Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA

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Background: The human immunodeficiency virus type 1 (HIV-1) regulatory protein Rev is required for unspliced and incompletely spliced viral mRNAs to appear in the cytoplasm and thus for viral replication. Translocation of Rev from the nucleus to the cytoplasm is essential if Rev is to function. We wanted to identify inhibitors of this transport process because they would be potential antiviral agents.

Results: The *Streptomyces* metabolite, leptomycin B, and other antibiotics of the leptomycin/kazusamycin family were identified as inhibitors of the nucleo-cytoplasmic translocation of Rev at nanomolar concentrations. Rev-dependent export of mRNA into the cytoplasm is also blocked by leptomycin B, which inhibits Rev-dependent, but not Rev-independent gene expression in a short-term transfection assay. In primary human monocytes, leptomycin B suppresses HIV-1 replication.

Conclusions: Leptomycin B is the first low molecular weight inhibitor of nuclear export to be identified. Although it cannot be used therapeutically, it should serve as a valuable tool for dissecting nuclear export pathways.

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Keywords: HIV-1 Rev, leptomycin B, nuclear export

Received: **17 October 1996**
Revisions requested: **7 November 1996**
Revisions received: **28 January 1997**
Accepted: **29 January 1997**

Electronic identifier: **1074-5521-004-00139**

Chemistry & Biology February 1997, 4:139–147

© Current Biology Ltd ISSN 1074-5521

Introduction

The acquired immunodeficiency syndrome (AIDS) is a life-threatening disease caused by HIV-1 [1]. The HIV-1 genome encodes several regulatory proteins, in particular Tat and Rev, that are essential for virus replication [2,3]. Rev is necessary for the appearance in the cytoplasm of unspliced and singly spliced viral mRNAs that encode the structural proteins Gag, Pol and Env [4,5]. Rev is a nuclear protein [6] and it interacts with a highly structured RNA, the Rev response element (RRE), in the viral *env* gene [7–10].

Since Rev is a viral protein lacking a cellular counterpart, several different strategies have been employed to inhibit HIV-1 replication by inhibiting the function of the Rev protein. One strategy has been to use antisense phosphorothioate oligonucleotides against Rev to suppress HIV in chronically infected cells [11]. Using gene therapy approaches, *trans*-dominant mutants of HIV-1 Rev [12], dominant-negative mutants of the cellular Rev cofactor eIF-5A [13], intracellular expression of an anti-Rev single chain antibody construct [14], and RRE-decoys [15] have all been shown to inhibit HIV-1 infection in cells. In a more classical approach using low molecular weight substances, neomycin was found to block Rev–RRE binding and to antagonize Rev function [16] and an 8-alkylpyrimidinone was found to antagonize cellular Rev response [17].

Recently, we and others have demonstrated that the export of Rev from the nucleus to the cytoplasm is crucially important for its function and the export depends on the Rev activation domain [18–20], which serves as a nuclear export signal [21,22]. Rev translocates from the nucleus to the cytoplasm in HeLa and COS cells transfected with Rev under conditions where rRNA synthesis is inhibited by, for example, actinomycin D (AD). It is clear that the appearance of Rev in the cytoplasm reflects export and not *de novo* synthesis of Rev protein in the cytoplasm, because the reaction takes place in the presence of the protein synthesis inhibitor, cycloheximide [18–20]. Furthermore, dominant-negative mutants of Rev with mutations in the activation domain (nuclear export signal) are unable to leave the nucleus upon treatment with AD [18–20].

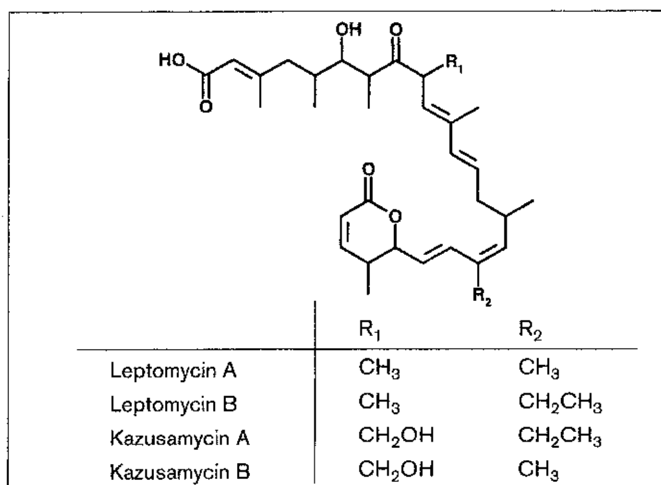
In a screening assay for low molecular weight inhibitors of Rev nuclear export, we have now found four antibiotics of the leptomycin/kazusamycin family that completely block this process at nanomolar concentrations. Leptomycin B (LMB) also inhibits Rev function and HIV-1 replication.

Results

LMB inhibits cytoplasmic localization of Rev, but not protein transport into the nucleus

Nucleo-cytoplasmic translocation of Rev induced by AD in HeLa cells transfected with Rev (HeLa-Rev cells) was used as a screen for inhibitors of Rev function. A streptomycete extract was identified with very potent inhibitory activity, and four compounds, leptomycins A and B and kazusamycins A and B (Fig. 1), were isolated as the active components.

Figure 1



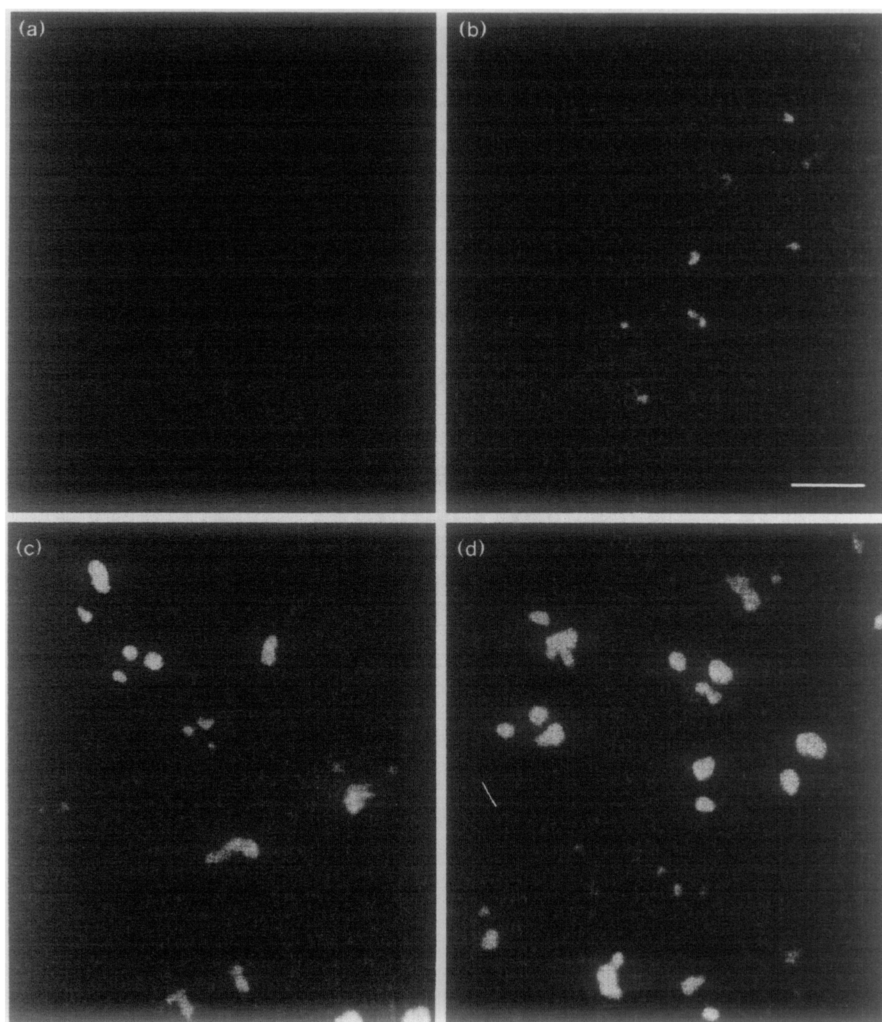
	R_1	R_2
Leptomycin A	CH_3	CH_3
Leptomycin B	CH_3	CH_2CH_3
Kazusamycin A	CH_2OH	CH_2CH_3
Kazusamycin B	CH_2OH	CH_3

Structures of leptomycin and kazusamycin antibiotics.

When HeLa-Rev cells were serum-starved, they did not produce appreciable amounts of Rev protein (Fig. 2a). Rev synthesis started within 1 h of serum addition, and the protein was observed to be in the nucleoli when a thin optical section (0.7 μm) was taken with the confocal microscope (Fig. 2b). But, a thicker optical section (1.8 μm) revealed that low levels of Rev were also always present in the cytoplasm (Fig. 2c). After 7 h, a high level of Rev was reached (Fig. 3a) and if, during the last 2 h of the incubation, AD was added, Rev was almost exclusively localized in the cytoplasm (Fig. 3b).

Upon incubation with LMB, the appearance of Rev in the cytoplasm was completely inhibited and Rev was localized instead to the nucleoplasm and the outer rim of the nucleoli (Fig. 3d). This nuclear retention was most obvious in the AD-treated cultures, but was also clearly visible in the absence of AD (Fig. 2d). In this experiment, the cells had been preincubated with 2 nM LMB for 1 h before the addition of serum and then further incubated

Figure 2



Rev localization in HeLa-Rev cells. HeLa-Rev cells were serum-starved for 48 h and fixed either **(a)** immediately or **(b, c)** after 1 h of serum addition to stimulate Rev synthesis. **(d)** Cells were preincubated with 2 nM LMB for 1 h before the addition of serum, after which they were incubated for a further 1 h. Cells were immunostained for Rev protein and examined by confocal microscopy as described in the Materials and methods section. Note that the thickness of the optical sections is 0.7 μm for (a,b) but 1.8 μm for (c,d). Scale bar is 10 μm.

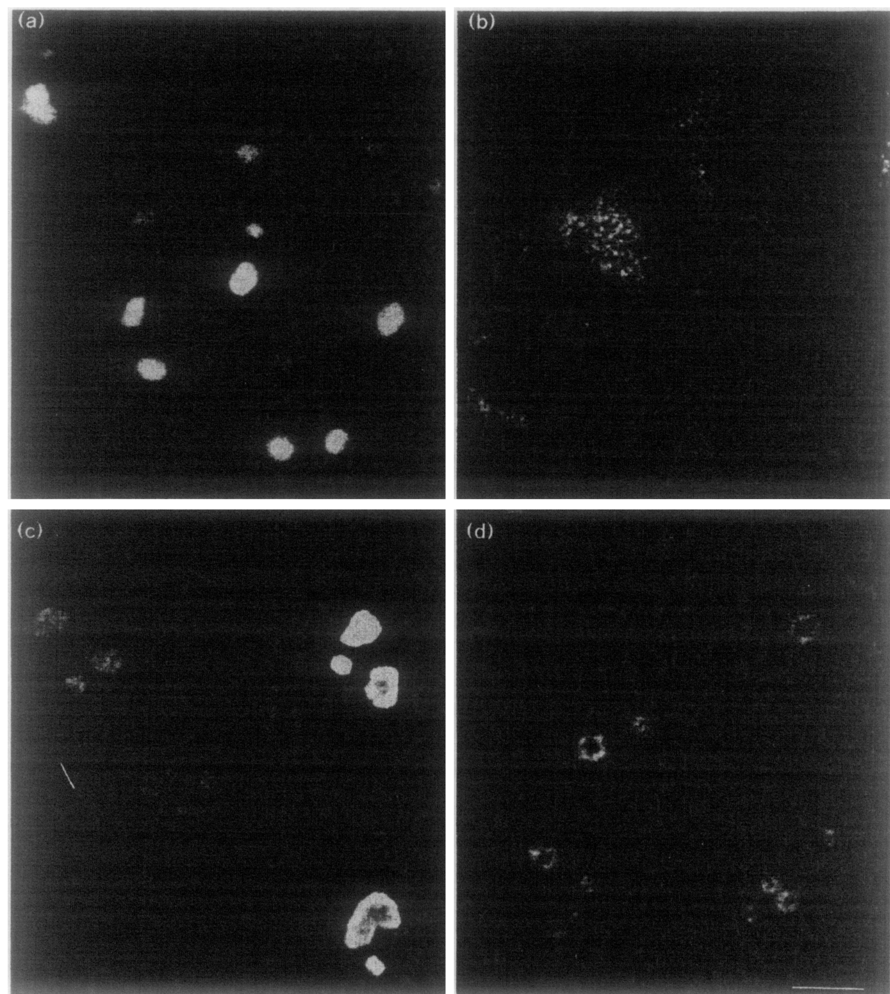
for 1 h. As revealed by the thick optical section of the confocal microscope (Fig. 2d), the low levels of Rev immunoreactivity seen in the cytoplasm in the absence of LMB (Fig. 2c) had completely disappeared. The presence of cycloheximide did not change either the nuclear export induced by AD [18–20] or its inhibition by LMB (data not shown). While the results depicted in Figures 2 and 3 were obtained with a total incubation time with the drug of 2 h and 7 h, respectively, the effect of LMB on nuclear accumulation of Rev could be observed as early as 30 min after the addition of the drug (data not shown).

In order to find out whether this effect was a general inhibition of nucleo-cytoplasmic trafficking, the effect of LMB on Rev nuclear import was investigated. HeLa-Rev cells were incubated with LMB for 1 h before the addition of serum to induce Rev synthesis. This incubation had no effect on the nucleolar localization of Rev, even after another 7 h of incubation (Fig. 3c). Taken together, the results shown in Figures 2 and 3 indicate that LMB specifically blocks nuclear export, but not import of HIV-1 Rev.

In order to characterize further the effect of LMB on nuclear protein import and in order to exclude the possibility that import would be inhibited at higher drug concentrations, an *in vivo* test system and an *in vitro* test system were used.

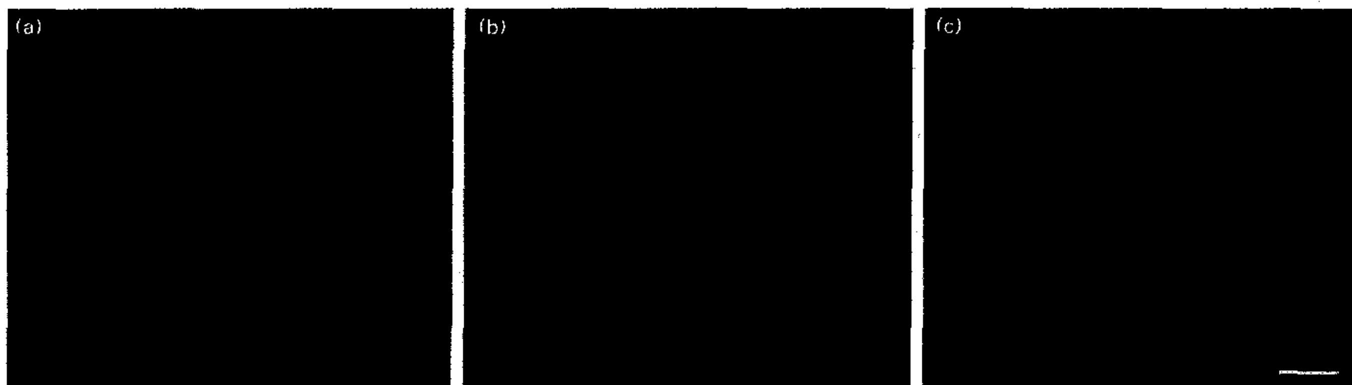
The p65 subunit of the transcription factor NFκB has been shown to translocate from the cytoplasm into the nucleus upon cell activation with, for example, tumor necrosis factor α (TNF-α) [23,24]. The protein NFκB uses a classical nuclear localization signal (NLS) that is masked by binding to IκB in the absence of stimulation [25,26]. When HeLa cells were grown in medium, NFκB p65 was localized in the cytoplasm (Fig. 4a). After incubation with TNF-α for 30 min, most of the protein had translocated to the nucleus (Fig. 4b). This nuclear import was not affected by the presence of 1 μM LMB 30 min

Figure 3



Effect of LMB on the subcellular localization of Rev. HeLa-Rev cells were serum-starved for 48 h and then stimulated to synthesize Rev by the addition of serum. After 7 h, cells were fixed and immunostained for Rev protein as described in the Materials and methods section. **(a)** Control cells incubated with medium only; **(b)** cells incubated with 1 $\mu g\,ml^{-1}$ AD during the last 2 h; **(c)** cells pre-incubated with 2 μM LMB for 1 h in serum-free medium and then with complete medium containing LMB for 7 h; and **(d)** cells incubated with medium containing 2 μM LMB for 7 h and 1 $\mu g\,ml^{-1}$ AD during the last 2 h. The thickness of the optical sections is 0.7 μm. Scale bar is 10 μm.

Figure 4

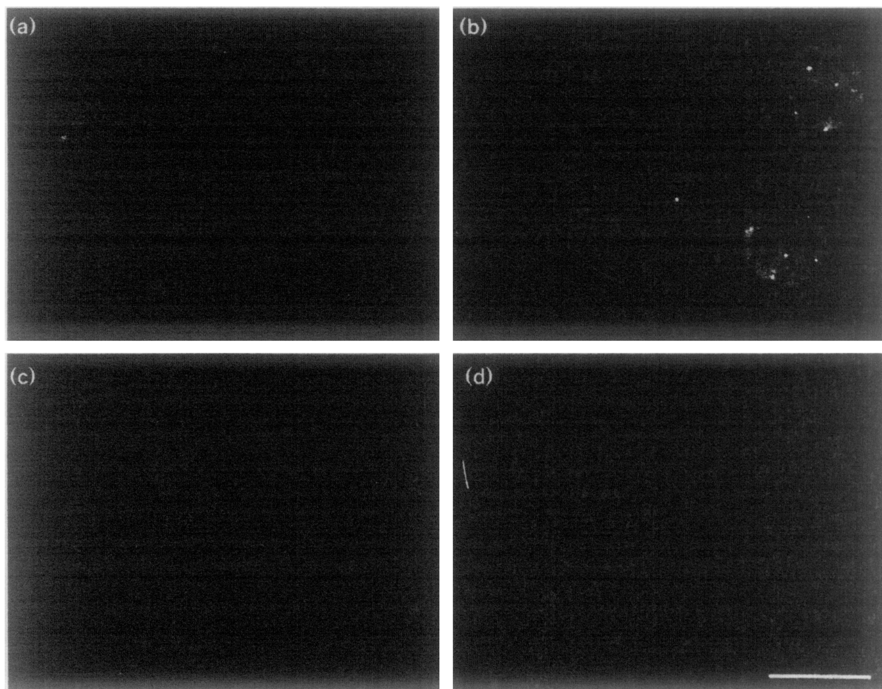


Nuclear import of NFκB p65 in the presence of LMB. HeLa cells were fixed and stained for NFκB p65 as described in the Materials and methods section. **(a)** Control cells incubated with medium only; **(b)** cells stimulated with 200 U ml^{-1} TNF-α for 30 min to induce nuclear localization of p65; and **(c)** cells incubated with 1 μM LMB for 30 min before and during the stimulation with TNF-α. Scale bar is 20 μm.

before and during the TNF-α treatment (Fig. 4c). Similarly, in COS cells the nuclear localization of SV40 Large T antigen was not affected even after a 48 h incubation with LMB (data not shown).

Nuclear import can be reconstituted *in vitro* in an assay using semi-permeabilized HeLa cells and a fluorescent import substrate, for example, FITC-labelled bovine serum albumin (BSA) coupled to a synthetic peptide containing the SV40 Large T antigen nuclear localization signal (FITC-BSA–NLS) [27]. After a 30 min exposure of this import substrate with digitonin-permeabilized HeLa cells in the presence of HeLa cell cytosol, the fluorescent protein was taken up by the nuclei (Fig. 5a). This import was not affected by the presence of 1 μM LMB (Fig. 5b), but was almost completely abolished by the addition of the import inhibitor GTPγS [28] (Fig. 5c) or by performing the reaction at 0°C (Fig. 5d), which prevents the active transport process involved in nuclear import [27].

Figure 5



In vitro nuclear import assay in semi-permeabilized HeLa cells. Digitonin-permeabilized HeLa cells were incubated with HeLa cytosol and FITC-BSA–NLS nuclear import substrate as described [27]. **(a)** Import of FITC-BSA–NLS; **(b)** in the presence of 1 μM LMB; **(c)** in the presence of 0.5 μM GTPγS; and **(d)** at 0°C. Scale bar is 15 μm.

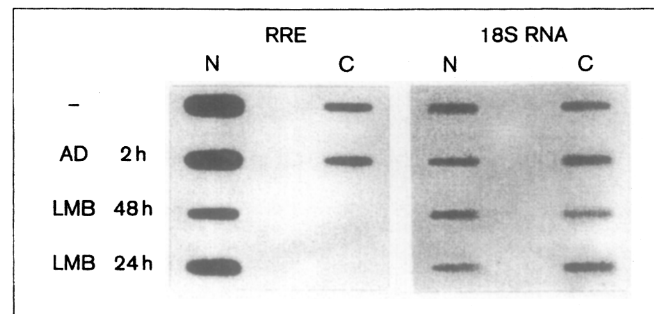
Taken together, the *in vivo* and *in vitro* import results show that LMB has no effect on protein import, even at concentrations more than 1000-fold higher than those required for the inhibition of Rev export (Table 1).

An inhibitory effect on Rev export was found not just for LMB but also for leptomycin A and kazusamycins A and B. The concentrations required for a 50% inhibition of transport are given in Table 1, together with the values for the antiproliferative effect after 7 h, 24 h, 48 h and 72 h. While there appeared to be a therapeutic window after short periods of incubation (7 h and 24 h), the concentrations required for inhibition of Rev export and 50% inhibition of cell growth after 72 h were almost identical. Therefore, for most subsequent experiments, incubation times shorter than 72 h had to be chosen in order to evaluate a Rev-specific effect. Since LMB was the most abundant and the most potent of the four *Streptomyces* metabolites, it was used for all additional experiments.

LMB inhibits the appearance of RRE-containing mRNA in the cytoplasm

In order to find out whether inhibition of Rev export from the nucleus would, as a consequence, prevent the appearance of RRE-containing mRNA in the cytoplasm, COS cells were co-transfected with expression plasmids for Rev and for Tat. The Tat plasmid contains an RRE, so its expression is dependent on Rev function [29]. The cells were treated with LMB for 24 h or 48 h, and cytoplasmic and nuclear RNA were prepared. Slot blot analysis was performed using a probe specific for RRE and a probe for 18S RNA to control the RNA loading of the blot (Fig. 6). In a parallel experiment, transfected COS cells were analyzed for the subcellular localization of Rev protein (Fig. 7). In the control cultures and the cells treated with AD for 2 h, both nuclear and cytoplasmic RRE-containing RNA could be detected (Fig. 6). Using immunofluorescence, untreated control cultures showed mostly nucleolar and nuclear Rev, but also some cytoplasmic Rev (Fig. 7a). While AD induced complete cytoplasmic accumulation of the Rev protein after 2 h (Fig. 7b), it had no effect on the localization of RRE-containing RNA (Fig. 6). In the LMB-treated cultures, however, nuclear but not cytoplasmic RRE could be detected, and the amount of 18S RNA was identical to the controls (Fig. 6). The immunolocalization of Rev in the LMB-treated cells shows that the protein is sequestered in the nucleus both in the absence (Fig. 7c) and in the presence (Fig. 7d) of AD during the last 2 h of the 48 h exposure to the drug. The immunofluorescence results obtained after 24 h of LMB treatment (data not shown) were identical to those shown in Figure 7. These transient transfection results indicate that LMB can block not only Rev export to the cytoplasm, but also export of the RRE-containing RNA that depends on Rev for its transport.

Figure 6



Nucleo-cytoplasmic distribution of RRE-containing mRNA in the presence of LMB. COS cells co-transfected with pcREV and pgTAT were incubated for 48 h in medium alone (–), in the presence of 1 μg ml^{-1} AD during the last 2 h (AD, 2h), in the presence of 2 μM LMB for the total incubation time of 48 h (LMB, 48h), or during the last 24 h (LMB, 24h). Nuclear (N) and cytoplasmic (C) RNA were prepared as described in the Materials and methods section and equal amounts of RNA were probed for RRE-containing RNA and 18S RNA.

Table 1

Inhibition of Rev translocation and proliferation by leptomycin and kazusamycin antibiotics.

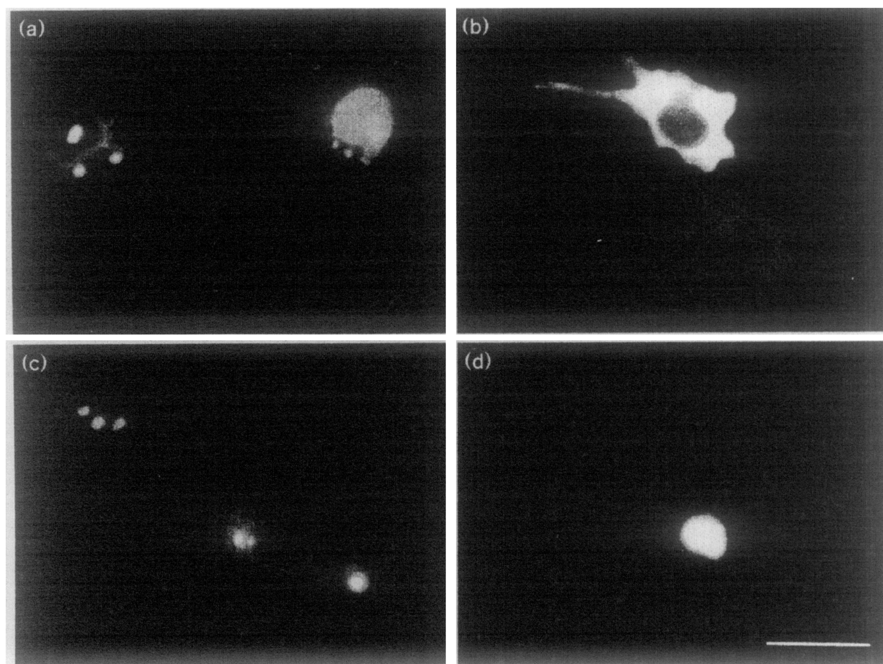
	IC_{50} for Rev translocation (nM)*				IC_{50} for proliferation (nM)†			
	7 h	24 h	48 h	72 h	7 h	24 h	48 h	72 h
Leptomycin A	0.6	0.6	2.5	3.5	>20	13.8	5.5	2.6
Leptomycin B	0.1	0.2	0.3	0.6	>20	12.6	1	0.9
Kazusamycin A	0.8	0.8	1.4	2.5	>20	10	3.7	2.9
Kazusamycin B	6.3	6.3	6.3	20	>20	>20	7.8	7.4

HeLa-Rev cells were seeded into 96-well plates and allowed to adhere overnight. They were then incubated with drugs for the time periods indicated. For the evaluation of Rev transport inhibition, AD was added to a final concentration of 1 mg ml^{-1} during the last 2 h of incubation. This addition had no effect on the cell number. Cells were fixed and immunostained for Rev, and, after microscopic examination, further incubated with sulforhodamine B to determine cellular protein, as described in the Materials and methods section. Results were calculated from triplicate experiments. *Concentrations at which Rev nucleo-cytoplasmic transport was inhibited by 50%. †Concentration which caused a 50% reduction in cell number as compared to untreated controls (IC_{50}).

LMB inhibits Rev-dependent, but not Rev-independent gene expression

If RRE-containing RNA is not transported to the cytoplasm, Rev-dependent gene expression should be suppressed, and, if this effect is specific for Rev, a Rev- independent plasmid should be efficiently translated in the presence of LMB. COS cells were co-transfected with pcREV and pDM128 (CAT plasmid, Rev-dependent) and pBC12/CMV/βGal (Rev-independent). After 24 h, they were incubated with different concentrations of LMB for another 24 h, and then harvested. CAT and β-galactosidase levels were determined and divided by the cellular protein content to correct for

Figure 7



Effect of LMB on the subcellular localization of Rev in transiently transfected COS cells. COS cells co-transfected with pcREV and pgTAT were incubated **(a)** for 48 h in medium alone; **(b)** in the presence of 1 μg ml^{-1} AD during the last 2 h; **(c)** with 2 nM LMB for 48 h and **(d)** with 2 nM LMB for 48 h and 1 μg ml^{-1} AD during the last 2 h. Cells were then fixed and immunostained for Rev as described in the Materials and methods section. Scale bar is 40 μm.

variations in cell number (which after this short-term incubation did not show any significant growth inhibitory effect of the drug). The results shown in Figure 8 are representative values of three separate experiments. While Rev-dependent CAT expression was maximally suppressed at 2 nM LMB, concentrations up to 20 nM had no effect on Rev-independent β-galactosidase expression. Rev-dependent CAT expression was only suppressed to about 37% of untreated controls (27% and 40% in the other two experiments), because the drug had been added after 24 h when the cells had already started synthesizing CAT. In a separate set of experiments, where LMB was added 3 h after transfection, Rev-dependent CAT expression could be reduced to 10% of the control values by 2 nM LMB, while Rev-independent CAT expression from a RSV–CAT construct was unaffected by 20 nM LMB (data not shown).

LMB inhibits HIV-1 replication in primary human monocytes

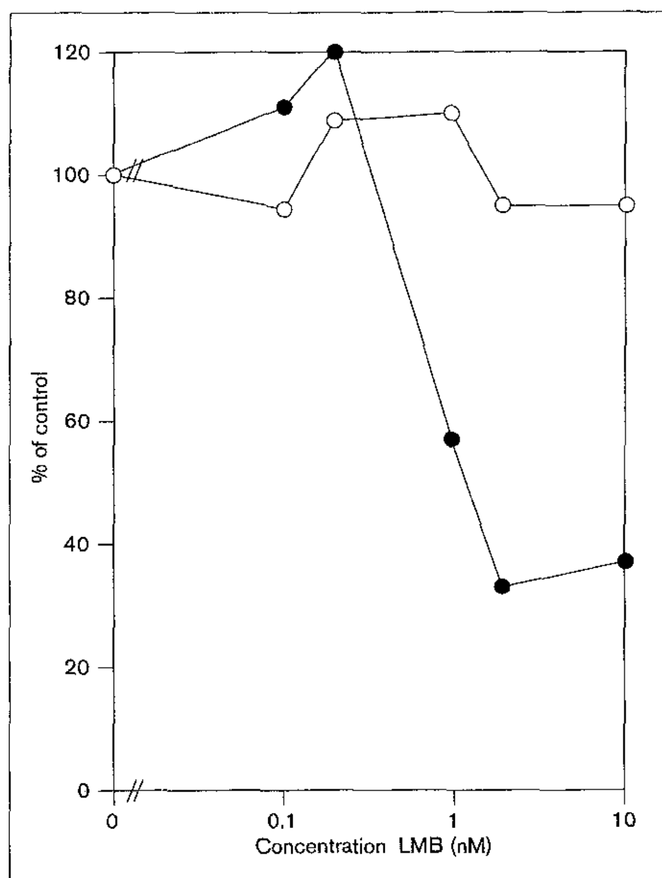
Due to the cytotoxic effect of LMB after 72 h of incubation, it was difficult to find a test system in which to assess its effect on HIV-1 replication. Most HIV-1 assays are performed in actively growing cells and take longer than 72 h. However, primary human monocytes, which do not replicate during several days of incubation, remain metabolically active and capable of propagating HIV. The results of a 72 h incubation of monocytes infected by the BaL strain of HIV-1 with LMB are shown in Table 2. Secretion of the 24 kDa Gag protein (p24) was used as a measure of HIV-1 replication. LMB at 0.6 nM significantly inhibited p24 production without any appreciable effect on the metabolic rate of the cells, as assessed by MTT staining (see the Materials and methods section). In another test system, HeLa-CD4 cells infected with HIV, a similar inhibition of p24 production could be observed at 2 nM LMB after 24 h (data not shown).

Discussion

In an attempt to identify a specific inhibitor of the nucleocytoplasmic translocation of the HIV-1 Rev protein, we isolated leptomycins A and B and kazusamycins A and B from extracts of a streptomycete strain. These drugs had originally been characterized as antifungal [30–32] and antitumor antibiotics [33–36]. They induce abnormal condensation of nuclei [37] and arrest the cell cycle of yeast and mammalian cells in both G1 and G2 phases [37,38]. Their mechanism of action is still unknown, however. Investigation of LMB-resistant mutants in the fission yeast *Schizosaccharomyces pombe* led to the identification of a LMB resistance gene that encodes a protein similar to the mammalian P-glycoproteins that confer multidrug resistance [39]. More specifically, another target gene in *S. pombe* for LMB is the gene that encodes the essential nuclear protein crm1, which is required for maintaining higher order chromosome structure, correct gene expression and cell growth [40]. Crm1 is a negative regulator of pap1, an AP–1-like transcription factor in *S. pombe*, which is responsible for the expression of a 25 kDa protein (p25). LMB induced the overproduction of p25 in wild-type cells, probably due to activation of pap1 through repression of crm1. The molecular mechanism for crm1 action is still unknown, however [40].

Our results on the inhibition of HIV-1 Rev nuclear export raises the intriguing possibility that this target gene of LMB

Figure 8



Effect of LMB on gene expression. COS cells were co-transfected with pcREV, pDM128 and pBC12/CMV/βGal. After 24 h, graded concentrations of LMB were added for another 24 h. Cells were then harvested, and CAT (●) and β galactosidase (○) activities, as well as cellular protein content per well, were determined as described in the Materials and methods section.

or another, as yet unidentified, target gene, is involved in nucleo-cytoplasmic transport processes. Recently, it was shown that Rev exploits the transport pathway that is normally used for 5S rRNA and U1 snRNA [21], whereas mRNA , tRNA and ribosomal export are directed via a different route. The activation domain of Rev was characterized as the nuclear export signal by transport studies using dominant-negative mutants [18–20] and by linking the domain to a reporter protein [21,22]. Similar export signals were identified in the human T-cell leukemia virus type 1 (HTLV-1) regulatory protein Rex [41], mitogen-activated protein kinase kinase [42], protein kinase inhibitor α (PKIα) [22], the IκB protein [43], and in the amphibian protein TFIIIA, which binds to 5S rRNA transcripts and has been proposed to be involved in the efficient nuclear export of 5S rRNA in amphibian oocytes [44]. LMB could conceivably be a specific blocking agent for this nuclear export pathway. Our results indicate that Rev-dependent gene expression was blocked, while Rev-independent gene

Table 2

Effect of LMB on p24 production in HIV-infected primary human monocytes.

LMB (nM)	p24 (pg ml^{-1})	MTT (OD)
–	233±75	251±123
0.2	272±98	253±27
0.6	63.3±23.6	233±107
2	54±9.6	299±192
6	53±13	214±135

Primary human monocytes were infected with HIV-1 BaL for 48 h. LMB was then added for 72 h. Levels of p24 were determined in the supernatant, and cellular esterase activity was measured by the MTT method, as described in the Materials and methods section. Results are means ± S.D. of triplicate experiments. Values of p24 in untreated controls at the beginning of the 72 h incubation period were 42 pg ml^{-1}. Values of p24 for 0.6 nM, 2 nM and 6 nM LMB were significantly different from the untreated controls, as evaluated by a two-tailed t-test (p values <0.05, <0.02 and <0.02, respectively). There was no significant difference among the MTT values.

expression, which should occur via the mRNA export pathway(s), was unaffected over a period of 24 h. A block of the 5S rRNA and U1 snRNA transport systems would also explain the long-term toxicity of leptomycin B in tissue culture. Therefore, even though LMB blocks Rev export and function, and can inhibit HIV replication in primary human monocytes, LMB cannot be used therapeutically. It should, however, provide a valuable tool for dissecting nuclear transport pathways. The effects of LMB on the export of 5S rRNA, U1 snRNA and several nuclear proteins are currently under investigation.

Significance

The export of the Rev protein of HIV-1 from the nucleus to the cytoplasm of infected cells has been shown to depend on a specific nuclear export signal in the sequence of Rev [21,22] and homologous sequences have been found in several other proteins [22,41–44]. This suggests that these proteins may all be exported from the nucleus to the cytoplasm by a common pathway. In addition this pathway appears to be used by some RNA species (unspliced and singly spliced HIV-1 mRNA, 5S rRNA and U1 snRNA) [4,5,21], but not by the bulk of mRNA. We show here that leptomycin B (LMB) and three other structurally related antibiotics from *Streptomyces* can completely block export of Rev from the nucleus. These are the first low molecular weight compounds known to have this inhibitory function. LMB appears to be a specific probe for this pathway because it affects Rev-associated export, but not Rev-independent export processes. Furthermore, we show that LMB is a probe for nuclear export only, because the nuclear import of Rev and other nuclear proteins is unaffected by the drug. LMB should be a valuable tool with which to dissect nuclear export pathways of different protein and RNA species.

Materials and methods

Isolation and purification of leptomycin and kazusamycin antibiotics

Leptomycins A and B and kazusamycins A and B were isolated from the *Streptomyces* sp. strain A87-18203, which was deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1b, D-3300 Braunschweig, Germany (reference number DSM 7517). Ethyl acetate extracts of *Streptomyces* A87-18203 were fractionated by gel filtration on Sephadex LH-20 (Pharmacia). Four compounds were isolated by preparative HPLC as will be described elsewhere (Y. Wang *et al.*, unpublished observations). The structures and the stereochemistry were established by spectroscopic and chemical methods.

Rev nucleo-cytoplasmic translocation assay

HeLa-Rev cells are HeLa cells stably transfected with an expression plasmid for HIV-1 Rev [29] and selected by co-transfection with the gene for dihydrofolate reductase. The original cells were a generous gift from B.R. Cullen and were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated fetal calf serum and 100 nM amethopterin. These cells were subcloned to achieve a high level of Rev expression in all cells, and clone 4D3E3 was used for all localization experiments.

Cells were seeded into 96-well plates (Nunc) and synchronized by serum starvation for 48 h. Rev synthesis was induced by the addition of serum. At this time, test compounds or streptomycete broth extracts were added in complete medium lacking amethopterin and incubated with the cells for a total of 7 h. During the last 2 h, actinomycin D (AD; Sigma) was added at 1 $\mu g\,ml^{-1}$ in order to induce complete cytoplasmic localization of Rev in the medium controls [18]. The cells were then fixed and immunostained for Rev protein using a mouse monoclonal antibody against Rev and a dichlorotriazinyl amino fluorescein (DTAF)-labelled goat-anti-mouse antibody as described elsewhere [18]. Inhibitors of Rev translocation into the cytoplasm were identified by microscopic examination of individual wells using a 32× objective on a Zeiss IM 10 inverted microscope equipped with epifluorescence optics. For the images shown in Figures 2 and 3, cells were grown on 8-well Lab-Tek chamber slides (Nunc), immunostained for Rev and examined in a BioRad MRC 600 confocal laser scanning microscope as described [18]. Results in Figures 2a, 2b and 3 are represented as 0.7 μm thick optical sections, while Figure 2c and 2d show optical sections of 1.8 μm thickness.

In order to assess the influence of LMB on Rev translocation from the cytoplasm into the nucleus, HeLa-Rev cells were serum-starved for 48 h, and the drug was added 1 h before the re-addition of serum.

COS cells in 6-well plates (Nunc) were transfected and treated with AD and LMB as described below (RNA transport assay) and immunostained for Rev. They were examined in an Olympus IX-70 inverted microscope equipped with a 100×(1.35 N.A.) objective and epifluorescence optics and photographed with a PM-30 camera (Olympus) using TMax 400 film (Eastman Kodak).

NFκB p65 nuclear import assay

HeLa cells (ATCC, Rockville, MD, USA) were grown in IMDM supplemented with 10% heat-inactivated fetal calf serum. They were seeded into 8-well LabTek chamber slides and allowed to adhere overnight. They were then stimulated by the addition of 200 $U\,ml^{-1}$ TNF-α (Boehringer Mannheim, Germany) for 30 min. LMB (1 μM) was added for 30 min before and during the stimulation. The cells were fixed and permeabilized with 4% paraformaldehyde and 0.5% Triton-X100, respectively. The cells were immunostained using a polyclonal rabbit antibody directed against NFκB p65 (Santa Cruz Biotechnology) and a tetramethylrhodamine-labelled goat-anti-rabbit antibody (Accurate Scientific) and examined by confocal laser scanning microscopy as described [18].

In vitro *nuclear-import assay*

The *in vitro* nuclear import assay was conducted as described for FACS analysis [27]. The suspension of nuclei was transferred to a glass slide and examined in the Olympus microscope as described above.

RNA transport assay

COS cells were seeded into T150 flasks (Nunc) and transfected with expression plasmids pcREV [29] and pgTAT [29] at 1 $\mu g\,ml^{-1}$ and 1.5 $\mu g\,ml^{-1}$, respectively, using DEAE-dextran and chloroquine [45]. LMB was added at 2 nM for 24 h or 48 h; in one sample, AD was added 2 h prior to the end of the experiment. Total incubation time after transfection was 48 h. Nuclear and cytoplasmic RNAs were prepared according to [46], and slot blot analysis was performed using probes specific for RRE (5′-CCT GTA CCG TCA GCG TCA TTG ACG CTG CGC-3′) and 18S RNA (5′-GCA CCA GAC TTG CCC TCC AAT GGA TCC TCG-3′).

Reporter gene assays

COS cells were co-transfected with pcREV (1 $\mu g\,ml^{-1}$), a Rev-dependent chloramphenicol acetyl transferase (CAT) indicator gene (pDM128; 1.5 $\mu g\,ml^{-1}$) [47] and a Rev-independent gene for the expression of β-galactosidase (pBC12/CMV/βGal; 1 $\mu g\,ml^{-1}$) [48] in 6-well plates (Costar). 24 h after transfection, different concentrations of LMB were added to the cells, and incubation was continued for another 24 h. The cells were prepared for the simultaneous determination of CAT and β-galactosidase activities as described [49]. Cellular protein was determined from an aliquot of the suspended cells, digested in 0.5 M NaOH, using the BioRad protein assay.

HIV-1 p24 Gag protein production in primary human monocytes

Five-day adherent monocyte cultures were prepared from the blood of healthy, HIV-negative donors [50]. The cells were maintained in 48-well plates (Costar). They were infected with HIV-1 BaL strain for 48 h, and LMB was then added at different concentrations. Three days after substance addition, p24 Gag protein was measured in the supernatant using a commercially available ELISA kit (Coulter). The cell number per well was determined using the MTT method [51].

Sulforhodamine B assay

In order to determine the effect of the drugs on cell growth, HeLa-Rev cells were seeded into 96-well plates at 5000 cells per well and allowed to adhere overnight. They were then incubated with test compounds for 7 h, 24 h, 48 h or 72 h, fixed with 3.7% formaldehyde/2% sucrose in PBS and stained with sulforhodamine B (Sigma) as described [52].

Acknowledgements

We would like to thank D. Bevec for performing the slot blot analysis, H. Jaksche for conducting the *in vitro* nuclear import assay, E. Pursch for carrying out the experiment with HIV-1 infected monocytes, and D. Bevec and A.P. Winiski for critically reading the manuscript.

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