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Nuclear import of HIV-1 integrase is inhibited *in vitro* by styrylquinoline derivatives*

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Running Title: Styrylquinolines inhibit HIV-1 integrase import

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List of nonstandard abbreviations: HIV-1, human immunodeficiency virus type 1; PIC, preintegration complex; IN, integrase; NLS, nuclear localization signal; SQ, styrylquinoline; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]propanesulfonate; BSA, bovine serum albumin

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ABSTRACT

Nuclear import of HIV-1 preintegration complexes (PICs) allows the virus to infect non-dividing cells. Integrase (IN), the PIC-associated viral enzyme responsible for the integration of the viral genome into the host cell DNA, displays karyophilic properties and has been proposed to participate to the nuclear import of the PIC. Styrylquinolines (SQs) have been shown to block viral replication at non-toxic concentrations and to inhibit IN 3'-processing activity *in vitro* by competiting with the DNA substrate binding. However, several lines of evidence suggested that SQs could have a post-entry, pre-integrative antiviral effect in infected cells. In order to gain new insights on the mechanism of their antiviral activity, SQs were assayed for their ability to affect nuclear import of HIV-1 IN and compared to the effect of a specific strand transfer inhibitor. Using an *in vitro* transport assay, we have previously shown that IN import is a saturable mechanism, thus showing that a limiting cellular factor is involved in this process. We now demonstrate that SQs specifically and efficiently inhibit *in vitro* nuclear import of IN without affecting other import pathways whereas a specific strand transfer inhibitor does not affect IN import. These data suggest that SQs not only inhibit IN-DNA interaction but would also inhibit the interaction between IN and the cellular factor required for its nuclear import.

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INTRODUCTION

Nuclear import of HIV-1 genome into the nucleus of infected cell is an essential step for HIV-1 replication. In contrast to other lentiviruses such as oncoretroviruses that require mitosis to replicate, HIV-1 is able to infect cells independently of mitotic nuclear envelope breakdown (Katz et al., 2003; Lewis et al., 1992; Weinberg et al., 1991). Following entry of the virus into the cell, the viral capsid seems to uncoat rapidly and the virion core is released into the cytoplasm of infected cell. The genomic HIV-1 RNA is reverse transcribed into linear double-stranded DNA and viral components are re-organized into a large nucleoprotein complex, the preintegration complex (PIC), composed of the viral DNA and viral and cellular proteins. Viral DNA is then actively imported into the nucleus through the nuclear envelope of interphase cell (Bukrinsky et al., 1992). HIV-1 DNA is ultimately integrated into host DNA, which ensures expression and perpetuation of the viral genome. This process is carried out by the viral integrase (IN) which represents therefore a legitimate target for new inhibitors as combination therapy with reverse transcriptase and protease inhibitors failed to eradicate viral replication and to prevent emergence of drug resistant strains. The integration process catalyzed by IN has been well described (Brown, 1997). It requires two distinct catalytic steps. In the first step, called 3'-processing, two nucleotides are removed from each 3'-end of the viral DNA. In the second step, the strand transfer reaction, the 3'-processed viral DNA ends are covalently joined to the target DNA. However, IN not only catalyzes the integration of HIV-1 DNA into the host genome but has also been proposed to participate in PIC import (Fouchier and Malim, 1999; Whittaker et al., 2000). It has been clearly shown that IN displays karyophilic properties since it accumulates in the nucleus of transfected cells as well as infected cells (Depienne et al., 2000; Gallay et al., 1997; Petit et al., 2000; Pluymers et al., 1999). The tight association between IN and viral DNA within PICs supports IN as a good

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candidate for nuclear import of viral DNA (Farnet and Haseltine, 1991; Miller et al., 1997). Moreover, the nuclear localization of IN is an absolute prerequisite for the accomplishment of its integration function in the viral cycle. Consequently, IN is an interesting target for novel drugs inhibiting its nuclear import as well as its integration activity. IN import is still poorly understood. Despite several attempts to identify a genuine transferable nuclear localization signal (NLS) in IN primary sequence, it rather seems that the three-dimensional structure of IN might be required for its nuclear import. Moreover, we previously reported that IN import is not mediated by a classical import pathway but the precise mechanism of this atypical import remains to be elucidated (Depienne et al., 2001). Many HIV-1 IN inhibitors have already been identified (for review, see Pommier et al., 2000), including a recently described new family of inhibitors corresponding to styrylquinoline (SQ) compounds (Mekouar et al., 1998; Zouhiri et al., 2000). Several SQs possess antiviral activity in cell culture (Bonnenfant et al., 2004; Mekouar et al., 1998; Zouhiri et al., 2000). *In vitro*, these compounds are competitive inhibitors of IN binding to DNA which therefore block the 3'-processing activity of the enzyme (Deprez et al., 2004). These compounds bind to the recombinant enzyme, thereby impairing the recognition of its DNA substrate (Deprez et al., 2004). In contrast to strand transfer inhibitors that affect specifically the integration step in infected cells (Hazuda et al., 2000), SQ derivatives block the viral replication at an earlier step. Several lines of evidence pointed to a post entry, pre-integrative effect; quantitative PCR determination of viral species showed a normal level of viral RNA in cells infected in the presence of the drug while a strong decrease of the amount of full-length reverse transcribed DNA was observed in total cell extracts. IN was confirmed to be the probable target as resistant viruses bearing mutations within the IN sequence were isolated following long-term virus culture in the presence of an active SQ derivative (Bonnenfant et al., 2004). In order to gain new insights on the mechanism of antiviral activity, these compounds were assayed for their ability to affect

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nuclear import of HIV-1 IN and compared to the effect of a specific strand transfer inhibitor. Using an *in vitro* transport assay, we show here that SQs specifically inhibit nuclear import of IN without affecting other import pathways, whereas a specific strand transfer inhibitor has no effect on IN import.

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MATERIALS AND METHODS

Cells and culture conditions

Adherent or S3 suspension HeLa cells were maintained in exponential growth in Dulbecco's Modified Eagle's Medium supplemented with 10 % fetal calf serum.

Preparation of HeLa cell cytosolic extracts

HeLa cell cytosol was prepared as described by Paschal and Gerace (Paschal and Gerace, 1995). 10^9 exponentially growing HeLa S3 cells were collected by centrifugation at 300 *g* for 5 min. Cells were washed twice with phosphate-buffered saline and once with lysis buffer (5 mM Hepes pH 7.4, 5 mM potassium acetate pH 7.4, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT and the following protease inhibitors: 10 μg/ml each aprotinin, leupeptin, pepstatin and 200 μg/ml Pefabloc SC (Roche)). The cell pellet was resuspended in one volume of lysis buffer and disrupted in a tight fitting stainless steel homogenizer (as judged by phase contrast microscopy). The homogenate was diluted with 0.1 volume of 10X transport buffer (transport buffer: 20 mM Hepes pH 7.4, 110 mM potassium acetate pH 7.4, 2 mM magnesium acetate, 0.5 mM EGTA, 1 mM DTT and protease inhibitors) and centrifuged at 40,000 *g* for 30 min at 4°C. The supernatant was further centrifuged at 100,000 *g* for 1 h. The resulting supernatant (~10 mg/ml as measured with the protein assay kit from Bio-Rad) was aliquoted, frozen in liquid N_2, and stored at -80°C.

Preparation of transport substrates

Recombinant IN produced in *E. Coli* was a generous gift from S. Escaich (Avantis Pharma, Ivry, France). 200 μg of recombinant IN was added to 1 mg of N-hydroxysuccinimide ester modified Cy3 (Amersham Biosciences) resuspended in 0.1 M sodium borate pH 9. The

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reaction was allowed to proceed for 2 hours at room temperature. Labeled IN was separated from unconjugated dye by extensive dialysis against a buffer containing 3 mM sodium phosphate pH 7.4, 1 mM EDTA, 0.3 M NaCl, 0.1% CHAPS, 10% glycerol and 1 mM DTT. Molar concentration of the labeled protein was estimated by measuring its absorbance at 280 nm and correcting the calculated value for the absorbance of the Cy3 dye at 280 nm according to the manufacturer instructions.

BSA (1 mg) was labeled with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (85 µg; FLUOS, Roche) in 100 mM sodium borate pH 8.5. Free fluorochrome was removed by chromatography on a Sephadex G-50 column equilibrated with 50 mM sodium borate pH 7.6. Resulting fluorescein-BSA was concentrated using centricon 30 K (Amicron) and coupled to sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate as a crosslinker (500 µg; Sulfo-SMCC, Pierce) in 50 mM sodium borate pH 7.6. Excess crosslinker was removed by gel filtration on a Sephadex G-50 column equilibrated in 100 mM sodium phosphate pH 6.0. Fluorescein-BSA-SulfoSMCC was concentrated and finally crosslinked to a peptide containing the SV40 large T antigen NLS (cgggDEVKRKVED; 1 mg) in 100 mM sodium phosphate pH 6.0. Noncoupled pepetide was eliminated by gel filtration on a Sephadex G-50 column equilibrated in transport buffer and resulting BSA-NLS-Fluorescein (BSA-NLS-Fluo) was concentrated.

Nuclear import assay

Digitonin-permeabilized HeLa cells were prepared according to Adam et al. (Adam et al., 1990). Cells were plated onto glass coverslips 24 h before the assay and grown to 80% confluence. Cells were washed once in phosphate-buffered saline containing 0.5 mM $CaCl_2$ and 1 mM $MgCl_2$ and once in transport buffer before permeabilization with 55 µg/ml digitonin (Sigma) in transport buffer for 5 min at 4°C. Nuclear import assays in the absence of

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cytosolic extracts were performed in transport buffer containing an energy-regenerating system (1 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, and 8 U/ml creatine phosphokinase), 25 µl BSA (10 mg/ml in transport buffer) and 1 µg/ml of Cy3-coupled recombinant IN in a total volume of 50 µl. For nuclear import assays in the presence of cytosolic extracts, BSA was replaced by 25 µl of HeLa cell cytosolic extracts (~10 mg/ml) and 15 µg/ml of BSA-NLS-Fluo was added to the 50 µl reaction mix. The assays in the presence of IN inhibitors were performed using the tested molecules at concentrations ranging from 10 µM to 100 µM. Transport reactions were allowed to proceed at 30°C for 30 min. Cells were then washed with transport buffer and fixed with 2% paraformaldehyde and 0.1% glutaraldehyde. Coverslips were subsequently mounted in phosphate-buffered saline containing 50% glycerol.

Images were acquired with a Leica DMRB epifluorescence microscope equipped with a CCD camera (Princeton) controlled by Metaview software (Universal Imaging Corporation). For each condition, fluorescence intensity per surface unit was quantified in 150 to 300 nuclei from 3 independent experiments using Image J software.

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RESULTS AND DISCUSSION

IN import is specifically inhibited by active SQ *in vitro*.

Active and non-active members of the SQ family of inhibitors represented on Figure 1 were defined according to their ability to inhibit IN integration activity *in vitro* (Bénard et al., 2004; Deprez et al., 2004; Polanski et al., 2002; Zouhiri et al., 2000). The archetypal L-731,988 strand transfer inhibitor (Hazuda et al., 2000) is also shown. The anti-integrase effect of khd161, fz41 and BioA53 was linked *in vitro* to the presence of the salicylic motif on the quinoleine ring (Bénard et al., 2004). In contrast, fz117 represents a structural analog lacking the 8-hydroxyl group and devoid of anti-integrase activity (Deprez et al., 2004). Similarly, addition of a carboxyl group at position 10 prevents khd227 to affect the enzyme activity (Polanski et al., 2002).

We previously characterized properties of IN nuclear import using an *in vitro* transport assay (Depienne et al., 2001). In this experimental system (figure 2A), plasma membrane of HeLa cells is selectively permeabilized with digitonin, which at low concentrations selectively perforates the plasma membrane thus releasing soluble cytosolic components but does not affect the integrity of the nuclear envelope (Adam et al., 1990). Indeed digitonin preferentially perforates the plasma membrane compared to internal cellular membranes due to its proportionally higher cholesterol content (Colbeau et al., 1971). A fluorescent import substrate can readily enter the resulting leaky plasma membrane and its uptake into the nucleus can be followed by fluorescence microscopy. Using this assay, we have previously reported that Cy3-labelled IN (IN-Cy3) rapidly accumulates into nuclei of digitonin-permeabilized cells. This accumulation does not result from passive diffusion but rather from an active transport that occurs through nuclear pore complexes. This import results from a saturable mechanism indicating that a limiting cellular factor is involved in this process.

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However, we found that IN import is not mediated by a classical import pathway involving karyopherin β family members nor the Ran GTPase and more generally does not require addition of cytosolic extracts. Cellular factor(s) involved in this atypical pathway thus likely remain(s) associated with the nuclear compartment of permeabilized cells. In contrast to classical import pathways, IN import requires energy in the form of ATP hydrolysis.

We thus used this minimal system to evaluate the effect of SQ derivatives on IN import compared to the specific strand transfer inhibitor L-731,988. The import reaction was performed in the presence of increasing concentrations of active SQs, non-active SQs or L-731,988. For each condition, IN import was quantified by measuring fluorescence intensity per surface unit in 150 to 300 nuclei from 3 independent experiments. Nuclear import of IN was clearly and specifically inhibited by the active SQ derivatives fz41 and khd161 in a dose-dependant manner with an IC_{50} of about 30 μM for fz41 and 55 μM for khd161 (figure 2B and 2C). BioA-53 prevented only 20% of the nuclear import when used at 100 μM thus showing a poor inhibitory effect. However it should be noted that this compound displays a 10 fold lower inhibitory effect in catalytic activity assays as compared to fz41 and khd161 (data not shown). In contrast, neither the non-active SQs fz117 and khd227 nor the archetypal L-731,988 strand transfer inhibitor displayed any significant inhibitory effect on IN import at any tested concentration (figure 2B and 2C). Together, these results indicate that active SQs specifically inhibit IN import in digitonin-permeabilized cells in the absence of cytosolic extracts, whereas non active SQs and the strand transfer inhibitor L-731,988 do not display any significant effect on this transport.

Active SQ inhibit specifically IN import *in vitro* in the presence of cytosolic extracts.

To test whether SQs affect selectively nuclear import of IN or prevent more generally nuclear import pathways, we tested the effect of SQ derivatives on the import pathway mediated by

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the classical basic nuclear localization signal (NLS) of the SV40 large T antigen. Transport of proteins containing the SV40 large T antigen NLS is insured by a specific heterodimeric receptor, importin α/β1, as well as the small GTPase Ran. Therefore, *in vitro* nuclear import of such proteins requires addition of exogenous cytosolic extracts to digitonin-permeabilized cells (Adam et al., 1990). The import assay of both fluorescein-labeled Bovine Serum Albumin fused to the SV40 large T antigen NLS (BSA-NLS-Fluo) and IN-Cy3 was thus performed in the presence of HeLa cell cytosolic extracts in the same cells. As previously, import reactions were performed in the presence of increasing concentrations of active SQs, non-active SQs or L-731,988. BSA-NLS-Fluo and IN-Cy3 import was quantified by measuring fluorescence intensity per surface unit in 150 to 300 nuclei from 3 independent experiments. None of the tested molecules presented any significant inhibitory effect on import mediated by the classical SV40 large T antigen NLS (figure 3B and 3C) whereas in the same cells, nuclear import of IN was specifically inhibited by fz41 (IC_{50} 17 μM), khd161 (IC_{50} 33 μM) and BioA-53 (IC_{50} 60 μM) in a dose-dependant manner (figure 3B and 3C). The discrepancy of inhibitory activity of BioA-53 measured in the absence or presence of cellular extracts in the import assay likely corresponds to a stabilization of this compound by the extracts *in vitro*. Similarly to results observed in the absence of cytosolic extracts, neither the non-active SQs fz117 and khd227 nor the strand transfer inhibitor L-731,988 were able to affect IN import in the presence of cytosolic extracts at any concentration tested (figure 3B and 3C). Together, these results indicate that active SQs specifically inhibit IN nuclear import in digitonin-permeabilized cells in the presence of cytosolic extracts and do not affect other import pathways such as the classical basic NLS-mediated transport.

Together, these data show that IN import is specifically inhibited by active SQs in digitonin-permeabilized cells and suggest that SQs would inhibit the interaction between IN and cellular factor required for its nuclear import. SQs have been previously reported to be

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competitive inhibitors for IN binding to DNA *in vitro*, that block the 3'-processing activity of the enzyme (Deprez et al., 2004). The *in vitro* effect of SQs on both IN integration activity and nuclear import, could be explained either by a binding of SQs on distinct functional sites of IN or alternatively by a binding on a unique site controlling both functions. We recently found that IN mutants bearing mutations on the V165 residue are resistant toward fz41 (Bonnenfant et al., 2004). This region could be involved in the interaction of IN with LEDGF/p75, a factor recently proposed to be involved in the subcellular localization of IN (Maertens et al., 2003). This region is also located in the vicinity of the actual active site of the enzyme and mutations of the V165 residue are known to alter the catalytic activity of recombinant IN, thereby pointing to a possible dual effect of the binding to a unique site (Limon et al., 2002). Nevertheless, we cannot rule out a simultaneous binding to different sites on the enzyme as we recently demonstrated by molecular modeling (Deprez et al., 2004). The fact that SQs are less active against nuclear import than against IN catalytic activity would favor this second hypothesis.

In contrast to SQs, the strand transfer inhibitor L-731,988 did not affect IN import in digitonin-permeabilized cells, supporting a distinct binding mode for SQs and L-731,988 to IN. In agreement with this result, viruses resistant to SQs have been selected and found to present either a single mutation (C280Y) or a double mutation (V165I, V249I) in the IN sequence (Bonnenfant et al., 2004). These mutated amino-acids are different from those conferring resistance to L-731,988 (T66I and/or S153Y, M154I and/or T66I) (Hazuda et al., 2000), clearly indicating that these two types of inhibitors use distinct binding sites on IN. IN catalyzes the insertion of a donor DNA into an acceptor DNA. Pommier and colleagues proposed that both DNA substrates could bind distinct adjacent sites on IN and that mono-functional β-diketo acids such as L-731,988, that specifically inhibit the strand transfer reaction, would bind selectively the acceptor DNA site on IN (Marchand et al., 2002). In

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contrast, SQs, that preferentially inhibit 3'-processing in a competitive manner (Deprez et al., 2004), would have a higher affinity for the donor DNA site of IN. If SQs preferentially bind an unique site on IN affecting both integration activity and nuclear import, the binding of SQs to the donor DNA site on IN would inhibit the binding of the cellular factor required for its nuclear import and we could expect that binding of donor DNA would also prevent nuclear import in vitro.

SQs now represent a major tool for the identification of the IN import factor, just as the antifungal antibiotic leptomycin B constituted a major clue to the identification of the Nuclear Export Sequence receptor Crm1. In turn, characterization of the IN import pathway should lead to the identification of new antiviral target.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Chemical structure of the compounds tested for their effect on IN import.

Khd161, fz41 and BioA53 are active SQs, whereas fz117 and khd227 are non-active members of the SQ family. Active and non-active SQs are defined according to their ability to inhibit IN integration activity *in vitro*. L-731,988 is a specific strand transfer inhibitor.

Figure 2. IN import is specifically inhibited by active SQ *in vitro* in the absence of cytosolic extracts.

(A) Schematic representation of the *in vitro* import assay. Plasma membrane of HeLa cells is selectively permeabilized with digitonin, thus leading to the release of cytosolic components without affecting the nuclear envelope integrity. Nuclear import of Cy3-labeled IN (IN-Cy3) can be reconstituted in this system in the presence of an energy-regenerating system.

(B) HeLa cells were digitonin-permeabilized and then incubated for 30 min at 30°C with IN-Cy3 in the presence of an energy-regenerating system and increasing concentrations of different compounds as indicated. Cells were subsequently fixed and analyzed by direct fluorescence. Images were acquired with an epifluorescence microscope equipped with a CCD camera.

(C) For each experimental condition shown in (B), fluorescence intensity per surface unit was quantified in 150 to 300 nuclei from 3 independent experiments using Image J software. The resulting values were expressed as a percentage of the untreated control value.

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Figure 3. Active SQ inhibit specifically IN import *in vitro* in the presence of cytosolic extracts.

(A) Schematic representation of the *in vitro* import assay in the presence of cytosolic extracts. Nuclear import of Cy3-labeled IN (IN-Cy3) and fluorescein-labeled Bovine Serum Albumin fused to the classical Nuclear Localization Signal of the SV40 large T antigen (BSA-NLS-Fluo) can be reconstituted and followed in the same digitonin-permeabilized HeLa cells in the presence of an energy-regenerating system and cytosolic extracts.

(B) HeLa cells were digitonin-permeabilized and then incubated for 30 min at 30°C with IN-Cy3 and BSA-NLS-Fluo in the presence of an energy-regenerating system, cytosolic extracts and increasing concentrations of different compounds as indicated. Cells were subsequently fixed and analyzed by direct fluorescence. Images were acquired with an epifluorescence microscope equipped with a CCD camera.

(C) For each experimental condition shown in (B), fluorescence intensity per surface unit was quantified in 150 to 300 nuclei from 3 independent experiments. The resulting values were expressed as a percentage of the untreated control value.

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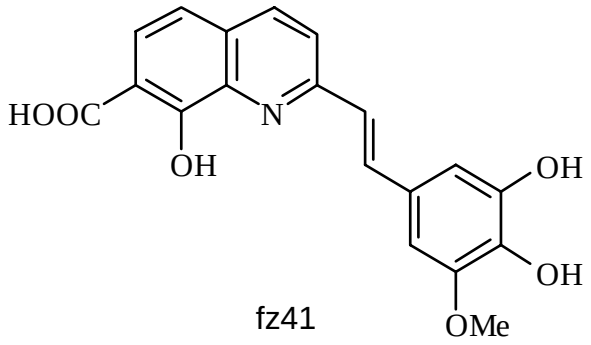
Figure 1



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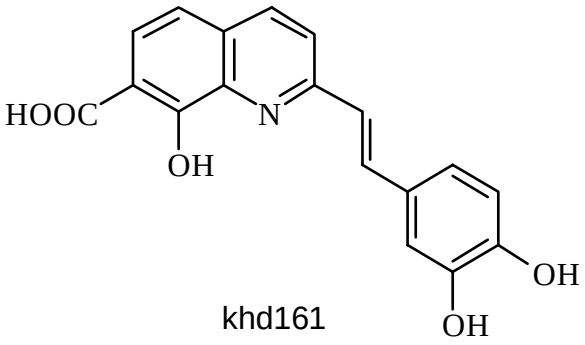

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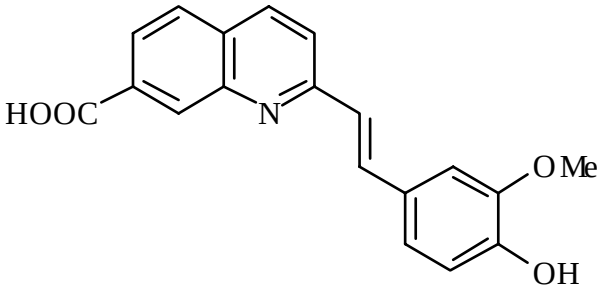

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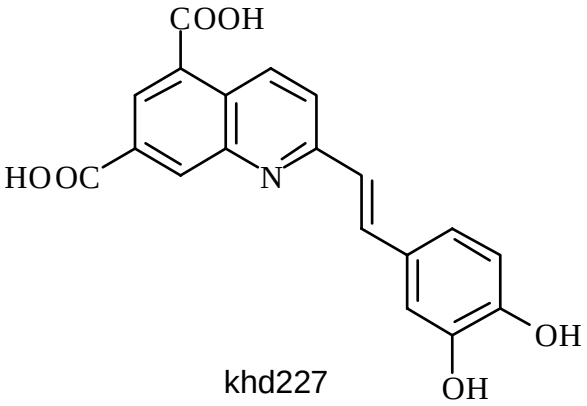

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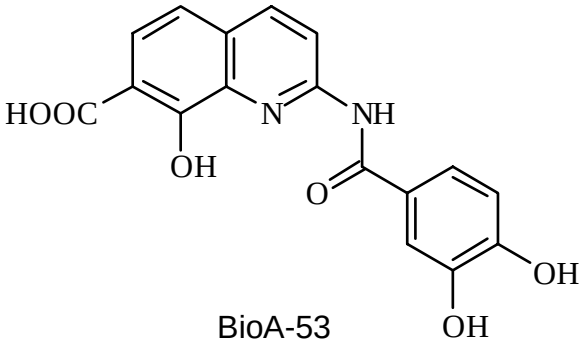
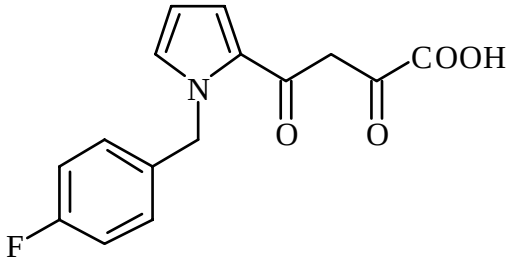


BioA-53



L-731,988

Figure 2

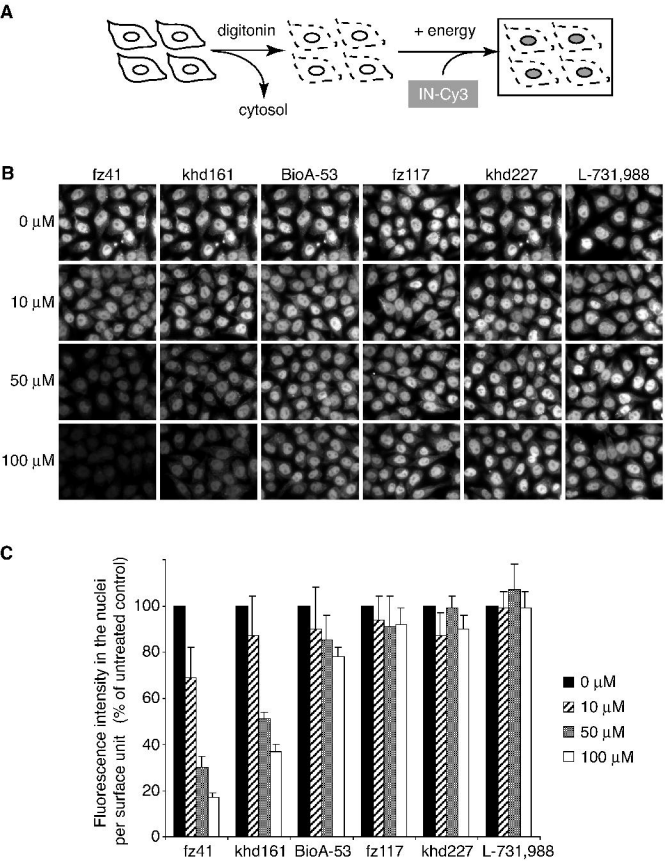

A
digitonin
+ energy
cytosol
IN-Cy3
B
fz41
khd161
BioA-53
fz117
khd227
L-731,988
0 µM
10 µM
50 µM
100 µM
C
Fluorescence intensity in the nuclei per surface unit (% of untreated control)
0
20
40
60
80
100
fz41
khd161
BioA-53
fz117
khd227
L-731,988
0 µM
10 µM
50 µM
100 µM

Figure 3

A
digitonin
cytosol
+ energy
+ cytosolic extracts
IN-Cy3
BSA-NLS-Fluo
B
fz41
fz117
L-731,988
fz41
fz117
L-731,988
0 µM
10 µM
50 µM
100 µM
IN-Cy3
BSA-NLS-Fluo
C
IN-Cy3
BSA-NLS-Fluo
Fluorescence intensity in the nuclei per surface unit (% of untreated control)
0
20
40
60
80
100
120
fz41
khd161
BioA-53
fz117
khd227
L-731,988
fz41
khd161
BioA-53
fz117
khd227
L-731,988
0 µM
10 µM
50 µM
100 µM