3.1 Materials

To achieve the aims and objectives of the proposed research work following materials and methods were followed throughout the course of the study.

3.1.1 Plant material

*Momordica balsamina* seeds received from the Bhagalpur University, Bhagalpur, Bihar and were cultivated in Botanical garden, Punjabi University, Patiala. The voucher specimens were deposited in the herbarium, Department of Botany, Punjabi University, Patiala.

3.1.2 Chemicals

Ion exchange gel (CM sepharose fast flow) was obtained from Sigma (St. Louis, US). Superdex G75 prepacked column (10/300 GL) were purchased from Amersham Biosciences Co., Piscataway, USA. DNA isolation kit was obtained from QIAGEN (GmbH, Hilden, Germany). DNase I was procured from Promega (Madison, USA). Platinum supermix for PCR reaction was obtained from Invitrogen. Primary CD4⁺ T cell isolation kit II was obtained from MACS Miltenyi Biotec (GmbH, Gladbach, Germany). p24 ELISA kit procured from NIH AIDS Research & Reference Reagent Program, Germantown, MD, USA. All chemicals used were of analytical or higher grade.

3.1.3 Cell Lines and Drugs

Human T cell line (Jurkat) was obtained from ATCC; Manassa, VA, USA and maintained in RPMI-1640 medium (Life Technologies) supplemented with 10% heat inactivated fetal calf serum (FCS), 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 200 mM glutamine. The PBMCs (peripheral blood mononuclear cells) were isolated from buffy coats of healthy seronegative blood donors in accordance with institutional guidelines of the ethical committee of the University of Geneva (Switzerland). Primary CD4⁺ T cells were purified from peripheral blood mononuclear cells (PBMCs) of healthy donors and maintained in RPMI-1640 complete medium. 3’-Azido-3’deoxythymidine (AZT) and phytohemagglutinin (PHA) were purchased from Glaxo Smith Kline and Sigma respectively. IL-2 was procured from Roche, Indianapolis, IN, USA. Adherent cells MDCK (Madin Darby Canine Kidney) and A549 were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% heat inactivated FCS, penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹) and glutamine (200 mM).
3.1.4 Viruses

Virus stocks were stored in aliquots at -80°C. HIV-1 stock (R9 strain) was initially produced by transient transfection of 293T cells with CaCl$_2$. Virus replication was scored either by monitoring production of viral p24 capsid protein by ELISA assay, or by monitoring the Reverse Transcriptase enzymatic activity in the producer cells supernatant. For single-round infections, we used an HIV-1 deleted for the Env gene and pseudotyped with the surface G protein of vesicular stomatitis virus (VSV). The infectivity titer of R9 virus (HIV-1; p24: 540 µg ml$^{-1}$) was 1.3 × 10$^8$ IU ml$^{-1}$. HIV-GFP+Nef+pMDG (p24: 125 µg ml$^{-1}$) titer was 4.3 × 10$^8$ IU ml$^{-1}$. Influenza A/PR8/34 (H1N1) strain was produced by infection of MDCK cells (Madin Darby Canine Kidney) at a moi (Multiplicity of infection) of 0.001, followed by culture for 72 h in serum-free Opti-MEM (Minimum essential medium; Invitrogen) supplemented with 1 µg ml$^{-1}$ TPCK-treated trypsin (Sigma).

3.1.5 Protein Analysis and Antibodies

Cells were lysed with RIPA (Radio-Immunoprecipitation Assay) buffer. Lysates were then pre-cleared (13’000 rpm for 10 minutes) and their protein content was quantified with the BCA kit (Thermo Scientific), and were subjected to 12% SDS-PAGE. Antibodies serving for the detection against actin (Millipore), M1 (abcam) were of mouse origin. Gag p55 and p24 were detected with the mouse monoclonal antibody made by Bruce Chesebro and Kathy Wehrly (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, US) (Toohey et al., 1995). HRP-conjugated horse anti-mouse (Bio-Rad Laboratories) IgG antibodies were used for Western blots.

3.1.6 Microbial cultures

The bacterial strains used for antimicrobial activity were Serratia marcescens 97, Bacillus subtilis 1790, Pseudomonas fluorescens 103, Klebsiella pneumonia 109, Bacillus cereus 1301 and Staphylococcus xylosus. All cultures were revived from stocks obtained from Microbial Type Culture Collection and Gene bank (MTCC). To obtain cultures, the bacteria were inoculated into the nutrient broth and incubated overnight at 37°C.
3.2 Methods

3.2.1 Cytological studies

For carrying cytological studies, appropriate sized flower buds of *M. balsamina* were collected. For the meiotic studies, flower buds were fixed in Carnoy’s fixative (6 part ethyl alcohol: 3 parts chloroform: 1 part glacial acetic acid) for 24 h. After fixation, the material was transferred to 70% ethanol for meiotic studies. For chromosomal preparations, anthers were squashed in 1% acetocarmine. Chromosome counts were confirmed from large number of PMCs (Pollen mother cells) at different stages of meiosis.

3.2.2 Preliminary Phytochemical analysis

A qualitative phytochemical analysis was carried out using dried *M. balsamina* fruit as follows:

3.2.2.1 Test for flavonoids: Two methods were used to determine the presence of flavonoids (Sofowara, 1993). Dilute ammonia solution (5 ml) was added to 1 ml of aqueous extract followed by addition of few drops of concentrated H$_2$SO$_4$. A yellow colouration observed in the extract indicated the presence of flavonoids. The yellow colouration disappeared on standing.

Few drops of 1% ammonium sulphate were added to 1 ml aqueous extract. A yellow colouration was observed indicating the presence of flavonoids.

3.2.2.2 Test for tannins: Two methods were used to determine the presence of tannins. Bluish-black colour formation on addition of few drops of 5% ferric chloride to 1 ml of aqueous extract indicates the presence of tannins.

Few drops of iodine solution were added to 1 ml extract. Formation of brownish, bluish-black colour indicates the presence of tannins (Trease and Evans, 1996).

3.2.2.3 Test for glycosides (Keller-Killani test): 5 ml of filtrate was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml concentrated H$_2$SO$_4$. A brown ring obtained at the interface indicates the presence of cardiac glycosides (Trease and Evans, 1996).
3.2.2.4 **Test for terpenoids (Salkowski test):** Five ml of extract was treated with 2 ml of chloroform, and 3 ml concentrated H$_2$SO$_4$ was carefully added to form a layer. A reddish brown colouration of the interface formed showed the presence of terpenoids (Sofowara, 1993).

3.2.2.5 **Test for saponins:** 0.5 g of the extract was separately stirred in a test tube, foaming which persisted on warming was taken as evidence for the presence of saponins (Trease and Evans, 1996).

3.2.2.6 **Test for alkaloids:**

**Mayer’s test:** To a few ml of the filtrate, a drop of Mayer’s reagent was added by the side of the test tube. A reddish brown precipitates indicates that the test is positive.

**Wagner’s test:** A drop of Wagner’s reagent was added to few ml of the filtrate. Formation of white or cream precipitates indicates the presence of alkaloids (Trease and Evans, 1996).

3.2.2.7 **Test for phytosterols or steroids:** 1ml of filtrate was treated with 2 ml acetic anhydride followed by addition of few drops of concentrated H$_2$SO$_4$. An array of colour change indicates the presence of phytosterols or steroids (Trease and Evans, 1996).

3.2.2.8 **Test for phlobatannins:** Deposition of red precipitate when an aqueous extract was boiled with 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatannins.

3.2.2.9 **Test for anthraquinones:** Powdered sample was boiled with 10% HCl for few minutes, filtered and allowed to cool. This was then partitioned against equal volumes of chloroform. Formation of pink colour in the aqueous layer on addition of 100% ammonium solution indicated the presence of anthraquinones (Trease and Evans, 1996).

3.2.2.10 **Test for carbohydrates:** To 0.5 ml of the filtrate, 0.5 ml of Benedict’s reagent was added. The mixture was heated on boiling water bath for 2 min. A characteristics red colour precipitate indicates the presence of sugar (Benedict, 1908).

3.2.2.11 **Test for resins:** 1 ml of the filtrate was treated with 5 ml of boiling ethanol and filtered through Whatman filter paper. To this 4 ml 1% HCl was added. Resinous formation indicated the presence of resins.
3.2.3 Antimicrobial activity of plant extract

Methanolic extract was prepared by adding 1g of air-dried powder *Momordica balsamina* leaf in 100 ml of methanol in a conical flask and kept on a rotary shaker at 200 rpm for 72 h. After 72 h the supernatant was collected and the solvent was evaporated on water bath and final volume was dissolved in DMSO (Dimethyl Sulphoxide) and stored at 4°C in an airtight bottle for evaluating antimicrobial activity. The agar well-diffusion method was used for antimicrobial activity by measuring the diameter of zone of inhibition. Nutrient agar plates were swabbed (sterile cotton swabs) with 24 h old broth culture of the *Serratia marcescens* 97, *Bacillus subtilis* 1790, *Pseudomonas fluorescens* 103, *Klebsiella pneumonia* 109, *Bacillus cereus* 1301 and *Staphylococcus xylosus*. The overnight broth cultures (100 µl) were used so as to ensure the concentration of these organisms to contain 1 x 10^6 cfu ml⁻¹. The cultures were spread on the solidified agar surface using sterilized spreader. A sterile cork borer was used to prepare two wells, each measuring 8 mm in diameter. About 100 µl of *M. balsamina* methanolic extract was added into the wells and allowed to diffuse at room temperature for 2 h. DMSO (Dimethyl Sulphoxide) used as a positive control in another well. The plates were incubated at 37°C for 18-24 h.

3.2.4 Extraction of Protein

*Momordica balsamina* seeds (20g) were decorticated and grounded by mortar and pestle to a powder form. The powder was homogenized in ice-cold 50 ml 150 mM NaCl. The mixture was stirred gently at 4°C for 1 h. The slurry was filtered through muslin cloth and centrifuged at 10,000×g for 20 min at 4°C. The resulting supernatant contained a layer of seed fat, which was removed by filtration through 0.45 µm filter (Millipore, US). The clear supernatant was collected and used for purification of protein.

3.2.5 Purification of protein

Various methods of protein purification were employed for achieving target protein (Figure 3.1).

3.2.5.1 Ammonium sulfate fractionation and dialysis

Crude *M. balsamina* extract was taken and then required quantity of ammonium sulfate was added slowly to obtain a various saturation levels (20%, 40%, 60% and 80%).
The clear supernatant was precipitated with the slow addition of solid ammonium sulfate with constant stirring using a magnetic stirrer at 4°C. The precipitated proteins were separated by centrifugation at 10,000 rpm at 4°C for 20 min. The supernatant was removed and resulting precipitate was dissolved in 15 ml of 10 mM phosphate buffer, pH 6.5 (Buffer A) and refrigerated for further analysis. Precipitated proteins were transferred into dialysis tube and dialyzed against 10 mM phosphate buffer (pH 6.5) at 4°C. Dialysis was carried out overnight and buffer was changed several times to increase the efficiency of the dialysis. The dialysate was further purified by Ion-exchange chromatography.

3.2.5.2 Ion exchange chromatography

Chromatography is the most commonly and widely used means of purifying proteins and separating small molecules. Ion exchange chromatography requires that a protein contains a net ion charge under experimental conditions. As a result, the protein will displace a lower molecular weight ion from an ion exchange matrix and become bound. Separation of proteins by ion exchange chromatography requires differential binding of proteins to ion exchange matrix by electrostatic forces. After proteins are applied to an ion exchange, those proteins which have no affinity for matrix are removed during wash of column. Then, adsorbed proteins were removed in an elution step by raising counter ion (salt) concentrations. In step elution, salt concentration is increased in distinct steps. A gradient elution utilizes a gradient maker to establish a smooth (continuous) increase in salt concentration. Ion exchange separation is carried out mainly in column packed with an ion exchanger. There are basically two types of ion exchangers:

Cationic exchanger: possesses negatively charged groups. These will attract positively charged cations. e.g. SP- Sepharose, CM-sepharose.

Anionic exchanger: possesses positively charged groups that will attract negatively charged groups. e.g. Q-Sepharose.

CM-sepharose column: CM-sepharose fast flow is a weak cation exchanger. CM Sepharose fast flow supplied by Pharmacia and with excellent flow properties and high capacity for proteins of all pI values. The ion exchange group is a carboxymethyl group (-O-CH₂COO-) which remains charged and maintains consistently high capacities over the entire working range, pH 4–13. Suspension of CM-sepharose dissolved in Buffer A and poured gently in column (20 cm × 1.5 cm; Pharmacia or GE Healthcare, US) through the sides to avoid bubble formation. The CM-sepharose was allowed to settle to make bed
and then the column was washed with 5 column volume of Milli Q water. The column was settled and washed with Buffer A for equilibration.

The dialyzed protein solution was loaded onto a CM-sepharose fast flow column (20 cm × 1.5 cm), which was equilibrated with five column volumes of buffer A. The column was then washed with the same buffer at a flow rate of 1 ml min⁻¹ until no protein eluted. About 60-70% of the contaminating proteins were removed, whereas balsamin bound to CM-sepharose fast flow was thus retained on the column. Bound proteins were eluted with a linear gradient of 0-0.4 M NaCl in 10 mM phosphate buffer, pH 6.5 (Buffer B). The fractions that contained low-molecular weight proteins (based on SDS-PAGE analysis) were pooled. The fractions were concentrated by ultrafiltration membrane (Amicon Ultra-15 10 kDa, US).

### 3.2.5.3 Gel filtration chromatography

Gel filtration chromatography separates proteins according to their size. The gel filtration matrix contains pores which permit the buffer and smaller proteins to enter but exclude larger proteins and protein complexes. The largest proteins emerge from the column first since they have the smallest volume to pass through before reaching the end of the column. Medium sized proteins can enter the larger size pores of the matrix, and so they reach the end of the column later. Small proteins are able to enter all pores, and they have the largest volume to pass through before emerging from the column last.

**Superdex 75 column:** Superdex 75 (10/300 GL) pre-packaged column (Amersham Biosciences Co., Piscataway, USA) was used for further purification. The column was equilibrated with Buffer A before the sample was loaded, until the baseline reached. The concentrated fraction (250 µl) was loaded carefully onto a superdex 75 column. The protein was eluted with 10 mM phosphate buffer, pH 6.5 at a flow rate of 0.5 ml min⁻¹ and the fractions (0.5 ml) were collected. The eluted fractions that showed N-glycosidase activity were pooled, and concentrated by ultrafiltration and stored at -20°C.

### 3.2.5.4 Ultrafiltration

Concentration of protein by ultrafiltration proceeds by forcing the liquid in a protein solution through a membrane which retains the protein of interest and efficient selective rejection of other solutes. In ultrafiltration, solutes, colloids or particles of dimensions larger than the specified membrane cut off are quantitatively retained in solution, where as solutes smaller than uniform minute pores pass unhindered with
solvent through supportive membrane substructure. Ultra filtration membrane offers a selection of macrosolute retention ranging from 500-30,000 MW as calibrated with globular macrosolutes. Each membrane is characterized by its normal ‘cut off’ i.e. its ability to retain molecules larger than those of given size.

Concentrating protein solutions by use of a filtering system designed for micro centrifuge Ultraspin cones (Millipore, US) consist of an insert fitted with a membrane filter suitable to hold back molecules greater than 10 kDa. This insert fits into the ultra spin cones in which effluent is collected.

**Protocol**

1. To the upper chamber of ultra spin cones, added 2 ml of pooled fraction from gel filtration chromatography.

2. The cones were spun at 4000 rpm for 20 min under refrigerated conditions.

3. The concentrated fraction was collected from the upper chamber by gently flushing the filter for deposited proteins using a micropipette.

4. The concentrated fraction was checked for $N$-glycosidase activity and protein concentration.

**3.2.6 Estimation of protein concentration**

Protein concentration of crude extract and fractionated sample at each purification step was quantified using Bradford method (1976). The protein concentration of the sample was then calculated from the BSA standard curve.

**3.2.7 SDS-PAGE**

The molecular weight of the purified protein i.e balsamin was determined by SDS-PAGE performed according to the procedure of Laemmli (Laemmli, 1970), using a 12% resolving gel. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 (CBB). The molecular mass of the balsamin was determined by comparison with the protein markers; myosin (250 kDa), phosphorylase b (148 kDa), bovine serum albumin (66 kDa), glutamate dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa) and aprotinin (6 kDa).
Strategy for the Purification of Balsamin

Crude Extract

Ammonium Sulphate precipitation

Dialysis

Ultrafiltration (Amicon Ultra-15 10 kDa, US)

Ion Exchange chromatography (20 cm × 1.5 cm)

Gel Filtration chromatography (Superdex 75 (10/300 GL))

Figure 3.1 Diagrammatically representation of purification strategy
3.2.8 Molecular or Biological characterization of the purified protein

3.2.8.1 rRNA N-glycosidase activity assay

The assay was carried out as previously described (Bagga et al., 2003a; May et al., 1989). The RNA N-glycosidase activity of balsamin was carried out by its ability to specifically depurinate 28S rRNA and produce a characteristic 400-base fragment on aniline treatment. Rabbit reticulocyte lysate was taken as a source of ribosome. Rabbit reticulocyte lysate (50 µl) was treated with different concentrations of balsamin (0, 10, 20 and 50 µg ml⁻¹) and saporin-6 (0, 2.5, 5 and 10 µg ml⁻¹), incubated at 30ºC for 30 min. The reaction was terminated by the addition of 10 µl of 10% SDS (w/v) and 170 µl of water. Total rRNA was extracted with trizol reagent (Biorad). The RNA pellet was dissolved in 20 µl of water and divided into two parts. One part was treated with 10 µl of freshly prepared aniline acetate, pH 4.5, whereas the other part was left untreated. The samples were incubated at 60ºC for 3 min, aniline was evaporated under vacuum and treated RNA was dissolved in 10 µl of water. The aniline-treated and untreated samples were electrophoresed on 2% agarose gel for 1.5 h at a constant voltage of 60 V. The gel was stained with ethidium bromide and the RNA was visualized on a UV-transilluminator.

3.2.8.2 Cell-free protein synthesis inhibition assay

The efficacy in inhibiting the protein synthesis was determined using a rabbit reticulocyte lysate-based in vitro translation assay system. The assay was performed according to the procedure described (Bagga et al., 2003a; Sambrook et al., 1989). In the cell-free translation assay, 30 µl of reaction mixture contained 10 µl of rabbit reticulocyte lysate, 375 mM KCl, 10 mM magnesium acetate, 15 mM glucose, 50 mM Tris-HCl, 5 mM ATP, 1 mM GTP, 1 mM amino acid mix without leucine, 1 µCi/µl [³H] leucine, 5 mg ml⁻¹ creatine phosphokinase, 2 mg ml⁻¹ creatine phosphate and 6 µl of different concentration of balsamin. BSA was used as a negative control to check whether any other protein besides balsamin has some effect on translation. The reaction was carried out at 30ºC for 60 min and stopped by adding NaOH (250 µl, 1N) containing H₂O₂ (0.2%). After further incubation at 37ºC for 10 min, the proteins were precipitated with trichloroacetic acid (15%) on ice for 30 min and harvesting was done on 26 mm glass fiber filters (Whatman). The dried filters were counted using a liquid scintillation counter.
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(Perkin Elmer, MD, USA). ID_{50} represents the concentration of toxin that inhibited *in vitro* protein synthesis by 50%. Saporin (a type I RIP) from *Saponaria officinalis* was used as a positive control (Bagga *et al.*, 2003b).

3.2.8.3 N-terminal sequence

The sequence of first 12 amino acids from N-terminal end was determined by Edman degradation method using an automated protein sequencer. Protein was subjected to SDS-PAGE using 12% separating gel. Blotting was performed at 350-400 mA for 35 min using polyvinyl fluoride membrane. After transfer, the membrane was stained and de-stained and then washed extensively with Milli-Q water. Protein blots were loaded on Blott™ cartridge (reaction chamber) and amino acid sequencing was conducted with protein sequencer (Applied Biosystems PROCISE 491 cLC).

3.3 Biophysical characterization of balsamin

3.3.1 Mass spectrometric analysis

3.3.1.1 In gel trypsin digestion

The protein spots from coomassie stained gel was cut into 1 mm³ pieces and transferred into a sterile micro centrifuge tube. In gel trypsin digestion was done as per the described protocol (Promega). The resulting peptide mixture was separated by reverse phase chromatography (Tempo™ nano LC system, Applied Biosystems) using a C_{18} column (Pep Map). Peptides were separated using a 70 minute linear gradient from 5% to 98% acetonitrile in 0.1% formic acid with a flow rate of 400 nl min⁻¹. The eluting peptides were ionized by electrospray ionization and analysed by QSTAR XL system (Applied Biosystems, USA). Nanospray ionization was carried out using an ion spray voltage of 900. The progress of each run was monitored by recording the total ion current (TIC) for positive ions as a function of time in the m/z range of 400-1600 for MS and 140-1600 for MS/MS. The spectrum was acquired in an information dependent manner utilizing the Analyst QS 2.0 software acquisition features to generate raw data in the wiff format. The other parameters set were: interface temperature, 50°C; curtain gas flow, 1.13 L min⁻¹; declustering potential, 60V; focusing potential, 280V; and declustering potential 2, 15V.
3.3.1.2 Protein identification

MS/MS (Tandem mass spectrometry) analysis and data base searching was performed using Protein Pilot™ software (version 2.0, Applied Biosystems). All Protein Pilot™ software data base searches were performed using Paragon™ database algorithm for searching in through mode. Modifications considered were oxidation of methionine and carbamidomethylation of cysteine while searching. Search was further refined to include peptides with charged state from +2 to +3 and limited to *Momordica charantia*. The peptide mass tolerance range was ± 1.2 Da and fragment mass tolerance was ± 0.6 Da.

3.3.2 CD spectroscopy

CD experiments were performed on Jasco J-815 spectropolarimeter. CD spectrum of the purified balsamin (1 mg ml\(^{-1}\)) prepared in a sodium phosphate buffer (10 mM, pH 6.5) was measured in the far-UV range (200-240 nm) in 1 mm path length cuvettes. Analysis of the protein CD spectrum was performed using Spectra manager™ software (Jasco).

3.4 Bioinformatics Analysis

3.4.1 Homology Modelling

Homology modelling is a powerful tool for predicting the 3D structure of a protein. In homology modelling, a known 3D structure of a macromolecule (template) is used to derive the structure of an unknown macromolecule (target). Homology modelling rely on the similarity between the target sequence and at atleast one known structure, and still remains the most used and reliable method to predict the 3D structure of a target protein with an accuracy that can be comparable to a low-resolution, experimentally determined structure. The steps in the modelling process include the identification of proteins with known 3D structure that are related to each other. Homology modelling was carried out in four sequential steps (Figure 3.2):

3.4.1.1 Identification of known macromolecule structure (template) related to the sequence to be modelled macromolecule (target)

3.4.1.2 Alignment of the target sequence with the template sequence
3.4.1.3 Building of the model

3.4.1.4 Assessment of the model

**3.4.1.1 Identification of known macromolecule structure (template) related to the sequence to be modelled macromolecule (target)**

The identification of template related to target sequence has to consider that comparative modelling is only applicable when sequence identity between target sequence and the template protein structure is at least 30%, as below this cut-off the accuracy of models decrease sharply, mainly as a result of a rapid increase of the errors in the alignment step (Sander and Schneider, 1991). The identification of a suitable template with respect to target sequence (balsamin) is carried out by PDB BLAST. This method gives output an alignment between the target sequence and the template sequence: the similarity between the target and the template, the reliability and accuracy of the alignment are expressed by a score that allows an easy comparison between alternative alignments for the same sequences.

**3.4.1.2 Alignment of the target sequence with the template sequence**

Sequence alignment is a method, where the primary amino acid sequence of the template is aligned with a sequence of the target (balsamin) in order to find structurally or functionally conserved region. The alignment obtained in the previous step could be refined using other programs, like Clustal W (Thompson et al., 1994), that take into consideration the secondary structure elements and the folding of the template.

**3.4.1.3 Building of the model**

Once the structural template has been selected and sequence has been aligned with the target sequence, the next step is building of the model. The Clustal W alignment obtained in the previous step has been submitted to Swiss-model for the building of model of balsamin. The three dimension (3D) model was then built automatically using Swiss-model program. The program is able to automatically derive the 3D structure from the balsamin sequence aligned with the target sequence when these are given as input. In this 3D modelling process one of the crucial step is to provide an appropriate aligned
sequence, since even very little differences in the gap opening positions might produce large changes in the final 3D structure.

**3.4.1.4 Assessment of the model**

Once the balsamin model has been generated by Swiss model, and before using them to docking, it is essential to validate the 3D structure in terms of two parameters: the accuracy of the model and the geometric/physico-chemical values. Regarding the accuracy, it is important that the quality can be expected is related to the percentage of sequence identity between the target and the template. Usually models are divided in high (50% sequence identity), medium (30-50%) and low accuracy (below 30%). In the model assessment phase, it is important to consider the physico-chemical/geometrical parameter that should follow the Ramachandran plot.

**3.4.2 Docking studies**

Docking, as a computational tool, allows the investigation of the binding between macromolecular targets and potential ligands to form noncovalent protein-ligand complexes and in general could be considered as an energy-optimization problem (Trotov and Abagyan, 2001) with two components: the search and the score (Kuntz et al., 1994). The docking studies were carried out on Docking server software using adenine as a ligand. Adenine was used as a ligand because balsamin (ribosome-inactivating protein from *M. balsamina*) binds with rRNA adenine and removes adenine residue from 28S ribosome in an irreversible manner and inhibits protein synthesis (Kaur et al., 2011b). Balsamin and template (alpha MMC) were docked with adenine so as to investigate possible interactions with adenine residue. Docking calculations were carried out using DockingServer (Bikadi and Hazai, 2009). The MMFF94 force field (Halgren, 1998) was used for energy minimization of ligand molecule (adenine) using DockingServer. Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. Docking calculations were carried out on balsamin model. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools. Affinity (grid) maps of 20×20×20 Å grid points and 0.375 Å spacing were generated using the Autogrid program (Morris et al., 1998). AutoDock parameter set- and distance-dependent dielectric
functions were used in the calculation of the vander Waals and the electrostatic terms, respectively.

Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method (Solis and Wets, 1981). Initial position, orientation, and torsions of the ligand molecules were set randomly. Each docking experiment was derived from 10 different runs that were set to terminate after a maximum of 250000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied. For template (alpha momorcharin; 1F8q) and balsamin, same docking procedure followed. Bioinformatics tools were used for this study mentioned in table 3.1.

Figure 3.2 Schematic representation of Bioinformatics study
Table 3.1: Bioinformatics tools used in this study

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3.5 Therapeutic applications

Therapeutic activity of balsamin was checked on R9 virus (HIV-1) and HIV-GFP+Nef+pMDG. The human immunodeficiency virus (HIV) is a member of the genus *Lentivirus* in the *Retroviridae* family that causes Acquired Immune Deficiency Syndrome (AIDS). HIV-1 deleted for the *env* gene and pseudotyped with the surface G protein of vesicular stomatitis virus (VSV). VSVG-HIV-1Δenv has deleted HIV envelope and can only infect cells in a single round. Therefore, it does not undergo a full replication cycle but produces HIV-1 p24<sup>gag</sup> that accumulates in cells. VSVG-HIV-1Δenv was produced by co-transfection of 293T cells with pHIV-GFP, a plasmid containing the full length genome except for a deletion in *Env*, *Vif* and *Nef*, where green fluorescent protein is cloned (a gift of D. Littman, New York University School of Medicine, New York, NY, USA), and pMDG, a plasmid encoding for the VSVG protein. VSVG-HIV-1Δenv was
produced in the presence of Nef. HIV belongs to the Retroviridae family, which groups together RNA viruses that require the action of the Reverse transcriptase (RT) enzyme to transform their RNA genome to DNA and thereby integrate it into the host cell genome and replicate.

Within the retroviruses, HIV belongs to the lentiviruses, which generally take long time to produce adverse effects in infected patients. Genetically, HIV differentiates into two strains: HIV-1 and HIV-2. These strains have 40 to 60% protein homology, are transmitted in the same way and both produce AIDS (Acquired Immunodeficiency Syndrome) indistinctly (Reeves and Doms, 2002). Compared to HIV-2, HIV-1 is more virulent, more infectious and constitutes the majority of HIV infectious globally. HIV-2 is largely found in West Africa, but it has the relatively lower capacity for transmission and pathogenicity (Ariën et al., 2005).

Further we checked antiviral activity of balsamin against influenza virus. Influenza viruses are single stranded RNA viruses belonging to the family of orthomyxoviruses. This virus is responsible for seasonal epidemics and high mortality pandemics in weakened individuals, as well as in young infants. Influenza virus is responsible for periodic widespread epidemics, which have taken the form of respiratory diseases with cold-like symptoms, but also sometimes serious disease with high mortality rates (Kao et al., 2010). Schematic representation of therapeutic application of balsamin is mentioned in figure 3.3.
Materials and Methods

Extraction/ Purification and MS analysis

Figure 3.3 Schematic representation of therapeutic application of balsamin

Momordica balsamina

Protein synthesis inhibition

Cytotoxicity

Inhibition of HIV-1

Inhibition of influenza virus
3.5.1 HIV-1 viral particles quantification

The HIV-1 replication inhibition was assayed by two methods; RT assay performed as per protocol (Aiken and Trono, 1995) and p24 antigen was analyzed by HIV-1 p24\(^{CA}\) Antigen Capture Assay kit from AIDS & Cancer Virus Program, NCI-Frederick, MD. In the presence of 3.57 µM balsamin, Jurkat cell line (7 × 10\(^5\) cells/ml) was infected with R9 (HIV-1) at a multiplicity of infection (moi) of 0.01. Well without virus infection (only balsamin) used as a negative control. After 8 h of virus post-infection, the cells were washed with 1x PBS to remove unbound virus and incubated with or without balsamin in a culture medium. Each two days, an aliquot of cell-free supernatant for each sample was harvested and filtered through 0.45 µm pore-size nitrocellulose membrane (Spin-X; Corning) for determination of p24 and reverse transcriptase assay. On the 11\(^{th}\) day of post-infection, cells were collected and stored at -80°C for further Western blot to analyse p24 and actin expression level. All experiments were performed in duplicate.

3.5.1.1 RT Assay

The assay was carried out in RT buffer containing 50 mM Tris-Cl, pH 7.9, 75 mM KCl, 5 mM MgCl\(_2\), 0.5 M EDTA, 10% NP-40 and Poly (A)-oligo dT solution, 10U ml\(^{-1}\). Virus was lysed in 1% Triton and incubated for 20 min at room temperature. RT mix was prepared by adding 4 µl of 1 M DTT and 40 µl 3H-TTP per ml of RT buffer. Sample (5 µl) was distributed in 96-well microtiter plate and 10 µl of complete “RT mix” was added and incubated at 37°C for 1 h. PBS was used as a negative control. After incubation, samples were loaded on a DEAE filter and washed three times with SSC (Saline-sodium citrate) 2x for 5 min. Later on 1x wash with 70% ethanol for 1 min and dried in oven for 20 min. Put DEAE filter in plastic bag and scintillation liquid was added. The reading was taken on Microbeta Trilux (Perkin Elmer) and result expressed in cpm ml\(^{-1}\) (Aiken and Trono, 1995).

3.5.1.2 p24 assay

The effect of balsamin on HIV-1 replication \textit{in vitro} was also measured by p24 expression using capture ELISA as HIV-1 p24\(^{CA}\) Antigen Capture Assay kit from AIDS & Cancer Virus Program, NCI-Frederick. This assay was used to determine the capsid protein concentration of HIV-1 (p24\(^{CA}\)) of samples derived from cell cultures infected
with Human immunodeficiency Virus-1 (HIV-1). HIV-1 p24 expression was assayed in cell-free supernatants harvested on each two days up to 11th day of post-infection.

Virus was lysed in 1% Triton and incubated at room temperature for 20 min, centrifuged at 3000 rpm for 3 min. Samples (100 µl) were added in 96-well plate, which was already coated with a monoclonal antibody to HIV-1 p24CA and blocked with a 0.5% casein solution. Incubate at 37°C for two hrs. After incubation, 100 µl Primary antibody (Rabbit anti-HIV (MN) p24; 1/300) was added and incubated at 37°C for 1 h. Wash plate with ELISA buffer provided with kit, secondary antibody (Goat anti-rabbit IgG (H+L)-HRP) was added and incubated for 30 min at room temperature. Reaction was stopped by adding 100 µl 1N HCl and reading was taken at 450/650 nm.

3.5.1.3 Western Blotting

Cells were collected on the 11th day of post-infection and lysed with RIPA (Radio-Immunoprecipitation Assay) buffer for the extraction of protein. Protein concentration was calculated with BCA method. Samples were subjected to SDS-PAGE and wet transfer was carried out in 1× Tris-glycine transfer buffer. Transfer was checked with the incubation of PVDF membrane in Ponceau Red for 5 min on rocker. Membrane was washed extensively with water until the water was clear and protein bands were well-defined. After the visualization of protein bands on membrane, the membrane was destained completely with 1× PBS/0.2% tween-20 and proceeds with blocking of membrane. Blocking the membrane/blot prevents non-specific background binding of the primary and secondary antibodies to the membrane.

Membrane was incubated in non-fat milk (5%) for blocking for 1 h at 4°C. Membrane was rinsed for 5 seconds in PBS/0.2% tween 20. Primary antibody (HIV-1 p24 monoclonal antibody; NIH AIDS reagent program) was added onto the membrane and incubated for 1 h on rocker. After incubation, membrane was washed three times with 1× PBS tween for 5 minutes each with agitation. Later on, membrane was incubated in secondary antibody (HRP-conjugated horse anti-mouse; 1/3000; Bio-Rad Laboratories) for 1 h with agitation. An enhanced chemiluminescent substrate (Thermo Scientific) for detection of HRP-conjugated secondary antibodies was used onto blot and incubated for 5 min at room temperature. Image was developed on X-ray film (Amersham). Actin was used as loading control to check that the lanes in gel were evenly loaded with sample and to compare between the expression levels of a protein (p24, p55 and p41) in different samples.
3.5.2 Cytotoxicity and IC₅₀

3.5.2.1 Cytotoxicity

Jurkat cells were seeded at a density of $7 \times 10^5$ cells/ml in a 12 well plate. After that cells were treated with different concentrations of balsamin in duplicate and incubated at 37°C. The wells without balsamin were used as positive control. Cell suspension was collected on each two days until 7th day of incubation. Each two days, cell viability was evaluated by counting cells in the presence of Trypan blue solution.

3.5.2.2 IC₅₀

Jurkat cells were seeded ($7 \times 10^5$ cells/ml) in 12 well plate. Seven different concentrations of protein sample were added to the cells and then incubated at 37°C. The wells without protein were used as a positive control. In the presence of different concentration of protein, cells were infected with HIV-1 at a multiplicity of infection (moi) of 0.1. After 8 h of post-infection cells were washed with PBS to remove free virus and then again resuspended in complete RPMI fresh medium. On 3rd day of post-infection cell-free supernatant was collected to determine reverse transcriptase assay as described before. The inhibition of HIV-1 replication by 50% (IC₅₀) was determined from dose-response curve.

3.5.3 Antiviral assay of Primary CD4⁺ T cells

The antiviral Primary CD4⁺ T cells assay was based on RT assay on the inhibition of HIV-1 replication, which was quantified in culture supernatant. The PBMCs (Peripheral blood mononuclear cells) were isolated from buffy coats of healthy seronegative blood donors in accordance with institutional guidelines of the ethical committee of the University of Geneva (Switzerland). CD4⁺ T cells were purified from PBMCs after Ficoll gradient separation with CD4⁺ T cell isolation kit II (Miltenyi Biotec), in accordance with the manufacturer’s instructions and maintained in RPMI 1640, supplemented with 10% FCS, 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin. Later on CD4⁺ T cells were activated by PHA-L (1 µg ml⁻¹) and IL-2 (20 ng ml⁻¹) prior to infection.

Primary CD4⁺ T cell ($7 \times 10^5$ cells/ml) were infected with HIV-1 at 0.1 multiplicity of infection (moi). AZT was included as positive control and three different protein (balsamin) concentrations were used. The wells without protein were used as a
negative control. After 8 h of post-infection, cells were washed with PBS and resuspended in fresh RPMI medium and cultured in the presence and absence of protein. AZT was also added in the appropriate well. On 3rd day of post-infection cell-free supernatant was collected and filtered through 0.45 μm pore-size nitrocellulose membrane (Spin-X; Corning) to determine RT assay as described before. In parallel, cells were collected from the same sample on the 3rd day of post-infection and lysed with RIPA (Radio-Immunoprecipitation Assay) buffer for the extraction of protein. The expression level of actin (as a loading control) and p24 were evaluated by western analysis of cell extract on 3rd day of post-infection. All experiments were performed with three different donors.

The cellular cytotoxicity of balsamin on primary CD4+ T cells was determined. Primary CD4+ T cells (7 × 10^5 cells/ml) were seeded in 12 well plate and treated with different concentrations of balsamin. The wells without balsamin were used as a positive control. Cell suspension was collected and cell viability was evaluated until day 3rd of incubation by counting cells in the presence of Trypan blue solution.

3.5.4 HIV-1 Single Round Infectivity Assay

Single round infectivity assay used to determine the effects of anti-viral agents on particular stages of the viral life cycle (Figure 3.4). In order to test whether single round assay was sufficient for detection of HIV, viruses were generated that would be defective in the Env gene. In order to generate a population of defective virus, virions were produced that would contain the envelope of another retrovirus and the Gag and Pol proteins and genome of HIV (pseudotypes). Virus was generated that would be defective in the Env gene. With the deletion in the envelope gene that includes sequences necessary to bind CD4. This deletion precludes synthesis of the transmembrane protein by changing the reading frame.

3.5.4.1 Primary CD4+ T cells

HIV virions in which Env had been deleted were used to make single round infectious particles. Therefore, it does not undergo a full replication cycle, but produces HIV-1 p24gag. Primary CD4+ T cells (8 × 10^5 cells/ml) were pretreated with different concentrations of protein and with AZT as a positive control. The cells without protein were included as a negative control. It was then infected with DNase-treated HIV-GFP+Nef+pMDG at a multiplicity of infection of 1 and 0.2. After 8 h of post-infection
cells were washed with PBS and cultured with different concentration of protein. Forty-eight hours of post-infection, the cell-free supernatant was harvested and filtered through 0.45 µm pore-size nitrocellulose membrane (Spin-X; Corning) to determine reverse transcriptase activity assay. In parallel, cell lysates were prepared for western blot analysis. The p24 and actin expression level were evaluated by Western blot analysis.

3.5.4.2 Jurkat cell line

Jurkat cell line (7 × 10^5 cells/ml) was pretreated with 3.57 µM of balsamin. The cells without protein were included as a negative control. HIV-1 was treated with DNase and incubated at 37°C for 30 min. Jurkat cell line was then infected with DNase-treated HIV-1 at a multiplicity of infection of 1 and 0.2. Heat inactivated virus was used as a negative control. After 8 h of post-infection cells were washed with Phosphate Buffer Saline and cultured with 3.57 µM of balsamin. Forty-eight hours of post-infection, the cell-free supernatant was harvested and filtered through 0.45 µm pore-size nitrocellulose membrane (Spin-X; Corning) to determine reverse transcriptase activity assay. In parallel, viral DNA was isolated with DNeasy kit (Qiagen) and individually amplified by PCR using primers targeting so-called late reverse transcripts. In parallel, the cell-free supernatant was harvested and filtered through 0.45 µm pore-size nitrocellulose membrane (Spin-X; Corning) to determine reverse transcriptase assay as described procedure.

**Primers**

The primers for amplification of proviral DNA were U5.2 (forward: 5’-TGTGTGCCCCGTCTGTGTGT-3’) and O.psi1b primers (reverse: 5’-GAGTCCTGCGTCCGAGGAGC-3’). The amplification of actin with actin primers (forward Act1: 5’-TCACCCACACTGTGCCCATCTACGA-3’; reverse Act2: 5’-CAGCGGAACCGCTCATTGCGAATGG-3’) served as an input control.

**PCR reaction**

PCR reaction (20 µl) was carried out with platinum super mix (Invitrogen), containing 18 µl of 1x platinum supermix, 0.5 µl of each forward and reverse primers (10 µM) and 1 µl of DNA. Reaction was carried as initial denaturation at 95°C for 10 min, followed by 30 cycles each denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, followed by final extension at 72°C for 7 min and final cooling at 4°C. The amplified PCR product was resolved on 1% agarose gel.
Figure 3.4 Schematic representation of Single-round infectivity assay
3.5.5 Anti-influenza virus activity

3.5.5.1 Influenza infections

A549 cells were split in 6 well plates and infected with influenza virus (PR8). The virus infected cells were incubated in the presence of different concentration of protein. The wells without protein were used as a negative control. After 16 h of incubation, cells were twice washed with PBS and suspended in OPTI-MEM medium. After 24 h of PBS wash viral supernatant was collected and centrifuged at 3000 rpm for 3 min. The cleared supernatant was collected and then treated with 5 µg ml⁻¹ of TPCK-treated trypsin to activate the hemagglutinin protein and titration was performed. In parallel, cells were detached using PBS-EDTA. Virus replication was assessed by monitoring either accumulation of influenza M1 (matrix) protein in infected cells, or by titrating the viral infectious output present in supernatant.

3.5.5.2 Influenza virus titration

The titration of viral supernatants was performed by infecting MDCK cells plated in 48 well plates with serial dilutions of the viral supernatant. 20 hours later, cells were washed once with PBS, fixed directly in the plate with 100% methanol at -20 degrees for 10 minutes, washed once with PBS, and incubated for 30 minutes at room temperature in PBS 1% BSA. Infected cells were then revealed by immunofluorescent staining with a FITC-coupled anti-NP (#8257F from Millipore, at a 1/500th dilution in PBS) for 45 minutes at room temperature, followed by three PBS washes. Titer was computed by scoring the numbers of green cells under a fluorescence microscope. Cell lysate were prepared from A549 cells and subjected to western analysis.

3.5.5.3 Protein analysis

A549 cells were detached from dishes either by pipetting or by 10 mM PBS-EDTA treatment, and subsequently lysed with RIPA buffer. Lysates were pre-cleared (13’000 rpm tabletop spin for 10 min), their protein content was quantified with the BCA kit (Thermo scientific), and they were subjected to standard SDS-PAGE. The expression level of M1 (matrix protein) and actin (as a loading control) was evaluated by western blot analysis. Experiments were performed in duplicates.