Inhibition of T-tropic HIV Strains by Selective Antagonization of the Chemokine Receptor CXCR4

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Summary

Bicyclams are a novel class of antiviral compounds that are highly potent and selective inhibitors of the replication of HIV-1 and HIV-2. Surprisingly, however, when the prototype compound AMD3100 was tested against M-tropic virus strains such as BaL, ADA, JR-CSF, and SF-162 in human peripheral blood mononuclear cells, the compound was completely inactive. Because of the specific and potent inhibitory effect of AMD3100 on T-tropic viruses, but not M-tropic viruses, it was verified that AMD3100 interacts with the CXC-chemokine receptor CXCR4, the main coreceptor used by T-tropic viruses. AMD3100 dose dependently inhibited the binding of a specific CXCR4 monoclonal antibody to SUP-T1 cells as measured by flow cytometry. It did not inhibit the binding of the biotinylated CC-chemokine macrophage inflammatory protein (MIP) 1α or MIP-1β, ligands for the chemokine receptor CCR5 (the main coreceptor for M-tropic viruses). In addition, AMD3100 completely blocked (*a*) the Ca^{2+} flux at 100 ng/ml in lymphocytic SUP-T1 and monocytic THP-1 cells, and (*b*) the chemotactic responses of THP-1 cells induced by stromal cell–derived factor 1α, the natural ligand for CXCR4. Finally, AMD3100 had no effect on the Ca^{2+} flux induced by the CC-chemokines MIP-1α, regulated on activation normal T cell expressed and secreted (RANTES; also a ligand for CCR5), or monocyte chemoattractant protein 3 (a ligand for CCR1 and CCR2b), nor was it able to induce Ca^{2+} fluxes by itself. The bicyclams are, to our knowledge, the first low molecular weight anti-HIV agents shown to act as potent and selective CXCR4 antagonists.

The bicyclam derivatives were described several years ago as potent and selective inhibitors of HIV type 1 and type 2 replication (1, 2). AMD3100, previously called JM3100 (2) or SID791 (3), exhibits anti-HIV potency at concentrations of 1–10 ng/ml, with a selectivity index ⩾100,000 (2). Based on time-of-addition experiments, the compound has been assumed to interact with the HIV fusion-uncoating process, but does not inhibit virus binding to the CD4 receptor (1, 2). AMD3100 blocks syncytium formation at a concentration that is 10–100-fold higher than the concentration required to inhibit virus infection (1). The *env* glycoprotein (gp)120 has been considered the major target molecule for this class of compounds because, for viruses that were made resistant to the bicyclams, a number of mutations accumulated in the gp120, especially in the V3-V4 region (3, 4).

Numerous publications over the last year have demonstrated the importance of chemokine receptors for HIV entry. Chemokines are chemotactic cytokines, which are classified as CC or CXC, depending on the positioning of conserved cysteine residues. Fusin/LESTR, now designated CXC-chemokine receptor 4 (CXCR4), mediates entry of T-tropic viruses (5, 6) which can be inhibited by its natural ligand, the CXC-chemokine stromal cell–derived factor 1α (SDF-1α) (7, 8). The CC-chemokine receptor, CCR5, mediates entry of M-tropic viruses (9–13) and the CC-chemokines regulated on activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP) 1α and MIP-1β have been shown to inhibit the replication of M-tropic viruses (14). Moreover, M-tropic *env* proteins can interact directly with CCR5 (15, 16).

In previous studies AMD3100 was shown to inhibit the replication of T-tropic HIV strains or clinical isolates in T cell lines (such as MT-4, MOLT-4, or CEM cells; references 1–4). While verifying whether AMD3100 was active against M-tropic viruses in PBMCs, we found that AMD3100 does not inhibit M-tropic viruses such as BaL, ADA, JR-CSF, and SF-162. Here we show that AMD3100 selectively inhibits the binding of a CXCR4-specific mAb, but not the binding of biotinylated human MIP-1α or MIP-1β. The bicyclam was also found to inhibit the Ca^{2+} flux and the chemotactic response induced by SDF-1α but not such effects induced by RANTES, MIP-1α, or monocyte chemoattractant protein 3 (MCP-3).

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Materials and Methods

Viruses, Cells, Cell Lines, and Cell Culture. The HIV-1 T-tropic viruses IIIB strain and RF strain, the HIV-2 T-tropic ROD strain, and the HIV-1 M-tropic strains BaL, SF-162, ADA, and JR-FL were all obtained through the Medical Research Council AIDS reagent project (Herts, UK). The HIV-1 T-tropic molecular clone NL4-3 was obtained from the National Institute of Allergy and Infectious Disease AIDS reagent program (Bethesda, MD). The $CD4^+$ lymphocytic SUP-T1 and the $CD4^+$ monocytic THP-1 cell lines were obtained from the American Type Culture Collection (Rockville, MD). PBMC from healthy donors were isolated by density gradient centrifugation and stimulated with PHA at 1 μg/ml (Sigma Chemical Co., Bornem, Belgium) for 3 d at 37°C. The activated cells (PHA-stimulated blasts) were washed three times with PBS, and viral infections were done as described by Cocchi et al. (14). HIV-infected or mock-infected PHA-stimulated blasts were cultured in the presence of 25 U/ml of IL-2 and varying concentrations of AMD3100, SDF-1α, and RANTES. Supernatant was collected at days 6 and 10, and HIV-1 core antigen in the culture supernatant was analyzed by the p24 ELISA kit from DuPont-Merck Pharmaceutical Co. (Wilmington, DE) and for HIV-2 detection the INNOTEST from Innogenetics (Temse, Belgium) was used.

Chemokines and mAbs. Recombinant human SDF-1α was purchased from PeproTech (London, UK) and human RANTES and human MIP-1α were purchased from R & D Systems, Inc. (Abingdon, UK). MCP-3 was chemically synthesized according to the published protein sequence (17). The biotinylated human MIP-1α and MIP-1β fluorokine™ kits were purchased from R & D Systems Inc. The mAb, termed 12G5, reacts specifically with the human CXCR4 and was initially provided by Dr. James A. Hoxie (University of Pennsylvania, Philadelphia, PA) and later purchased from R & D Systems Inc.

Analysis of CXCR4 Expression. SUP-T1 cells were incubated with AMD3100 or SDF-1α (at different concentrations) or PBS for different time periods (1 or 15 min) and at different temperatures (on ice or at room temperature) and the cells were washed once with PBS. The 12G5 mAb (10 μg/ml) was then added for 30 min at room temperature. The cells were washed twice in PBS and then incubated with FITC-conjugated goat anti–mouse Ab (Caltag Labs, San Francisco, CA) for 30 min at room temperature and washed twice in PBS. The binding of the biotinylated human MIP-1α was performed according to the protocol of the manufacturer. Cells were analyzed by a FACScan® flow cytometer. The percentage of positive cells and the mean fluorescence intensity (MFI) values are indicated in each histogram. The region for positivity was defined using a control isotype mAb (Becton Dickinson, San Jose, CA). The percentage of inhibition of mAb binding in the presence of different concentrations of chemokine or compound was calculated using the MFI values, as previously described (18).

Measurement of Intracellular Calcium Concentrations and Chemotactic Assay. The determination of intracellular calcium concentrations $[Ca^{2+}]_i$ was carried out as previously described (19). In brief, THP-1 cells or SUP-T1 cells were loaded with Fura-2 (Molecular Probes, Leiden, The Netherlands). Fura-2 fluorescence was measured in a luminescence spectrophotometer, fitted with a water-thermostatable, stirred four-position cuvette holder (Perkin-Elmer, Norwalk, CT). Cells were first stimulated with dilution buffer (control) or AMD3100 or 12G5 mAb at different concentrations. As a second stimulus, chemokines were used at an optimal concentration to induce a maximal $[Ca^{2+}]_i$ increase. The second stimulus was added 100 s after the first stimulus. The percentage of inhibition of the $[Ca^{2+}]_i$ increase in response to the second stimulus was calculated. Chemotaxis of THP-1 cells was measured in the microchamber assay (5 μm pore membrane) essentially as previously described (17, 20). For inhibition of chemokine activity by AMD3100, the compound was added to the cells just before transfer to the upper compartment of the chemotaxis chamber containing chemokine in the lower compartment (20). Chemotactic activities are expressed as indexes ± SEM (17).

Results

Antiretroviral Activity Profile of AMD3100. AMD3100 was active in PHA-stimulated blasts against T-tropic virus strains such as IIIB, RF, and NL4-3, and also against the HIV-2 ROD strain. The 50% inhibitory concentration (IC_{50}) was between 2 and 7 ng/ml (Table 1). Surprisingly, AMD3100 was completely inactive against four different M-tropic virus strains (IC_{50} >25 μg/ml; Table 1). These M-tropic virus strains mainly use the chemokine receptor CCR5, but some can also use other chemokine receptors such as CCR2b and CCR3 (but not CXCR4) to enter the target cells (9–13). As controls, SDF-1α and RANTES were included and, as can be seen in Table 1, there was no activity of RANTES (up to 1 μg/ml) against the T-tropic virus strains, whereas the IC_{50} of SDF-1α varied between 20 and 100 ng/ml against the T-tropic virus strains. The opposite activity profile of these two chemokines was observed with M-tropic viruses. Here, RANTES had IC_{50} values between 4 and 25 ng/ml, whereas SDF-1α was not active up to 1 μg/ml. AMD3100 was not active against several simian immunodeficiency virus (SIV) strains such as MAC, MND, and AGM in MT-4 or MOLT-4 cells (2), which use CCR5 rather than CXCR4 as the main coreceptor to enter human T cells (21).

AMD3100 Dose Dependently Inhibits the Binding of a CXCR4-specific mAb. Because of the specific and potent inhibitory effect of AMD3100 on T-tropic viruses and not on M-tropic viruses (or SIV), it was verified that AMD3100 interacts with CXCR4. The mAb 12G5 reacts specifically with the human CXCR4 protein and recognizes this receptor on many T cell lines such as the SUP-T1 cells (22). AMD3100 dose dependently interacted with the CXCR4 receptor, as shown in Fig. 1. Indeed, AMD3100 at 1 μg/ml completely inhibited the binding of the mAb 12G5 to CXCR4 on SUP-T1 cells, as measured by flow cytometry. At 100, 10, 1, and 0.1 ng/ml, AMD3100 still blocked the mAb binding for 79, 70, 24, and 9% respectively. SDF-1α competed, as expected, with the binding of the CXCR4 mAb to its receptor. SDF-1α inhibited the binding of the mAb for 83% at 2 μg/ml and for 54% at 200 ng/ml. Even when washed away before addition of the mAb, AMD3100 inhibited the binding of the CXCR4 mAb as efficiently as when the compound was present during the whole incubation period with the mAb. Adding AMD3100 only 1 min before the CXCR4 mAb still blocked the binding of the mAb as efficiently as adding the compound 15 min before the mAb (data not shown). Irrespective of whether the staining was

Table 1. *The Anti-HIV Activity Profile of AMD3100 Correlated with Coreceptor Use*

Strain	Coreceptor used	IC_{50} (ng/ml)		
		ADM3100	SDF-1α	RANTES
T-tropic				
HIV-1 IIIB	CXCR4	2	20	>1,000
HIV-1 RF	CXCR4	5	50	>1,000
HIV-1 NL4-3	CXCR4	3	100	>1,000
HIV-2 ROD	CXCR4	7	55	>1,000
M-tropic				
HIV-1 BaL	CCR5	>25,000	>1,000	25
HIV-1 SF-162	CCR5	>25,000	>1,000	5
HIV-1 ADA	CCR5 (CCR2b, CCR3)	>25,000	>1,000	10
HIV-1 JR-FL	CCR5 (CCR2b, CCR3)	>25,000	>1,000	4

Effect of AMD3100, SDF-1α, and RANTES on the replication of T-tropic and M-tropic HIV strains in PHA-stimulated blasts. Virus yield was monitored in the cell-free supernatant 6–10 d after infection by viral Ag ELISA. Results represent mean values for three separate experiments from three different PBMC donors.

performed on ice or at room temperature, identical results were obtained with AMD3100 for inhibition of the binding of the CXCR4 mAb.

In contrast, even at 25 μg/ml AMD3100 did not inhibit the binding of biotinylated human MIP-1α to THP-1 cells, whereas, as control, the anti-human MIP-1α blocking Ab included in the fluorokine™ kit almost completely blocked the binding of the biotinylated MIP-1α (Fig. 2). Identical results were obtained with the biotinylated human MIP-1β fluorokine™ kit (data not shown).

AMD3100 Specifically Blocks SDF-1α–induced Ca^{2+} Fluxes and Chemotaxis. We next examined the inhibitory effect of AMD3100 on the SDF-1α–induced increase in $[Ca^{2+}]_i$ (Ca^{2+} flux). Because the lymphocytic SUP-T1 cells did not respond in the Ca^{2+} flux assays to the CC-chemokines RANTES and MIP-1α, we used the monocytic THP-1 cell line, which is responsive to these chemokines. This allowed us to test the chemokine receptor specificity of AMD3100. In addition, the THP-1 cells were positive for CXCR4 expression, as measured by flow cytometry with the CXCR4 mAb (data not shown). THP-1 cells also dose dependently responded in the Ca^{2+} flux assay to SDF-1α, and half-maximal increases in $[Ca^{2+}]_i$ were obtained with 10 ng/ml (data not shown). As a control for receptor usage, 10 μg/ml of the CXCR4 mAb was found to completely inhibit the SDF-1α–induced Ca^{2+} flux and at 1 μg/ml of the mAb there was still 36% inhibition (data not shown). AMD3100 at 100 ng/ml completely blocked $[Ca^{2+}]_i$ increases induced by 30 ng/ml SDF-1α in both SUP-T1 and THP-1 cells (Table 2). Lower doses of AMD3100 (10 and 1 ng/ml) still conferred partial inhibition (35–69%) of Ca^{2+} increase induced by SDF-1α (Fig. 3; Table 2). In addition, the THP-1 cells responded to RANTES, MIP-1α, and MCP-3, but no inhibition whatsoever was seen when AMD3100 was added at 100 ng/ml before addition of the chemokines (Fig. 4; data not shown). Finally, to confirm the inhibitory effect of AMD3100 on functional CXCR4 binding and signaling, chemotaxis assays were performed. Similar to monocytes (23), THP-1 cells dose dependently responded to SDF-1α. On THP-1 cells, 100 ng/ml resulted in a half-maximal chemotactic index (11 ± 4; $n = 3$). The chemotactic effect of SDF-1α (100 ng/ml) was completely blocked in the presence of AMD3100 at 10 μg/ml (chemotactic index: 1.3 ± 0.6; $n = 3$), whereas 1 μg/ml of AMD3100 resulted in a partial reduction of the chemotactic index (4.3 ± 0.7; $n = 3$). AMD3100 alone induced no chemotactic response on THP-1 cells when tested at a concentration range from 0.01 to 10 μg/ml.

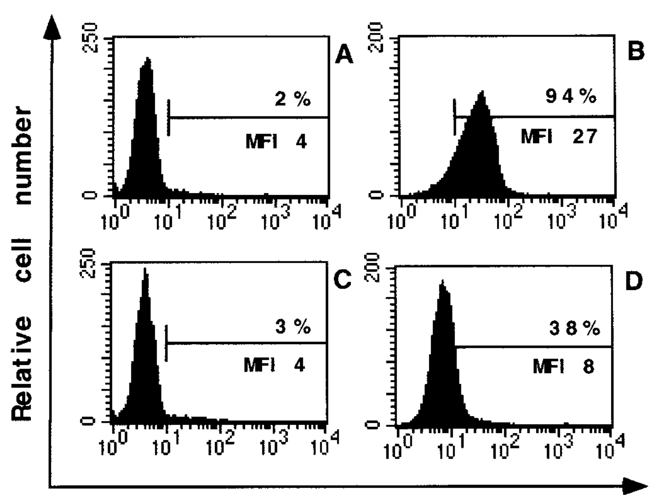

Figure 1. Inhibition of the binding of the anti-CXCR4 mAb to SUP-T1 cells in the presence of AMD3100 at 1 μg/ml (*C*) and SDF-1α (2 μg/ml; *D*). In *A* an isotype control mAb and in *B* the specific anti-CXCR4 mAb were used. The percentage of positive cells and the MFI values are indicated in each histogram.

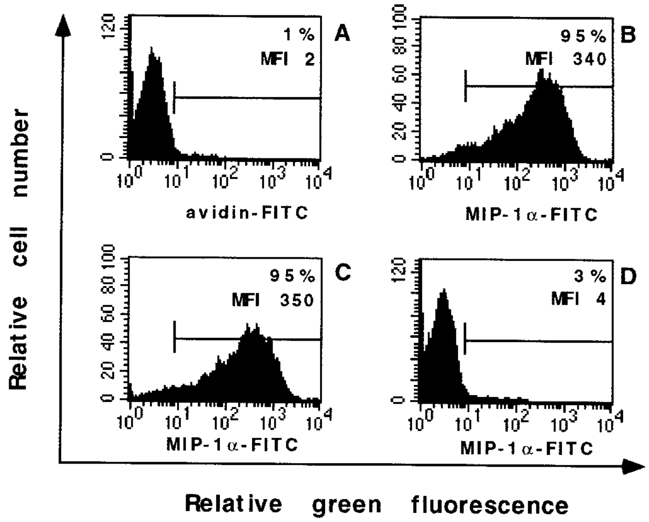

Figure 2. Lack of inhibition of the binding of biotinylated MIP-1α to THP-1 cells in the presence of AMD3100 (25 μg/ml; *C*). In *A* only the avidin-FITC was added, in *B* the biotinylated MIP-1α and avidin-FITC were added, in *C* AMD 3100 (25 μg/ml) was added, and in *D* the blocking Ab was added. The percentage of positive cells and MFI values are indicated in each histogram.

Discussion

CXCR4 is the coreceptor that promotes entry of T-tropic HIV strains (7, 8), whereas CCR5 allows entry of M-tropic HIV strains (9–13). SDF-1α, the natural ligand for CXCR4, has been shown to inhibit T-tropic viruses and primary HIV isolates through CXCR4 blockage (6, 7). AMD3100 is a bicyclam active against a broad range of T-tropic HIV-1 and HIV-2 strains (IC_{50}: 1–10 ng/ml), but not against M-tropic HIV-1 strains (Table 1) such as BaL, ADA, SF-162, and JR-FL (IC_{50} >25 μg/ml). AMD3100 is able to inhibit the replication of HIV-2 strains such as ROD, but these virus strains also use CXCR4 to enter the cells (24). In addition, AMD3100 is not active against several SIV strains (2), and recently it was demonstrated that not only the M-tropic SIV strains but also the T-tropic SIV strains use CCR5, and not CXCR4, as the main coreceptor to enter human T cells (21).

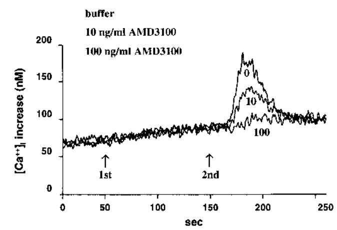

Figure 3. Inhibition of SDF-1α–induced Ca^{2+} fluxes in SUPT-1 cells by AMD3100 pretreatment (first stimulus; *1st*) at 100 ng/ml and 10 ng/ml. SDF-1α was given as second stimulus (*2nd*) at 30 ng/ml.

The specific antiviral activity profile of AMD3100 suggests that it might directly interact with the CXCR4 receptor. This study brings evidence to support this hypothesis, at both the level of binding and of signaling. AMD3100 not only inhibits the binding of CXCR4 mAb to its receptor (Fig. 1), it also inhibits the intracellular SDF-1α signaling in a dose-dependent fashion (Table 2). The CXCR4 mAb, 12G5, is reported to inhibit HIV-1 and HIV-2 infection at 1–20 μg/ml, although the ability of this mAb to block infection of T-tropic isolates of HIV-1 is highly dependent on the viral isolate and the target cell; occasionally, it is even inactive against T-tropic viruses (23). Very potent and far less variable antiviral activity is seen with AMD3100 (IC_{50}: 1–10 ng/ml, 50% cytotoxic concentration [CC_{50}] >100 μg/ml; reference 2), indicating a very strong interaction of AMD3100 with the CXCR4 receptor.

Although the interaction of the bicyclams with CXCR4 has been unequivocally demonstrated in this study, interference either with other (still unknown) CXCR4-like or other chemokine receptors used by SDF-1α cannot be excluded at this moment. At present there is no evidence that the CXCR4 mAb and SDF-1α can recognize receptors other than CXCR4. AMD3100 does not appear to interfere with CCR5 as there is no inhibitory effect of AMD3100 on the replication of SIV and M-tropic HIV-1 strains in PBMC. Moreover, AMD3100 does not inhibit the binding of either of the biotinylated CC-chemokines, MIP-1α (Fig. 2) and MIP-1β, nor does it block the Ca^{2+} flux induced by RANTES (Fig. 4) or MIP-1α, although it markedly inhibited the Ca^{2+} flux induced by SDF-1α (Fig. 3). The bicyclam also did not inhibit the Ca^{2+} flux induced by

Table 2. *Inhibition of SDF-1α–induced Ca^{2+} Flux by AMD3100 in SUP-T1 and THP-1 Cells*

Stimulus		SUP-T1 cells $[Ca^{2+}]_i$ increase				THP-1 cells $[Ca^{2+}]_i$ increase			
AMD3100 (first)	SDF-1α (second)	exp. 1	exp. 2	exp. 3	mean percentage of inhibition	exp. 1	exp. 2	exp. 3	mean percentage of inhibition
ng/ml	*ng/ml*		*nM*		*%*		*nM*		*%*
100	0	0	0	0		0	0	0	
100	30	<10*	<10	<10	⩾90	<8	<8	<8	>90
10	30	83	33	49	45	31	37	ND	69
1	30	ND	39	78	35	ND	56	ND	59
0.1	30	ND	ND	90	<10	ND	ND	ND	
0	30	110	89	91	0	87	136	65	0

*Results of individual Ca^{2+} flux assays are given.

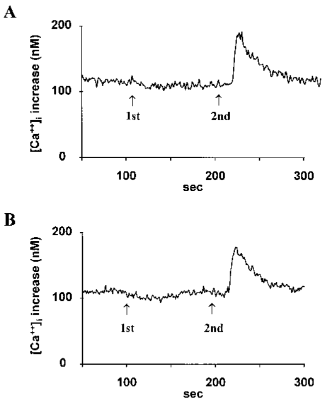

Figure 4. AMD3100 fails to inhibit Ca^{2+} fluxes induced by RANTES in THP-1 cells. Buffer was added in *A* and AMD3100 at 100 ng/ml in *B* as first stimulus (*1st*), then RANTES was added at 100 ng/ml as second stimulus (*2nd*).

MCP-3, a natural ligand for CCR1 and CCR2b (25). By itself AMD3100 did not induce $[Ca^{2+}]_i$ increases even at a concentration of 100 μg/ml (data not shown). The IC_{50} of AMD3100 required to inhibit binding of the CXCR4 mAb and to desensitize the SDF-1α–induced Ca^{2+} flux is between 1 and 10 ng/ml. This dose nicely correlates with the IC_{50} of the compound for the replication of T-tropic viruses in T cell lines and PBMCs, whereas a relatively higher concentration (10 μg/ml) was necessary to completely block SDF-1α–induced chemotaxis. This illustrates that AMD3100 can funtion as a potent antiviral compound in vitro (1, 2) and in vivo (100 ng/ml in plasma of SCID-hu mice is sufficient to reduce the viral load significantly; reference 26), rather than acting as an antagonist of leukocyte chemoattraction.

Some individuals who were repeatedly exposed to HIV infection and remained uninfected were found to be homozygous for a 32-bp deletion in the CCR5 (27–29). Perhaps mutations in CXCR4 and other coreceptors may also be identified in individuals who are less susceptible to HIV infection and/or in individuals who have been infected but do not proceed to AIDS, the so-called long-term nonprogressors (30), who have a predominance of M-tropic viruses. CCR5-binding viruses are important during early stages of infection, whereas the CXCR4-binding viruses emerge later in the progression to AIDS (31). AMD3100, because of its strong interaction with CXCR4, may become an important antiviral drug in vivo, because of its potential to block infection with T-tropic viruses, which in most cases precedes the decline in $CD4^+$ T cells and the development of AIDS.

Recently, derivatives of the CC-chemokine RANTES have been described as CCR5 antagonists with activity against M-tropic HIV-1 strains (32, 33). However, the bicyclams are the first low molecular weight chemicals among the anti-HIV agents described as CXCR4 antagonists.

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