (1986) Cancer Res. 46, 4472-4478.
8. Orlowski, S., Belehradek, J. Jr, Paoletti, C. and Mir, L. M. (1988) Biochemical Pharmacology 37, 4727-4733.
9. Biedler, J. L. and Riehm, H. (1970) Cancer Res. 30, 1174-1184.
10. Fujimoto, J., Higashi, H. and Kosaki, G. (1976) Cancer Res. 36, 2248-2253.
11. Lazo, J. S., Braun, I. D., Meandzya, B., Kennedy, K. A., Pham, E. T. and Smaldone, L. F. (1985) Cancer Res. 45, 2103-2109.
12. Grove, R. B., Eckelman, W. C. and Reba, R. C. (1973) J. Nucl. Med. 14, 917-919.
13. Nunn, A. D. (1976) J. Antibiot. 29, 1102-1108.
14. Novel, J. P., Renault, H., Robert, J., Jeanne, C. and Wicart, L. (1972) Nouv. Presse Méd. 1, 95-98.