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# Mechanism of inhibition of the human immunodeficiency virus type 1 by the oxygen radical generating agent bleomycin

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**Abstract**

Alternative targets of attack of the human immunodeficiency virus (HIV) are necessary in light of infection persistence due to onset of resistance after conventional reverse transcriptase and protease inhibitor therapy. We have recently shown that the cancer chemotherapeutic agent bleomycin (BLM) dose-dependently inhibits HIV-1 replication. The mechanism of this viral inhibition in vitro was investigated. Cell-free wild-type virions were affected directly by BLM in the presence of $H_2O_2$, as shown by a 38% decrease of viral infectivity. Viral inhibition by BLM did not proceed via NF-κB inhibition. The viral R/U5 DNA product was reduced by 70% without any effect on reverse transcriptase activity. In both a cell-free system as well as two-cell systems the antiviral dependence of BLM on iron and oxidant species was demonstrated. Bleomycin seems to inhibit HIV-1 replication through the same properties that make it a suitable anti-cancer agent. The results presented in this study describe a novel mechanism of HIV-1 inhibition with potential application in viral infections. The anti-HIV effects of BLM in patients receiving this drug in combination with HAART should be carefully monitored in order to evaluate the clinical significance of the findings described in this study.


*Keywords:* Bleomycin; HIV; Iron; Reactive oxygen species; Hypoxia

## 1. Introduction

The cytostatic agent bleomycin (BLM) is an iron chelator, with anti-tumor properties and since its discovery (Umezawa et al., 1966), it has been used to treat various malignancies, such as lymphoma, cervical carcinoma and germ cell tumor (Comis, 1992). In addition to damaging DNA, BLM has been shown to cause RNA damage (Carter et al., 1990; Holmes et al., 1993). The compound is a planar bi-functional glycopeptide containing a DNA binding site and an active redox site which contains a binding site for $Fe^{2+}$ (Lown and Sim, 1977; Sausville et al., 1976). DNA damage is believed to be initiated by its ability to form in the presence of $Fe^{2+}$ an activated ferrous complex with oxygen (Moseley and Chalkley, 1987). Ferrous ions from this activated complex donate an electron to oxygen resulting in reactive oxygen species (ROS) formation which cause oxidative damage to DNA and ultimate DNA strand cleavage. In addition to ROS production, the process of strand scission is believed to be facilitated by hydrogen abstraction from C-4′ of the deoxyribose sugar by the activated BLM-$Fe^{2+}$ complex (Lazo and Sebti, 1997). Bleomycin has been shown to preferentially cleave 5′-GC-3′ and 5′-GT(U)-3′ sequences (Hecht, 1994).

It has been considered for a long time that the iron- and oxidative radical- dependent DNA damage properties of this antibiotic form the basis of its antitumour properties. The DNA degrading action of the drug was shown to require oxygen (Onishi et al., 1975) and it could be readily terminated by the addition of strong metal-chelating agents such as EDTA and the iron chelator deferoxamine (DF) (Sausville et al., 1978). Bleomycin-treated patients are sensitive to high concentrations of inspired $O_2$ and as was shown in a study (Goldiner et al., 1978), patients who had received BLM

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seemed to be at greater risk of respiratory failure during the post-operative recovery period after surgery. Reducing inspired $O_2$ and decreasing fluids administered during surgery prevented mortality in subsequent patients (Goldiner et al., 1978). In a study whereby human sarcoma cells were exposed to BLM for 1 h at various oxygen concentrations (>1, 2.5, 5, 21 and 95%) the authors assessed cytotoxicity 5 days after drug exposure and found a progressive increase in cytotoxicity by increasing oxygen concentrations (Yamauchi et al., 1987).

Bleomycin has been safely used in combination with antivirals for the treatment of HIV-related lymphomas (Levine et al., 1996, 2000). We have previously demonstrated the dose-dependent antiviral properties of BLM in vitro, following p24 production in wild-type $HIV_{Ba\text{-}L}$ infected peripheral blood lymphocytes (PBL) as well as human monocyte-derived macrophages, incubated in the presence of BLM for a total of 5 days (Georgiou et al., 2000). With this study we firstly aimed at elucidating the mechanism of viral inhibition by BLM. Secondly, we wanted to establish whether the iron- and oxidative- conditions which contribute to the well known anti-tumour properties of BLM also apply for the antiviral properties of the drug.

## 2. Methods

### 2.1. Cell isolation

Peripheral Blood Mononuclear Cell (PBMC) fractions were isolated from heparinized blood from HIV-1-, HIV-2- and hepatitis B-seronegative donors (Bloodbank, Utrecht, the Netherlands) by Ficoll-Isopaque gradient separation. Cells were washed twice and monocytes in the PBMC fraction were allowed to adhere on fibronectin-coated flasks before the peripheral blood lymphocyte (PBL) fraction was collected. Peripheral blood lymphocyte fraction was of >85% purity by May-Grünwald-Giemsa-staining. Viability was >95% at the point of experiment initiation as determined by trypan-blue exclusion. Isolated PBL ($1 \times 10^6$/ml) were stimulated to proliferate for 3 days with 4 μg/ml phytohemagglutinin (PHA; Sigma Chemical Co., St. Louis, MO) in RPMI-1640 (FBS; Life Technologies Ltd., Paisley, Scotland) medium supplemented with 10% heat inactivated foetal bovine serum (Life Technologies Ltd., Paisley, Scotland) and 10 μg/ml gentamycin. After PHA stimulation the PBL were cultured in medium containing 10 U/ml human recombinant IL-2 (Boehringer, Mannheim, Germany). All incubations were carried out in flat-bottomed 96-well plates at 37 °C, 5% $CO_2$ and 95% air.

Jurkat cells were cultured at a concentration of $0.25 \times 10^6$ /ml for 2 days before initiation of each experiment to ensure that cells were growing in the log phase at the start of each experiment. Cells were grown in RPMI medium containing 10% FBS (Life Technologies Ltd., Paisley, Scotland) and 10 μg/ml gentamycin (Life Technologies Ltd., Paisley, Scotland).

### 2.2. Virion treatment with bleomycin

$HIV_{Ba\text{-}L}$ virus was treated for 2 h singly with either 100 μM $H_2O_2$ (Merck KGaA, Darmstadt, Germany), 0.7 μM BLM (Asta Medica, Brussels, Belgium) or a combination of $H_2O_2$ and BLM at 37 °C. The virions were subsequently washed twice by centrifuging at 14,000 rpm for 30 min to remove any trace of the compounds and used to infect freshly stimulated PBL at an MOI of 0.005. All virion incubations were treated in the same way, regarding washout of drug, thus assuming that the $TCID_{50}$ of the virus is the same in all different incubations. Cells were cultured in the absence of BLM or any other compounds, in medium containing 10% FBS, 10 μg/ml gentamycin and 10 U/ml human recombinant IL-2. After 7 days supernatant samples were taken for p24 antigen analysis. Virus in culture supernatant was inactivated in a final concentration of 0.05% empigen (Calbiochem-Novabiochem Co., La Jolla, CA) and after heating at 56 °C for half an hour. The presence of HIV-1 in the inactivated supernatant was monitored by checking the p24-core antigen using an enzyme-linked immunosorbent assay (ELISA), as previously described (Moore et al., 1990; McKeating et al., 1991). Any reduction in p24 antigen levels could be attributed to reduced infectivity of virions by BLM. As the different viral incubations were treated in the same way (twice washing) we assumed that any viral loss from washing would be equal in all eppendorf tubes where the incubations took place and any eventual differences in the infectivity of these treated virions would reflect the effect of the compound incubations. The amount of virus that we started off with was equal at all incubations.

### 2.3. Nuclear extract preparation for NF-κB measurements and EMSA

$7.5 \times 10^6$ Jurkat cells were incubated in the presence or absence of 2.1 μM BLM and in the presence or absence of phorbol 12-myristrate 13-acetate (PMA) (20 ng/ml) at 37 °C for 1 h. Nuclear extracts were prepared as described previously (Andrews and Faller, 1991). Protein content was determined (Bradford, 1976) and 5 μg nuclear protein per sample was used to determine NF-κB activity by an electromobility shift assay (EMSA). Nuclear extracts were incubated with a double stranded $^{32}$P-labelled probe containing the NF-κB binding motif from the HIV-1 long terminal repeat (LTR) (wild type 5′-AGCTTCAGAGGGGACTTTCCGAGAGG-3′). Incubation was performed at room temperature for 30 min in 20 μl (total volume) of 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 2 μg poly (dI-dC), 1 μg of bovine serum albumin, 100 mM NaCl and 1 ng of probe. Specificity of binding was controlled

by addition of 25, 50 or 100 ng of unlabelled wild-type or mutant (5′-AGCTTCAGAGCTCACTTTCCGAGAGG-3′) probes. For supershift experiments, nuclear extracts were pre-incubated for 10 min on ice with 2 μg of goat polyclonal immunoglobulin-G raised against the NF-κB subunit p50 (D-17) or p65 (C-20) (Santa Cruz Biotechnical, Santa Cruz, CA) before addition of the labeled probe. Samples were loaded on a 5% polyacrylamide gel and run until the free probe was at the end of the gel. Films were exposed to vacuum-dried gels at −70 °C in cassettes containing intensifying screens.

### 2.4. Construction of HIV-LTR constructs coupled to luciferase gene and luciferase measurements

A plasmid HIV-CAT construct was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pHIV-CAT from Dr. Gary Nabel and Dr. Neil Perkins (Nabel and Baltimore, 1987). The HIV-CAT plasmids contained *Hin*dIII and *Bam*HI restriction sites flanking the CAT gene. After the CAT gene was excised out of the plasmid the LUC gene contained in a pGL3-basic vector (Promega, Madison, WI) was ligated into the empty HIV vector. *E. coli* DH5aF′ were made competent with $CaCl_2$ and were subsequently transformed with the pHIV-LUC vector. pHIV-LUC was isolated from these transformants after overnight incubation using the Qiagen isolation kit (Qiagen Inc., Valencia, CA).

Jurkat cells ($5 \times 10^6$ cells) were transfected with 1 μg of a plasmid expressing the luciferase reporter gene, under the control of the HIV-LTR and co-transfected with 1 μg tat expression plasmid and 1 μg β-galactosidase plasmid as control for transfection efficiency. At 2 h after transfection the cells were stimulated with 20 ng/ml PMA and different concentrations BLM were added. After overnight incubations firefly luciferase activity was measured by the promega luciferase assay system (Promega Benelux, Leiden, The Netherlands) and related to β-galactosidase activity using the luminescent β-galactosidase detection kit (Clontech Laboratories Inc., Palo Alto, CA).

### 2.5. Reverse transcriptase (RT) measurements

Enzyme activity from lysed virions was measured by the formation of ds DNA from a template poly(A)-oligo(dT) RNA primer and a nucleotide mix containing digoxigenin- and biotin-labelled nucleotides (Roche Diagnostics, Almere, The Netherlands). RT activity in lysed virions was measured by BLM incubation with a poly(A)-oligo$(dT)_{15}$ template RNA primer and a nucleotide mix containing digoxigenin- and biotin-labelled nucleotides by a colorimetric assay (Roche Diagnostics, Almere, The Netherlands). Incubations were in the presence or absence of either 0.21 or 0.7 μM BLM and 0.25 μM $FeCl_3$ and 10 μM $H_2O_2$. DNA formation was measured after 5 h incubation at 37 °C. The sequence of addition of the compounds to the lysed virions was: BLM and $FeCl_3$ added together before addition to the DNA. Lastly, $H_2O_2$ was added to initiate the reaction.

### 2.6. DNA extraction and R/U5 measurements

PBL were infected for 2 h with the T-cell tropic strain $HIV_{AT}$ at an MOI of 0.02. After excess virus was washed away, 2.1 μM BLM was added for a further 3 h, after which time the cells were pelleted, taken up in DNA-STAT$^{TM}$ (CAMPRO Scientific, Veenendaal, The Netherlands) and stored at −20 °C until DNA isolation according to the Manufacturer's protocol. Primers used for strong-stop minus DNA (R/U5) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification have been reported elsewhere (Schmidtmayerova et al., 1998; Boven et al., 1999). The antisense primer was 5′-biotinylated and a probe designed for each set of DNA amplified was 5′-digoxigenin-labelled. PCR amplification conditions were denaturation at 94 °C for 5 min followed by 38 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 2 min. The DNA product was finally extended at 72 °C for 10 min. Five microliters of the amplified product was quantified using the digoxigenin-labeled probe, by means of a digoxigenin-detection ELISA (Roche Diagnostics, Almere, The Netherlands). Results are expressed as relative fluorescence units (rfu) as the ratio of R/U5 to GAPDH.

### 2.7. Cell-free DNA damaging properties of BLM

DNA from PBL infected for 5 days with $HIV_{AT}$ was isolated and the R/U5 product was amplified using the primers and PCR protocol as described in Section 2.6. Aliquots of this DNA product were used to perform different incubation combinations. The concentrations used in the incubations (in order of adding to the DNA) were 0.21 μM BLM, 0.25 μM $FeCl_3$ and 1 mM $H_2O_2$. All dilutions were prepared in sterile water. Incubations were for 1 h and were carried out at 37 °C. After incubations DNA was loaded on a 1.8% agar gel and visualized using ethidium bromide staining.

### 2.8. Luminol-enhanced chemiluminescence measurements

Luminol enhanced chemiluminescene by the xanthine oxidase system using hypoxanthine as substrate was measured in a Berthold luminometer (Autolumat LB 953). Final concentrations of reagents in reaction tubes were 18 mU/ml xanthine oxidase (Sigma), 540 μM hypoxanthine (Sigma) and 0.15 mM luminol. Iron(III) citrate, deferoxamine (DF; Novartis Pharma, Arnhem, The Netherlands), BLM were used in concentrations of 15 μM in various combinations as shown in Fig. 6. Catalase (CAT; Sigma) was used at a concentration of 500 μg/ml.

### 2.9. Co-incubations of BLM with deferoxamine (DF) or dimethylthiourea (DMTU) in HIV-infected PBL: antiviral dependence on iron and oxidative conditions

DF is an iron chelator and DMTU an $H_2O_2$ and $^{\bullet}OH$ scavenger (Fox, 1984; Curtis et al., 1988). For the BLM incubations in the presence of DF, PBL were infected for 2 h with HIV-$1_{AT}$ at an MOI of 0.02. The cells were then washed twice to remove excess virus and subsequently incubated with 2 μM DF. Two hours after DF pre-incubation 0.21 μM BLM was added to the medium for a total period of 2 days, at which time p24 levels were measured in the supernatants. For the BLM-DMTU (Sigma) co-incubations, PBL were infected for 2 h with HIV-$1_{AT}$ at an MOI of 0.001. After removal of excess virus the cells were incubated with 3 mM DMTU and 0.21 μM BLM. Five days after compound addition, samples were taken for p24 measurements. From previous experience, 2 μM DF within two days had no effect on cellular proliferation (data not shown). This is why we carried out shorter incubation periods with DF (2 days) and use a higher MOI to infect the cells.

### 2.10. Incubations under hypoxic conditions: antiviral dependence on oxidative conditions

PBL were infected with HIV-$1_{Ba\text{-}L}$ at an MOI of 0.005 before excess virus was washed away. The cells were split and half were placed in a conventional incubator with 20% oxygenated environments and the other half in an incubator adjusted to 10% oxygenated environment (Forma model 3337, Forma Scientific, Marietta, Ohio). Bleomycin was titrated and after 7 days supernatant samples were taken for p24 measurements. Cell viability was also monitored at the end of the incubations. The $IC_{50}$ (concentration achieving 50% p24 inhibition) and $CC_{50}$ (cytotoxic concentration where 50% loss in cell viability is noted) values of BLM were calculated using the computer software program CalcuSyn (Chou and Hayball, 1996).

## 3. Results

### 3.1. Virion infectivity is reduced by BLM

In light of studies reporting that the tertiary structure of HIV reverse transcriptase RNA (Carter et al., 1990; Holmes et al., 1993) as well as the RNA strand of a DNA–RNA heteroduplex (Morgan and Hecht, 1994) is a target for BLM, the possible effect of the drug on virion infectivity itself was checked, this time in a cell system. It could be speculated that any damage to the viral RNA or any weak disruption of viral proteins could result in reduced infectivity.

Interestingly, when we investigated the possibility of the drug affecting the virus itself (by a possible effect on viral RNA), this was confirmed. Treating virions for 2 h with BLM in the presence of hydrogen peroxide and subsequently infecting PBL in the absence of BLM for 7 days, resulted in a 38% reduction of p24 antigen (Fig. 1), implying that the infectivity of the virions had been altered by BLM during their 2 h incubation with the drug. In the absence of hydrogen peroxide during these first 2 h of virion incubation, BLM could not exert its effect, which is in agreement with the oxidative dependence of the drug (bar 3 compared to bar 1, Fig. 1, no statistically significant change). Hydrogen peroxide in itself did not result in a significant reduction of viral infectivity (bar 2 compared to bar 1, Fig. 1, no statistically significant change).

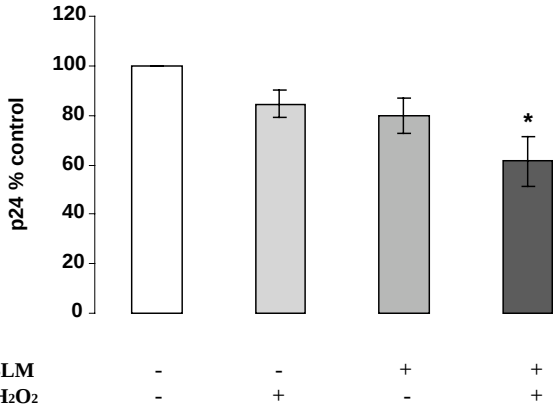


Fig. 1. Bleomycin (BLM) has a direct effect on virions, decreasing infectivity. Virions were pre-treated with the compounds indicated in each bar for 2 h and after twice compound-washout the virions were used to infect peripheral blood lymphocytes. Percentage of p24 was obtained 7 days after infection and 100% p24 represents the value obtained with untreated virions. Results represent the average of four experiments in duplicate. Repeated measures by analysis of variance (ANOVA) and Student's Newman–Keuls test were used to analyze the data. $^{*}P$ values below 0.05 were considered as significant.

### 3.2. NF-κB activation is not affected by BLM

Bleomycin, being an iron chelator, differently to the conventional iron chelator DF, will chelate iron and render it catalytically active (Lazo and Sebti, 1997). Iron catalyzes the Fenton reaction, whereby $Fe^{2+}$ will react with $H_2O_2$ to form the highly reactive hydroxyl radical (Fenton, 1894). Bleomycin could thus possibly be involved in the pathway of NF-κB activation by reducing the levels of available iron in the cell and thus decreasing the formation of the hydroxyl radical ($^{\bullet}OH$) generated by the Fenton reaction. On the other hand, theoretically, BLM could result in the activation of NF-κB due to the increase in ROS formation as a result of this iron chelation and the property of the activated BLM-$Fe^{2+}$ complex to generate ROS. Experiments were conducted to see whether NF-κB activation was in either way affected by BLM, directly by checking for NF-κB by EMSA (Fig. 2A) and indirectly by investigating whether overall proviral transcription was affected by the drug (Fig. 2B). No significant change in the p50/p65 signal, was observed (Fig. 2A), indicating that the pathways

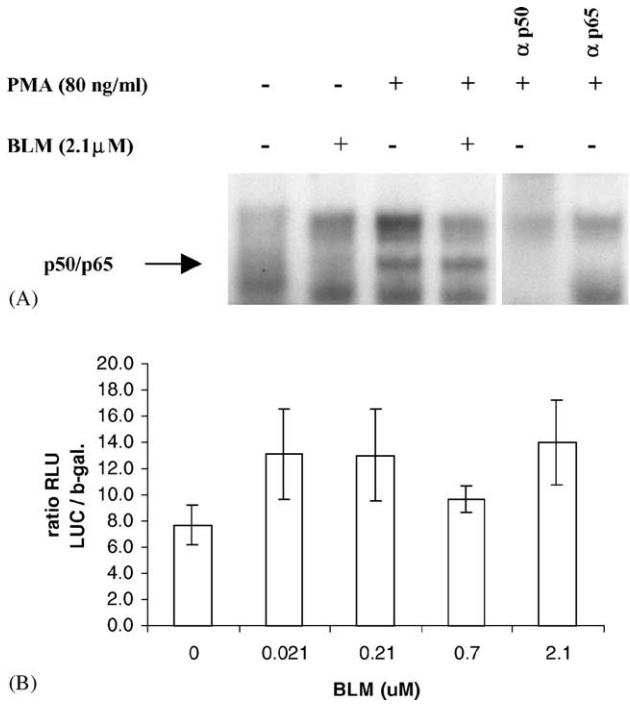


Fig. 2. (A) NF-κB activation is not influenced by bleomycin (BLM) in Jurkat cells. A total of $7.5 \times 10^6$ cells were incubated in the presence or absence of bleomycin and in the presence or absence of PMA stimulation. Nuclear extracts were prepared and NF-κB activity measured by gel-shift assay. (B) Viral transcription controlled by the HIV-LTR is not affected by BLM. Jurkat cells ($5 \times 10^6$ cells) were transfected with 1 μg of a plasmid expressing the luciferase reporter gene, under the control of the HIV-LTR and co-transfected with 1 μg tat expression plasmid and 1 μg β-gal plasmid as control for transfection efficiency ($n = 4$ in duplicate). Repeated measures by analysis of variance (ANOVA) and student Newman–Keuls test were used to analyze the data as well as Student's *t*-test.

leading to NF-κB activation remain functional in the presence of BLM.

The ability of HIV to replicate is dependent on NF-κB activation as the long terminal repeat (LTR) of the virus contains two adjacent NF-κB binding sites that play a central role in mediating inducible HIV-1 gene transcription (Fauci, 1996). Transcription efficiency in the presence of BLM in Jurkat cells transfected with the HIV-LTR coupled to a luciferase reporter gene was investigated, after overnight compound incubation. Compared to untreated cells no significant change in luciferase production was noted by BLM concentrations up to 2.1 μM (Fig. 2B), implying that NF-κB dependent proviral HIV-LTR transcription is not affected by BLM. The apparent enhancement of luciferase expression seen in Fig. 2B with various concentrations of BLM was not significant and could be attributed to differences in transcription efficiency between the cell incubations with the different concentrations. In addition, we have never recorded an increase in viral replication by any of the BLM concentrations investigated in Fig. 2B, which would justify any possible significant increase in LUC/β-gal signals (results not shown). From the findings it emerged that the drug exerts its antiviral effects at a stage other than proviral transcription and this does not involve inhibition of NF-κB activation as was suggested for the iron chelator DF (Sappey et al., 1995).

### 3.3. The reverse transcriptase enzyme is not affected by BLM

The possibility of BLM inhibiting the reverse transcriptase (RT) enzyme was investigated by monitoring the activity of this enzyme in cell-free incubations. Measuring the activity of this enzyme employs the build-up of a DNA product in the presence of the enzyme and labeled nucleotides provided in the assay kit (Roche Diagnostics, Almere, The Netherlands). Intensity of coloration relates to the amount of ds DNA formed and subsequently gives an indication to the amount of RT enzyme present in the corresponding incubations facilitating the reaction. The reactions proceeded under oxidative conditions, in the presence of 10 μM $H_2O_2$ and 0.25 μM $FeCl_3$. The amount of DNA product built-up as a result of reverse transcription in the presence of BLM within 5 h was the same as that which was achieved in the absence of BLM (Fig. 3).

A concentration of BLM up to 0.21 μM did not influence the activity of the RT enzyme in lysed virions (Fig. 3). BLM at 0.7 μM had a limited (15%) effect on the RT activity. Regarding the choice of ds DNA formed in the RT reaction, it has been shown that even though ferrous bleomycin is capable of binding both to d(ATATAT) and d(CGCGCG), only with the latter the conformation of the iron ligands changes, resulting in the preferential cleaving 5′-GpPy-3′ sequences (Sam et al., 1998). The template for the reaction in this assay kit consisted of a poly-(dA) oligo-(dT) RNA primer. Thus, the d(ATATAT) formed as a result of the RT reaction should not be affected by the activated ferrous BLM complex, enabling its measurement in this particular assay.

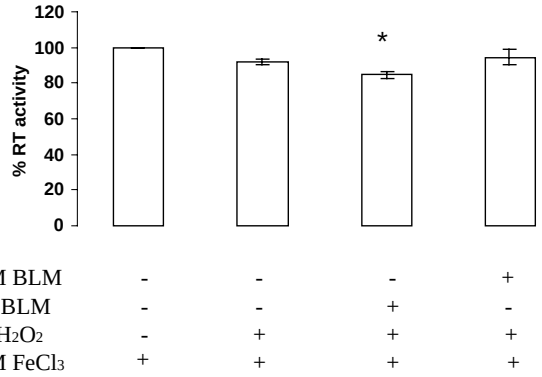


Fig. 3. Reverse transcriptase (RT) is functional in cell lysates incubated with BLM. RT activity was measured by BLM incubation with a poly(A)-oligo$(dT)_{15}$ template RNA primer and a nucleotide mix containing digoxigenin- and biotin-labelled nucleotides in lysed virions ($n = 3$ in duplicate, $^*P < 0.05$).

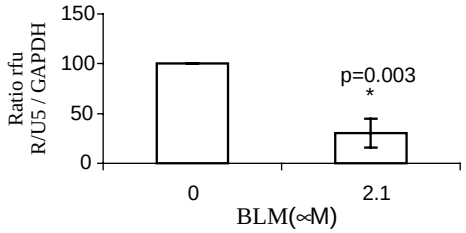


Fig. 4. BLM inhibits viral R/U5 formation. PBL were infected for 2 h with $HIV_{AT}$ at an MOI of 0.02. After excess virus was washed away the cells were incubated with BLM for an additional 3 h before DNA extraction. Results are expressed as relative fluorescence units as the ratio of R/U5 to GAPDH ($n = 3$ in duplicate).

### *3.4. Bleomycin affects the viral R/U5 DNA product formation*

Since the RT activity in the presence of BLM was found to be unaffected, we investigated the completion of the reverse transcription of viral RNA by quantifying for the R/U5 (strong-stop minus DNA) product, one of the early HIV DNA products formed after reverse transcription. Cell viability in these incubations was monitored and found to be >99% after compound incubation and at the point of DNA extraction. Viral DNA reduction values were represented as the ratio of R/U5 signal in relation to GAPDH levels (Fig. 4). Interestingly, the GAPDH levels isolated from samples incubated with BLM were not significantly different from control samples with no drug incubations (not shown). This would imply that GAPDH being the genomic control is not significantly affected by BLM. A 70% reduction of the R/U5 to GAPDH DNA ratio was observed in cells incubated with BLM, implying that even though the RT enzyme activity remained intact in the presence of the compound (Fig. 3), DNA formation following the completion of the RT step was affected (Fig. 4). R/U5 DNA was also measured 4 days after addition of a ten times lower concentration BLM (0.21 μM) than used in Fig. 4, and in the presence of 1 μM AZT to avoid re-infection. A 20% reduction in the signal was noted ($P < 0.025$) (ratio R/U5 to GAPDH of control cells = 10, ratio R/U5 to GAPDH of cells incubated with 0.21 μM BLM = 8 (not shown)).

### *3.5. DNA damaging properties of BLM in a cell-free system*

The DNA-damaging properties of BLM are believed to be the mechanism of the anti-tumor activity of BLM. The same properties could be responsible for damaging the viral product R/U5. We demonstrated this DNA damaging property of BLM in a cell-free system. Amplified R/U5 DNA product was incubated in the presence of 0.21 μM BLM with various combinations of 0.25 μM $FeCl_3$, 1 mM $H_2O_2$, 100 μM of the iron chelator DF or 3 mM of the oxygen radical scavenger DMTU. One hour after DNA compound incubations, the presence of the 132 bp R/U5 fragment was visualized in a 1.8% agarose gel. The results (Fig. 5A) showed that a combination of BLM, $FeCl_3$ and $H_2O_2$ resulted in complete disappearance of the DNA band (Fig. 5A, lane 9), whereas any of these compounds separately did not have an effect (Fig. 5A, lanes 2–4, respectively).

The DNA product of lane 6 was also damaged, that is following incubation with BLM and $H_2O_2$, with no external addition of iron. This implies that the PCR mix contained enough traces of iron to facilitate DNA strand breakages by BLM. The damage in the absence and presence of this external iron source (Fig. 5A, lanes 6 and 9, respectively) seems to be of the same magnitude. Addition of either 2 μM DF, either simultaneously with BLM to DNA and in the absence of Fe (Fig. 5A, lane 7) or as an iron chelator of the externally added $FeCl_3$ before addition to BLM (Fig. 5A, lane 10), resulted in complete restoration of the band, implying complete protection from the DNA-damaging properties of BLM once the iron source is removed from the environment. The difference between the incubations in lanes 10 and 11 is that in the latter the iron source was added together with BLM before addition of DF. In this lane restoration of the DNA band was not as effective as in lane 10 where the iron source is completely removed from the environment by first adding DF to the iron source before addition to BLM. Addition of DMTU in the absence (Fig. 5A, lane 8) of an external iron source resulted in protection from the DNA damaging properties of BLM. In the presence of an iron source only partial protection was noted (Fig. 5A, lane 12).

### *3.6. Reactive oxygen radical production by BLM: mechanistic dependence to oxidative conditions and iron*

In a $H_2O_2$ and $O_2^{\bullet -}$ generating- system employing hypoxanthine (HX) and xanthine oxidase (XO) the mechanistic dependence of BLM on oxidative conditions and iron is demonstrated (Fig. 5B). The addition of iron in the form of iron(III) citrate to the HX/XO system resulted in an increase in chemiluminescence from 3.68 to $5.4 \times 10^6$ cpm.

The addition of 15 μM BLM significantly enhanced the chemiluminescence from $3.68 \times 10^6$ to $5.7 \times 10^6$ in the absence of iron(III) and from $5.4 \times 10^6$ to $7.15 \times 10^6$ cpm in the presence of iron(III), pointing towards oxygen radical production by BLM. As this was able to proceed in the absence of external iron source, the presence of contaminant iron in the reaction medium seemed to be sufficient to enhance the production of oxygen radicals ($P = 0.002$ between chemiluminescence in the absence and presence of iron).

The addition of either CAT or DF in iron(III) citrate incubations reduced the chemiluminescence to a level of $0.83 \times 10^6$ and $0.88 \times 10^6$ cpm, respectively. The chemiluminescence increase noted in the presence of BLM was inhibited in the presence of DF (70% inhibition) and CAT (88% inhibition). When both DF and CAT were present in addition to BLM and iron, 90% inhibition was noted.

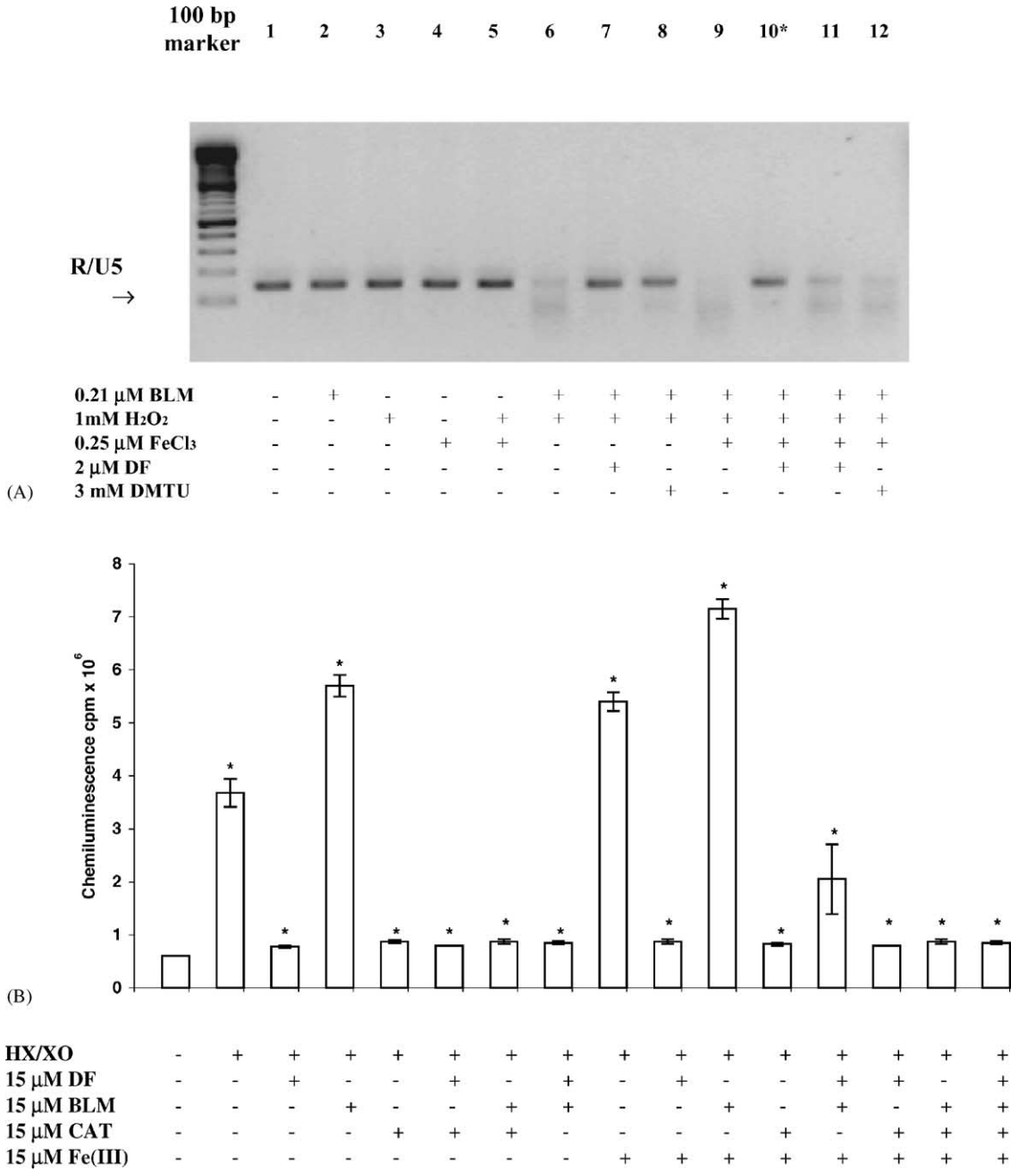


Fig. 5. (A) Representative experiment of DNA-damaging properties of bleomycin (BLM) in a cell-free system. DNA from PBL infected for 5 days with $HIV_{AT}$ was isolated and the R/U5 product was amplified. Aliquots of this product were used to perform different incubation combinations. The concentrations used in the incubations (in order of adding to the DNA) were 0.21 μM BLM, 0.25 μM $FeCl_3$ and 1 mM $H_2O_2$ followed by either 2 μM DF or 3 mM DMTU, except for ∗ lane 10 where DF and $FeCl_3$ were added together prior to addition to the rest of the reagents. (B) Luminol enhanced chemiluminescence using hypoxanthine (HX) as substrate and xanthine oxidase (XO) generating system. Deferoxamine (DF), bleomycin (BLM), catalase (CAT) and iron(III) citrate were each used in a concentration of 15 μM in various combinations ($n = 4$, Student's $t$-test, $^*P < 0.003$).

### *3.7. Viral replication inhibition by BLM is reversed after removing the iron and oxidative source from the cell culture system*

Co-incubations of BLM with either DF or DMTU, resulted in complete recovery of p24 antigen (Fig. 6), implying that in the absence of either iron or oxidative conditions, respectively, BLM is not able to exert its anti-viral properties. In these experiments cellular proliferation as well as viability were also monitored and found to be >99% (data not shown).

### *3.8. Bleomycin at hypoxic conditions*

The well documented anti-tumour properties of BLM are iron- and oxygen-dependent. We wanted to investigate the antiviral mechanistic dependence of BLM on oxygen and for

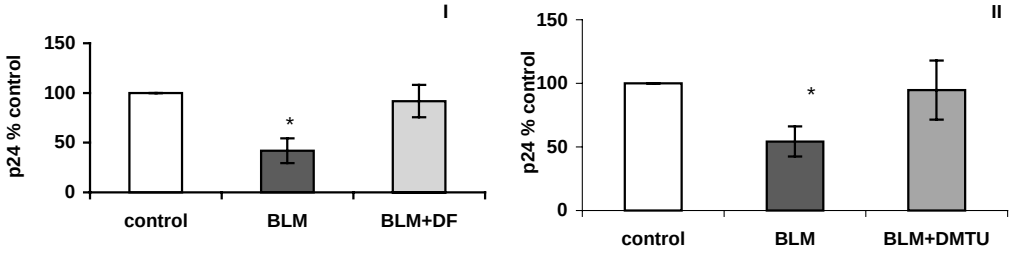


Fig. 6. The antiviral properties of BLM in HIV-1 infected PBL under iron- and oxidative-withholding conditions. For the BLM/DF co-incubations, stimulated PBL were infected for 2 h with HIV-$1_{AT}$ at an MOI of 0.02. The cells were then washed twice to remove excess virus and subsequently incubated with 2 μM DF. Two hours after DF pre-incubation 0.21 μM BLM was added to the medium for a total period of 2 days, at which time p24 levels were measured in the supernatants (I). For the BLM/DMTU co-incubations, PBL were infected for 2 h with HIV-$1_{AT}$ at an MOI of 0.001. After removal of excess virus the cells were incubated with 3 mM DMTU and 0.21 μM BLM. At 5 days after compound addition, samples were taken for p24 measurements (II). Repeated measures by analysis of variance (ANOVA) and Student's Newman–Keuls test were used to analyze the data. $^*P$ values below 0.05 were considered significant ($n = 3$ in duplicate for each set of incubations).

Table 1
Antiviral activity and cytotoxicity of bleomycin (BLM) against HIV-$1_{Ba\text{-}L}$ infected peripheral blood lymphocytes incubated with the drug either at 20% or 10% oxygen environments

| | Percent oxygen conditions | | |
|---|---|---|---|
| | 20% | 10% | *P* value (significance) |
| $IC_{50}$ (μM) | 0.37 ± 0.12 | 3.43 ± 1.02 | 0.041 |
| $CC_{50}$ (μM) | 7.52 ± 5.83 | 35.23 ± 4.22 | 0.018 |

PBL were infected with HIV-$1_{Ba\text{-}L}$ at an MOI of 0.005 for 2 h, at 20% oxygen conditions. Excess virus was washed away and the cells were split between the two incubators with the different oxygen conditions. After 7 days incubations with various concentrations of BLM, p24 supernatant samples were measured. Using the computer program Calcusyn (Chou and Hayball, 1996) the $IC_{50}$ (concentration achieving 50% p24 inhibition) and $CC_{50}$ (cytotoxic concentration where 50% loss in cell viability is noted) values of the drug were calculated. Results are the average of three experiments on three different PBL donors. Statistical analysis was done by Student's *t*-test.

this reason we observed activity of the drug in HIV-infected PBL grown in either 20 or 10% oxygen conditions. At 20% oxygen, the $IC_{50}$ of BLM was 0.37 μM and the $CC_{50}$ 7.52 μM (Table 1). These two values increased to 3.43 and 35.23 μM, respectively, representing a 9- and 5-fold increase in the $IC_{50}$ and $CC_{50}$ value of BLM, respectively, at hypoxic conditions (10% oxygen). The $IC_{50}$ and $CC_{50}$ values at 10% oxygen conditions were significantly higher than those recorded at 20% oxygen.

## 4. Discussion

Alternative targets of attack of the human immunodeficiency virus (HIV) are necessary in light of infection persistence due to onset of resistance after conventional reverse transcriptase, protease and, more recently, fusion inhibitor therapy. Recently, the potential usefulness of non-nucleoside anticancer chemotherapeutics as a novel approach to develop anti-HIV drugs has been reviewed (Sadaie et al., 2004). In this study, we describe the antiviral mechanisms underlying the oxygen-radical generating agent and anticancer chemotherapeutic agent, BLM. We postulate the following mechanism of HIV-1 inhibition by the drug: it is capable of reducing viral DNA situated in the host cytoplasm in a different manner than conventional reverse transcriptase inhibitors. DNA measurements of viral R/U5 and cellular GAPDH (represented in Fig. 4) after 3 h BLM incubations revealed unchanged GAPDH levels between HIV-infected cells with or without drug incubations, indicating that cytoplasmic viral DNA could be more readily accessible to BLM than integrated nuclear proviral DNA. Moreover, BLM was found to have antiviral effects not only on virus-infected cells but also directly on virions, in a cell-free system, decreasing their infectivity by 38%. It could be speculated that damage to the viral RNA or any weak disruption of viral proteins resulted in reduced virion infectivity.

We also report the antiviral dependence of the antitumour agent BLM on iron and oxidative conditions. Yamauchi et al. reported in 1987 a progressive increase in BLM cytotoxicity in human sarcoma cells 5 days after drug exposure by increasing oxygen concentrations. This is in agreement with our results when human PBL were incubated at environments of 10% compared to 20% oxygen concentration. The $CC_{50}$ of BLM was increased by a factor 5 in cells incubated at 10% oxygen levels, showing the mechanistic dependence of BLM to oxygen. At the same time, the antiviral capacity of BLM was decreased by a factor 9 at the lower oxygen environments (Table 1). We have also looked at the antiviral properties of BLM in a 1% oxygen environment. However, even at the highest drug concentration BLM used in the incubations (3μM), there was neither a reduction in p24 antigen production, nor any effect on cell viability (results not shown). Due to the lack of a dose-response effect at 1% oxygen conditions no $IC_{50}$ or $CC_{50}$ values could be calculated. The significance of the findings shown in Table 1 is of clinical interest and has to be taken into account in patients being treated with BLM. Increased oxygen intake during surgery, for example, could result in an increase in the toxicity of the drug.

This would imply that the well-known antitumour mechanism of the drug, namely the DNA and RNA damaging properties and ROS production in the presence of iron and oxidative conditions, could be the mechanism by which the drug exerts its antiviral action.

All approved anti-HIV drugs are aimed at blocking viral replication by inhibiting either HIV reverse transcriptase or protease enzymes and more recently fusion of virus with the cells. Combinations of these antiviral agents can control infection in many HIV-1-infected individuals; however, they do not eliminate viral replication in secondary lymphoid tissues. In addition, replication-competent virus persists in resting memory CD4 T-cells despite long term treatment, this making the outlook for possible eradication of the virus doubtful (Zhang et al., 2000). The development of new drugs that would target other events of HIV-1 replication or use alternative approaches may become advisable in the future. After the initial hope of virus eradication by HAART was not met, the word "eradication" in relation to HIV-1 was again recently mentioned (Yang et al., 2002). The possibility of employing existing cytotoxic drugs used in cancer therapy to inhibit HIV replication was touched on in the aforementioned article and we believe that the elucidation of the mechanism of action of the chemotherapeutic cytotoxic agent BLM as we report it in this study, reinforces the need to look into combinations of cytotoxic with antiviral agents in the treatment of HIV infection. The major concern so far with the clinical use of BLM is the cytotoxicity of this drug which could result in lung fibrosis in patients receiving a cumulative dose of higher than 450 mg (Blum et al., 1973). We do not exclude the usefulness of this alternative mechanism of viral degradation provided that administration of the drug is targeted. Liposomes have proven their potential to reduce the toxic effects of anticancer agents and to maintain or enhance their therapeutic effects (Hengge et al., 2001; Lasic and Papahadjopoulos, 1995). It could be speculated that low concentrations of the drug would already be effective in an HIV infection, since it was shown that HIV-infected patients have less antioxidant protection due to 30% lower glutathione concentrations in their plasma than in normal individuals (Buhl et al., 1989). We have recently shown that BLM synergizes with either zidovudine, ritonavir or indinavir in a two-drug combination to decrease HIV-1 replication in vitro (Georgiou et al., 2001); these findings further support the speculation that lower concentrations of the drug could be effective in vivo, in the presence of other antivirals. In addition and given the drug's dependence on iron and oxidative conditions, manipulation of these two parameters (for example, administration of an iron chelator) could also determine to some degree the level of drug susceptibility and control onset of toxicity.

This is the first study published describing the antiviral mechanism of BLM using HIV-1-infected human cells. This mechanism might potentially be a novel pathway for treating HIV infections. We believe that BLM or less toxic derivatives of the drug, as part of combination therapy on the one hand and the effects of the drug per se on HIV-related lymphomas on the other hand, merits further investigation.

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