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Isolation, Identification and Immunosuppressive Activity of a New IMM-125 Metabolite from Human Liver Microsomes. Identification of its Cyclophilin A-IMM-125 Metabolite Complex by Nanospray Tandem Mass Spectrometry

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The isolation from human liver microsomes and identification by electrospray mass spectrometry and tandem mass spectrometry of a new metabolite of IMM-125 resulting from the biotransformation of the amino acid 1 vinylic methyl group to a carboxylic acid, called the IMM-125-COOH metabolite, is described. It was found that the complex of this new metabolite with cyclophilin A is formed less easily than the corresponding cyclophilin A-IMM-125-CH_2OH main metabolite and cyclophilin A-IMM-125 complexes. However, when formed, the IMM-125-COOH metabolite–cyclophilin A complex requires more collision-induced dissociation (CID) to dissociate the complex than the complexes formed with the two other ligands. The nanospray tandem mass spectrum of the IMM-125-COOH metabolite–cyclophilin A complex (m/z 1755) gives rise to cyclophilin A–ligand complexes of m/z 1751 by elimination of CO_2 and of m/z 1749 by loss of CO_2 and H_2O or glycerol. Since immunosuppressive activity is known to be dependent on the formation of a binary complex between cyclophilin A and the drug and since the target for the binary complex was found to be the calcium- and calmodulin-dependent protein phosphatase, calcineurin, it could be interesting to measure for structurally related immunosuppressive drugs the CID energy necessary to dissociate the binary complexes in order to evaluate whether a correlation with the phosphatase activity could be derived. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: drug metabolism; IMM-125 metabolite; cyclophilin A complex; immunosuppressive activity; drug–receptor interaction; mass spectrometry

INTRODUCTION

Cyclosporin A (CyA) has been regarded as the prototype of a new generation of immunosuppressive agents but its adverse effects[1] on the kidney, the liver and the arterial blood pressure[2] limit its more widespread clinical use. Consequently, new analogues or other drugs with different structures[3] were developed with the aim of overcoming the adverse side-effects in the clinical use of CyA.

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IMM-125 is a new cyclosporin resulting from the hydroxyethyl modification of D-serine-8-cyclosporin that is obtained by fermentation of *Tolypocladium inflatum* in a medium enriched in D-serine. This modification does not alter the interaction of the cyclophilin complex with calcineurin since the modification is located between the cyclophilin binding domain[4] and the calcineurin interaction effector sector (Fig. 1). Like CyA, IMM-125 is a powerful inhibitor of lymphocyte proliferation *in vitro* and a very effective immunosuppressive agent *in vivo*.[5] This drug is also approximately eight times less lipophilic than CyA, which is reflected by a five times greater proportion of unbound IMM-125 in plasma compared with CyA.[6] Also, a significant amount of IMM-125 is excreted unchanged, representing ~ 18% of the administered dose in rat bile and 7% in urine, whereas the total excretion of unchanged CyA is

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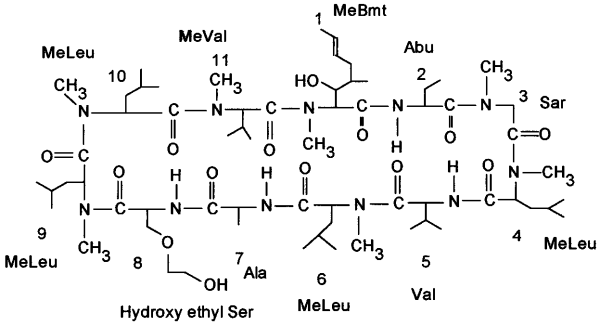

Figure 1. Structure of IMM-125.

$<1\%$.[7] Human liver cytochrome P450 3A is responsible for the biotransformation of the cyclosporin IMM-125.[8]

In this paper, we describe the isolation and identification by electrospray tandem mass spectrometry (MS/MS) of a new metabolite of IMM-125 isolated from human liver microsomes. The *in vitro* immunosuppressive activity using mixed lymphocyte reaction (MLR) and also the cyclophilin A–new metabolite complex relative to the main IMM-125 metabolite[9] and parent drug–cyclophilin A complexes are discussed in terms of the collisional energy necessary to dissociate the binary complex. The detection of non-covalent receptor–ligand complexes has already been described for the rapamycin–FKBP complex.[10]

EXPERIMENTAL

Chemicals and reagents

IMM-125 (M_r 1261.85) and cyclophilin A (CyPA, M_r 18 012), a specific cytosolic binding protein of IMM-125 and cyclosporin A, were kindly supplied by Novartis Pharma (Basle, Switzerland). Spectrograde solvents (hexane, dichloromethane and propan-2-ol) used in extraction or analytical procedures were purchased from Labscan (Dublin, Ireland) and acetonitrile from Acros Organics (Geel, Belgium). $NADP^+$, NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase grade II (5 g l^{-1}) were purchased from Boehringer Mannheim (Mannheim, Germany). Demineralized and filtered (Milli-Q water purification system; Millipore-Waters, Milford, MA, USA) water was used. All cell culture reagents were obtained from Gibco (Paisley, UK).

Human liver

A piece of liver from a healthy donor who had died in a traffic accident with a gift from a Belgian hospital surgical department.

Preparation of human liver microsomes

A piece of liver was weighed, washed with ice-cold 3 mmol l^{-1} imidazole homogenizing buffer containing 0.5 mol l^{-1} sucrose, blotted with filter-paper and minced with scissors. The minced liver was treated and fractionated according to the method of Amar-Costesec *et al.*[11] Protein and cytochrome P-450 concentrations were determined according to standard published procedures.[12,13]

IMM-125 microsomal incubation and extraction of metabolites

The NADPH-generating medium (2 ml) containing 1.76 mg of NADPH, 5.07 mg of $NADP^+$, 0.4 ml of $MgCl_2$ (0.5 mol l^{-1}), 30 mg of glucose 6-phosphate and 1.6 ml of Tris (pH 7.4) was preincubated in a Gallenkamp shaking incubator (Grant Instruments, Cambridge, UK) for 15 min at 37 °C in small Erlenmeyer flasks. To this solution were added 2.5 ml of human liver microsomes, 12 µl (5 g l^{-1}) of glucose-6-phosphate dehydrogenase (specific activity 350 kU g^{-1}) and 62.5 µg of IMM-125 dissolved in acetonitrile (25 µl). This mixture was incubated for 4 h at 37 °C. After the elapsed time and transfer of the incubation medium to a centrifuge tube, 7 ml of dichloromethane were added. After 2 min of vortex mixing, the sample was centrifuged for 10 min at 1150 *g*. The aqueous phase was discarded and the residue remaining after evaporation of the dichloromethane phase was dissolved in 1.5 ml of acetonitrile–water (3:7 v/v). The resulting solution was then washed with 1.5 ml of hexane (2 min of vortex mixing) and the hexane layer was discarded after centrifugation for 5 min at 1150 *g*. The acetonitrile–water phase was extracted again with 3 ml of dichloromethane. After 2 min of vortex mixing and centrifugation for 10 min at 1150 *g*, the aqueous phase was discarded and the dichloromethane layer was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 µl of propan-2-ol and the resulting solution was analyzed by HPLC.

HPLC

The HPLC system consisted of two LC10AD Shimadzu pumps, a Waters U6K injector, a variable-wavelength Pye Unicam (Cambridge, UK) LC–UV detector connected to an AST computer loaded with a Softron PC integration pack (Kontron, Zürich, Switzerland). IMM-125 metabolites were first separated on an Alltech (Ghent, Belgium) Rsil CN column (10 µm, 250 × 10 mm ID) using hexane–propan-2-ol (73:27) as the mobile phase. The flow-rate was adjusted to 3 ml min^{-1} and the UV detector was set at 208 nm. Under those conditions two groups of peaks were observed at retention times of 18–20 min (group 1) and 20–30 min (group 2). Eluate corresponding to group 1 was collected and subsequently rechromatographed on a Macherey-Nagel (Oensingen, Switzerland) Nucleosil C_8 column (5 µm, 250 × 10 mm ID) at 50 °C. The mobile phase was acetonitrile–water (55:45) and the flow-rate and UV detector settings were 2.5 ml min^{-1} and 208 nm, respectively: the oven temperature was 60 °C. Under these conditions, a metabolite peak was detected on the HPLC trace with a retention time of 4.3 min. The eluate

corresponding to this peak was collected. After evaporation of the mobile phase under reduced pressure, the residue was dissolved in acetonitrile, transferred to individual tubes preweighed on a semimicro balance (Precisia, Zürich, Switzerland) and evaporated to dryness under a stream of nitrogen. A quantity of 150 μg was accumulated to obtain sufficient amounts for evaluation of its *in vitro* immunosuppressive activity and for electrospray MS.

Electrospray MS/MS

Electrospray ionization (ESI) tandem mass spectra were obtained with an LCQ and MS^n instrument (Finnigan, San Jose, CA). The source voltage was 5.05 kV, the capillary voltage 25.76 V and the capillary temperature 220.10 °C. The octapole offsets 1 and 2 were set at 5.5 and 3.0 V, respectively, and the flow-rate of dry gas (N_2) was 8 l min^{-1}. The compounds (100 μg) were dissolved in acetonitrile–5 mM aqueous ammonium acetate (50:50) and the solution was infused with the aid of a syringe pump at a flow-rate of 5 μl min^{-1}.

Nanospray MS and MS/MS

All experiments were performed on a Finnigan LCQ quadrupole ion trap instrument equipped with a nanospray ESI source.[14] Cyclophilin A, IMM-125 and its metabolites were prepared in 1 mM acetic acid (HOAc, pH 4–5). The protein (250 mM) in water containing 5 mM NaCl and 5 mM HEPES buffer was diluted in 1 mM HOAc to a concentration of 15 μM. SDZ-IMM-125 and the metabolites were dissolved in acetonitrile (1.2 mM) and further diluted to a concentration of 30 μM in 1 mM HOAc. Subsequently, the binding protein CyPA and the ligands were brought together by doping equimolar amounts of the protein and the ligand to a concentration of 5 pmol μl^{-1} each in 1 mM HOAc containing 2.5% methanol.

Analyses were performed on 1 μl of these mixtures brought into a metallized glass capillary needle with a tip of inner diameter ~ 1 μm and were sprayed at 0.75 kV. The temperature of the sampling capillary in the ESI source was set at 200 °C. The ions of CyPA complexed with IMM-125 (*m/z* 1753) and the carboxylic acid (IMM-125-COOH) and also the hydroxymethyl metabolites (IMM-125-CH_2OH) appearing at *m/z* 1755 and 1754, respectively (complex charge state: 11), were selected with an *m/z* window of 5 Da and a maximum ion injection time of 500 ms. Collision induced dissociation (CID) energies were varied from 0 to 50% and the resulting product ions were mass analyzed from *m/z* 1000 to 2000 using averaging of five scans per spectrum.

Functional assays to determine the immunosuppressive effect

Peripheral blood mononuclear cells (PBMNC) were isolated from heparinized blood by density gradient centrifugation on LSM (Lymphocyte Separating Medium; Pharmacia, Uppsala, Sweden). Isolated PBMNC were resuspended in enriched medium: RPMI 1640 medium (Gibco, Merelbeke, Belgium), supplemented with 100 U ml^{-1} penicillin, 100 mg l^{-1} streptomycin, 10 mM glutamine and 20% heat-inactivated fetal calf serum (Biosys, Compiègne, France). Bidirectional mixed lymphocyte reactions were performed with 1×10^6 PBMNC of two major histocompatibility complex (MHC) incompatible donors per well in 96 U-well microplates (Falcon, Lincoln, NE, USA) containing 200 μl per well and incubated for 6 days at 37 °C, 5% CO_2 with 50 μl of the test compound solution at different concentrations (from 5000 to 1 ng ml^{-1}). After that time, 10 μl of a [^{3}H]thymidine solution (0.2 mCi ml^{-1}) (Amersham International, Amersham, Bucks., UK) were added to each well. Eight hours later, the ^{3}H incorporation was measured by liquid scintillation counting in a beta counter (LSD 6000 SE; Beckman Instruments, Fullerton, CA, USA). All results are the means of three experiments. In each experiment, immunosuppressive activity measured for each concentration was expressed as the mean of three wells. Standard deviations were always <15% of the mean. The immunosuppressive effect of the solvent [acetonitrile–water (50:50)] was also checked and found to be negative.

RESULTS AND DISCUSSION

The electrospray mass spectrum (Fig. 2) of metabolite rt4.3 (rt4.3 = metabolite of retention time 4.3 min, also called the IMM-125-COOH metabolite) reveals the presence of quasi-molecular ions of *m/z* 1292 $(MH)^+$, 1314 $(MNa)^+$ and 1330 $(MK)^+$ and quasi-fragment ions of *m/z* 1284 $(M - HCOOH)K^+$, 1270 $(M - CO_2)Na^+$ and 1248 $(M - CO_2)H^+$, indicating that the vinylic methyl group of the first amino acid of IMM-125 has been metabolized by the cytochrome P-450-dependent mixed function monooxygenase system to a conjugated carboxylic acid losing either CO_2 or formic acid. The fact that an amino acid 1 of IMM-125 was engaged in the metabolic process was confirmed by ESI-MS/MS of the quasi-molecular ions MH^+ (*m/z* 1292) (Fig. 3) and MNa^+ (*m/z* 1314) (not shown). Fragment ions of *m/z* 1292 were found to be at *m/z* 1274 $(M - H_2O)H^+$, 1230 $(M - HOCH_2CH_2OH)H^+$ or 1230 $(M - H_2O - CO_2)H^+$ and 1212 $(M - HOCH_2CH_2OH - H_2O)H^+$ (Fig. 4) and those of *m/z* 1314 were observed at *m/z* 1296 $(M - H_2O)Na^+$, 1286 $(M - CO_2)K^+$, 1270 $(M - CO_2)Na^+$, 1252 $(M - CO_2 - H_2O)Na^+$ and 1172 $(M - 142)Na^+$, proving that the vinylic methyl group of IMM-125 was metabolized to a primary alcohol, an aldehyde and finally to a conjugated carboxylic acid (Fig. 5). The recorded classical loss of 142 mass units characteristic of the cyclosporins confirms that IMM-125 amino acid 1 was engaged in the metabolic process. That the conjugated carboxylic acid new metabolite rt4.3 may be submitted to a decarboxylation process was also confirmed by the fact that a metabolite of *m/z* 1248 $(MH)^+$, which may be considered as an artefact metabolite, has been identified by fast atom bombardment and electrospray MS (unpublished results).

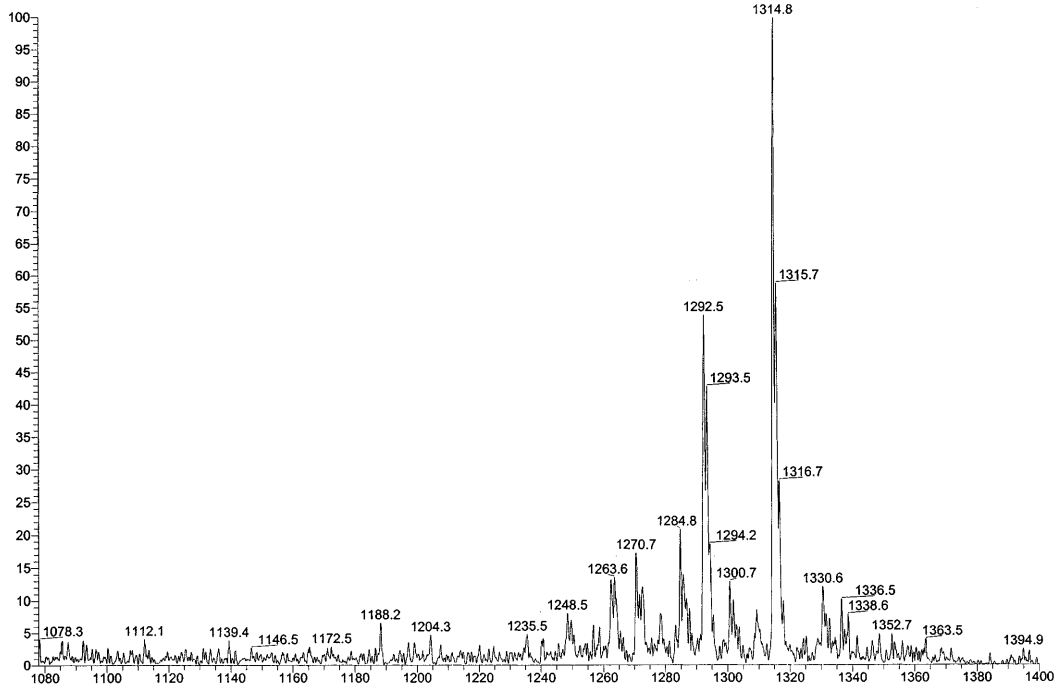

Figure 2. Electrospray mass spectrum of the IMM-125-COOH metabolite.

The mass spectrum of cyclophilin A (Fig. 6) and the masses of the complexes between the IMM-125-COOH metabolite rt4.3, the IMM-125-CH_2OH main metabolite resulting from the biotransformation of amino acid 1 vinylic methyl group to a hydroxymethyl group,[9] IMM-125 and cyclophilin A were obtained by nanospray MS and certain CID energies necessary to dissociate the drug-binding protein complexes were set (Fig. 7). It may be observed that under the described experimental conditions, the complex between the IMM-125-COOH metabolite [Fig. 7(B)] and cyclophilin A is less abundant than the corresponding complexes formed with IMM-125 [Fig. 7(A)] and the IMM-125-CH_2OH main metabolite [Fig. 7(C)]. Nevertheless, the IMM-125-COOH metabolite–cyclophilin A complex [Fig. 7(B)] seems to be more strongly bound than the two other receptor–ligand complexes since a 40% CID energy is necessary to dissociate the complex.

Fragmentations were also observed in the nanospray tandem mass spectrum of the IMM-COOH metabolite–

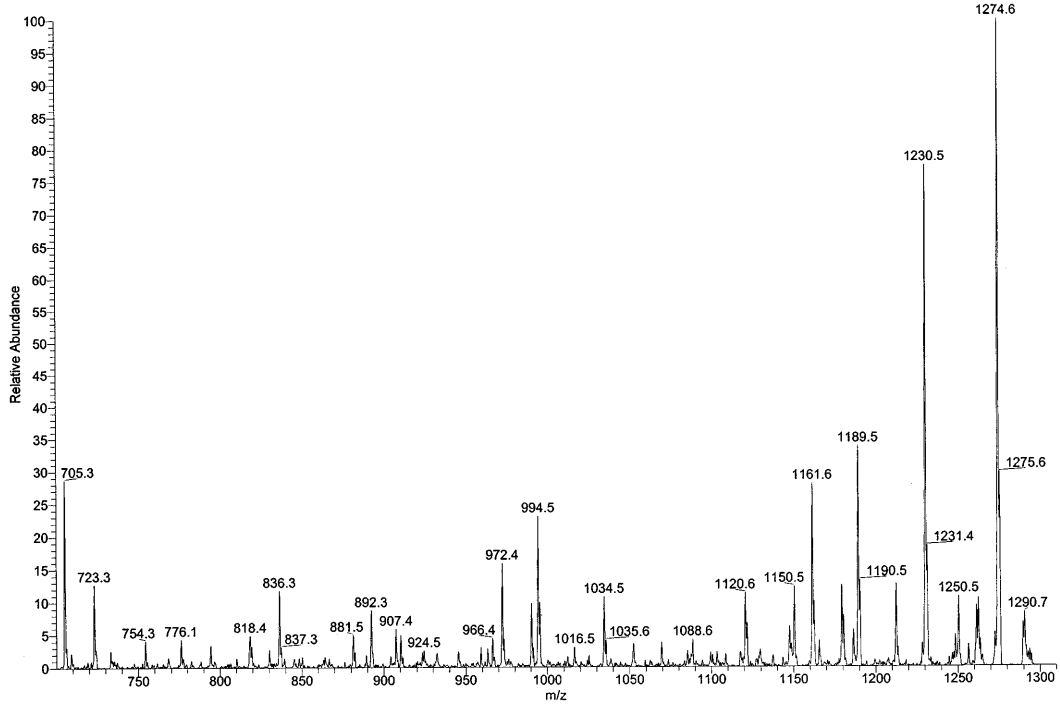

Figure 3. Electrospray tandem mass spectrum of the ion at m/z 1292.

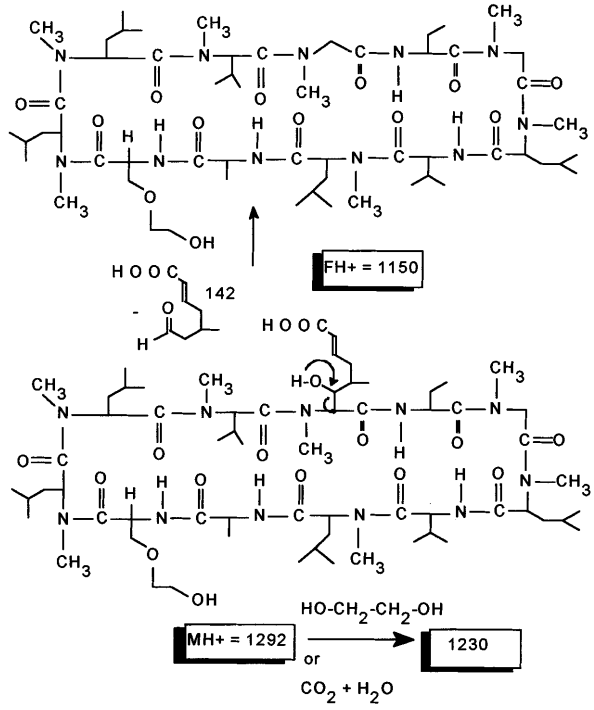

Figure 4. Fragmentation pathways of the IMM-125-COOH metabolite.

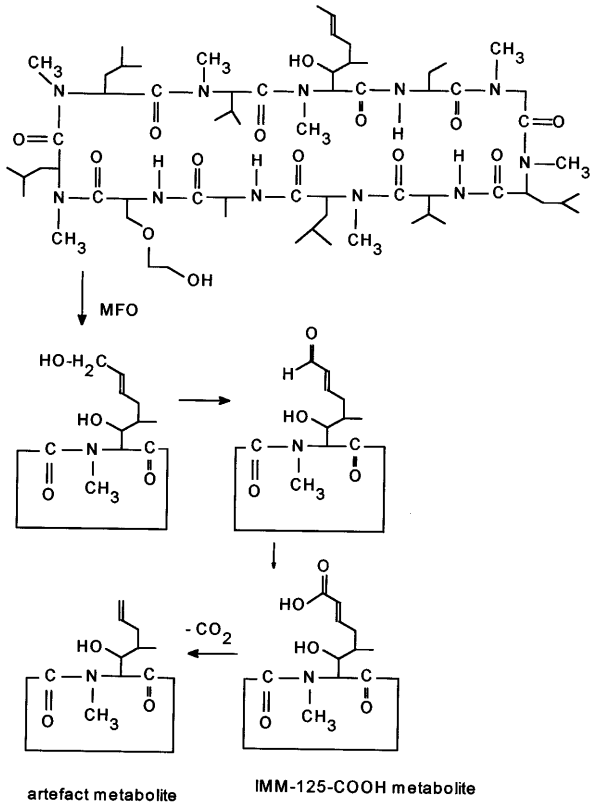

Figure 5. Metabolic pathways of the IMM-125-COOH metabolite.

cyclophilin A complex (*m/z* 1755). At 0% CID, the complex [Fig. 8(A)] is largely present compared with the uncomplexed cyclophilin A (*m/z* 1802). At 30% CID [Fig. 8(B)], a new peak at *m/z* 1751 resulting from the elimination of CO_2 [(18 010 + 1249)/11 = 1750.8] from the IMM-125-COOH conjugated carboxylic acid ligand is observed, in addition to the formation of a certain amount of free ligand (*m/z* 1293). When CO_2 and H_2O or glycerol is lost from the CyPA–ligand complex a peak of mass *m/z* 1749 is observed [18 010 + 1231/11 = 1749.2]. At 40% CID [Fig. 8(C)], the peak of *m/z* 1751 is almost absent and the presence of free ligands at

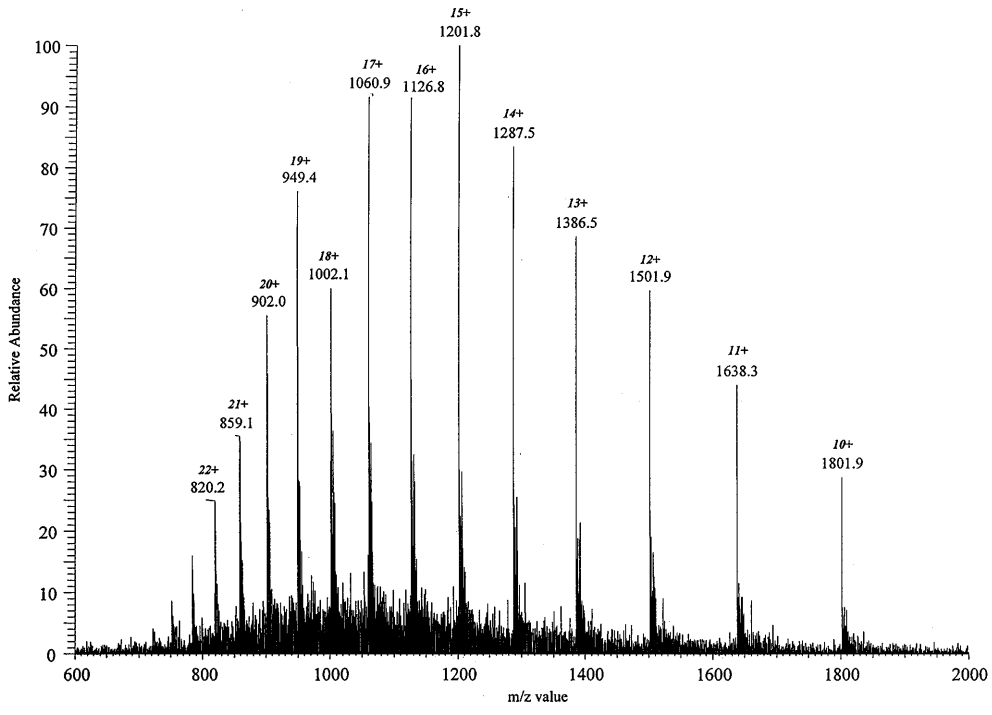

Figure 6. Electrospray mass spectrum of the IMM-125 binding protein, cyclophilin A

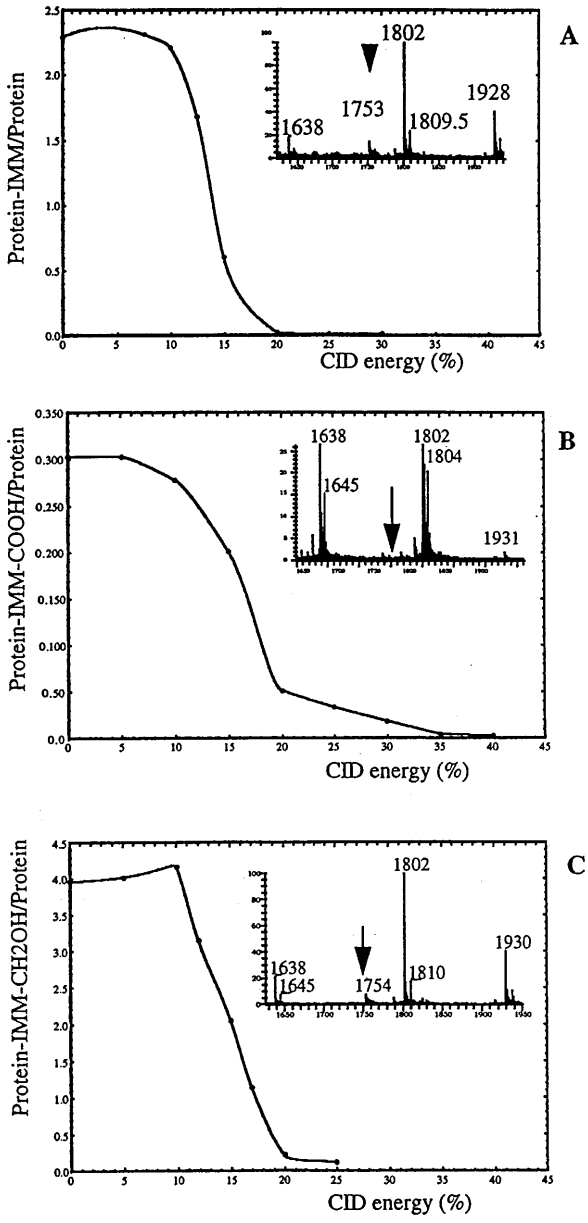

Figure 7. Nanospray mass spectra of (A) IMM-125, (B) IMM-125-COOH metabolite and (C) IMM-125-CH_2OH metabolite–cyclophilin A complexes. Relationship between the complexed/uncomplexed ratios and the CID energy for the three ligands.

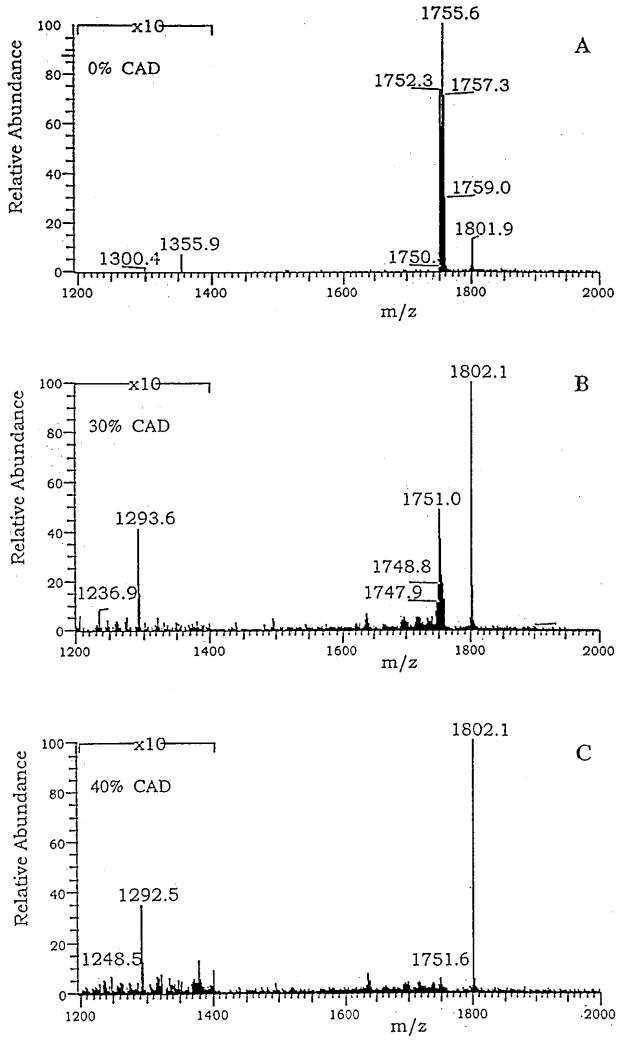

Figure 8. Relationship between the formation of peaks of *m/z* 1751 and 1749 and the CID energy.

m/z 1293 and 1249 (1239 – CO_2) as well as cyclophilin A (*m/z* 1802) are recorded.

The *in vitro* immunosuppressive activity expressed as the inhibition of the mixed lymphocyte reaction (Fig. 9) performed with peripheral blood mononuclear cells (PBMNC) of two major histocompatibility blood donors was found lower for the IMM-125-COOH metabolite than for IMM-125, indicating that the complex of this metabolite with cyclophilin A[15] is formed less easily and/or that the ternary cyclophilin A–IMM-125-COOH metabolite–calcineurin complex is formed with great difficulty owing to the structural modification of the IMM-125 binding region including amino acids 9, 10, 11, 1, 2 and 3.[15]

It may be concluded that IMM-125 under the influence of the cytochrome P-450 mixed function oxygenase system may be oxidized to an amino acid 1 carboxylic acid metabolite resulting from the biotransformation of the vinylic methyl group and possessing a lower *in vitro* immunosuppressive activity than the parent compound. The immunosuppressive activity is known[15] to be dependent of the formation of a binary complex between cyclophilin A and the drug. The target of this binary complex was found to be the calcium- and

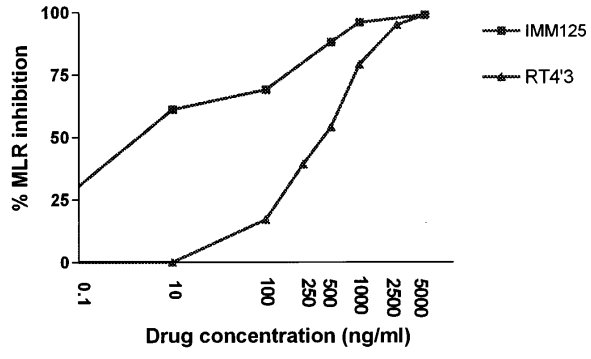

Figure 9. *In vitro* immunosuppressive activity of the IMM-125-COOH metabolite and IMM-125.

calmodulin-dependent protein phosphatase calcineurin, and this model strongly suggests that immunosuppressive activity is mediated through phosphatase inhibition. Since structural modifications resulting from metabolism may affect the binding affinities of the drug–cyclophilin A binary complexes and phosphatase inhibition, it could be interesting to measure for structurally related immunosuppressive drugs the CID energy necessary to dissociate the binary complexes in order to evaluate whether a correlation with the phosphatase activity could be derived.

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