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Conjugation of thymopentin (TP5) with lipoamino acid residues increases the hydrolytic stability and preserves the biological activity[1]

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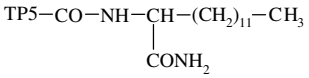
Three conjugates of thymopentin (TP5), an oligopeptide derived from the thymic hormone thymopoietin, with lipoamino acid (LAAs) have been obtained by solid-phase peptide synthesis. Both linear and dendrimer structures have been prepared to achieve enhanced lipophilicity. After incubation in foetal calf serum the lipophilic conjugates showed a higher stability to hydrolysis with respect to the parent drug. In a preliminary *in vitro* biological assay, LAA conjugates showed the ability to retain or improve the growth inhibitory activity of the parent peptide against a human lymphoblastoid cell line. The interaction of the prepared conjugates with 1,2-L-α-dimiristoylphosphatidylcholine multilamellar liposomes, chosen as a biological membrane model, was studied. The higher lipophilicity of TP5 conjugates was reflected in a better penetration through phospholipid bilayers, whose thermal behaviour was altered in a concentration-dependent way. Such enhanced affinity of TP5-LAA conjugates for this membrane model could anticipate a better interaction with cell membranes and, ultimately, an improved biological activity of compounds compared with the parent pentapeptide.

1. Introduction

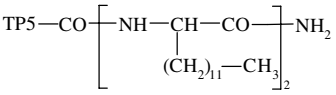
Development of drugs able to interact with the immune system, by stimulating the natural mechanisms of body defence (by induction of T-cell sub-populations) and recovering or increasing the original immune functions is important.

TP5 (**1**): H-Arg-Lys-Asp-Val-Tyr-OH (RKDVY)

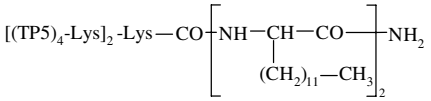
TP5-C14 conjugate (**2**):



TP5-bis(C14) conjugate (**3**):



TP5-LCP (**4**):



Schematic structure of TP5 and TP5-LAA conjugates **2–4**

In the last years, immunostimulating and immunoregulatory properties of several naturally occurring or synthetic peptides have been studied. In particular, chemical approaches to the preparation of thymus hormones allowed to isolate small peptides that retain the biological activity of natural proteins. The work of Goldstein et al. (Goldstein et al. 1979; Schlesinger and Goldstein 1975) led to the synthesis of the pentapeptide thymopentin (Arg-Lys-Asp-Val-Tyr; TP5) (**1**) which corresponds to the 32–36 amino acid sequence of the thymus hormone thymopoietin. TP5, also known as the thymosin fraction 5, affected lymphocyte activity *in vivo*, including cyclic nucleotide levels, production of T-dependent antibodies and migration inhibitory factor, and expression of cell surface maturation/differentiation markers (Goldstein et al. 1979; Goldstein and Audhya 1985). The therapeutic perspectives of these peptides have been recently reviewed (Singer et al. 1998; Gonser et al. 1999) and several clinical uses have been proposed, including the treatment of immune deficiencies, AIDS and malignancies (Aiuti et al. 1983; Conant et al. 1992; Rogalski et al. 1999). Combined chemo-immunotherapy in cancer seems to be more efficacious than chemotherapy alone, and the significant hematopoietic toxicity associated with chemotherapeutics can be reduced by the addition of immunostimulating agents (Bodey et al. 2000).

Like most peptides, TP5 shows absorption problems after oral administration and a very short half-life in human serum (Heizmann et al. 1996; Lee and Stavchansky 1998), which strongly reduce its therapeutic potential. Chemical modifications have been tested to obtain stabilised forms of TP5, such as selective amino acid changes, acylation or amidation of the peptide terminal amino acids, and introduction of hydrolytically stable amino groups (Heavner et al. 1986; De Graw et al. 1997). Recently, pH-sensitive chitosan nanoparticles have been described to enhance the hydrolytic stability of TP5 after oral administration (Zheng et al. 2006).

Among the possible approaches to reduce the enzymatic degradation in gastro-enteric environment and serum of peptide molecules, conjugation with lipophilic moieties has been proposed with success (Toth 1994; Toth et al. 1995; Kellam et al. 1998; Toth et al. 1999). In particular, our attention was directed to bind peptides to lipoamino acids (LAAs), a class of α-amino acid derivatives which can impart to drug molecules an enhanced lipophilicity and amphiphilicity (Toth 1994; Wong and Toth 2001; Pignatello et al. 2006a). LAAs combine the physico-chemical properties of both lipids and amino acids; because of their amphipatic structure, their linkage to drugs can facilitate their interaction with cell membranes and penetration across absorption membranes and biological barriers. Conjugation with LAA of GABA, thyrotropin releasing hormone (TRH) and luteinizing hormone-releasing hormone (LHRH) showed to prolong the residence time of peptides in serum and to positively affect their pharmacological activity (Hussain et al. 1991a, 1991b; Toth et al. 1994).

The present note describes the preparation of linear conjugates of TP5 with 2-aminotetradecanoic acid (**2**) and its dimer (**3**), as well as a lipid-core peptide system containing the same LAA residues (LCP) (**4**). These conjugates were obtained with good yields and purity by means of a classical solid-phase peptide synthesis (SPPS) technique, using Boc-chemistry strategies. The stability of selected terms in foetal calf serum (FCS), with respect to the corresponding pure peptide was evaluated by HPLC analysis.

The enhanced lipophilic character induced by the conjugation of TP5 with LAAs can significantly affect their interaction with and diffusion into biological membranes. To confirm such a feature, an anisotropic model for lipophilicity determination was used. A biomembrane model consisting of dimiristoylphosphatidylcholine (DMPC) multilamellar liposomes (MLVs) was chosen (Puglisi et al. 1998; Saija et al. 2002; Castelli et al. 2005) and the effects of TP5 and its conjugates on the thermotropic parameters of liposomes were evaluated by differential scanning calorimetric (DSC) experiments (Pignatello et al. 2006b).

2. Investigations, results and discussion

The SPPS procedure followed gave the wished TP5 monoconjugate with C_{14}-LAA (**2**) and its dimer **3**, as well as the LCP **4** with high purity and good yields. FAB-MS-coupled semi-preparative HPLC techniques were successfully used to obtain the pure conjugates.

A preliminary stability assay was carried out by incubating conjugates **2** and **4** in FCS. TP5 possesses a very short stability in human and mouse plasma (Bodey et al. 2000), which limits its in vivo efficacy. For instance, half-lives of 1.5 and 0.8 min have been reported in human and mouse heparinized plasma, respectively (Heavner et al. 1986).

In our experiments, incubation in FCS at 37 °C led to a rapid degradation of pure TP5, with a half-life of 1.4–1.8 min. Coupling LAA moieties to the peptide enhanced its hydrolytic resistance: the linear conjugate **2** displayed a half-life of 18 min, whereas the LCP derivative **4** degraded faster in the first 15 min of incubation (half-life$_I$ = 7.2 min), thereafter the hydrolysis proceeded slowly up to 24 h of incubation (half-life$_{II}$ = 29.4 min). To avoid interference with the degradation of TP5 molecule, the above half-lives values were determined by the evaluation of the reduction of HPLC peak areas related to the starting conjugates **2** and **4**.

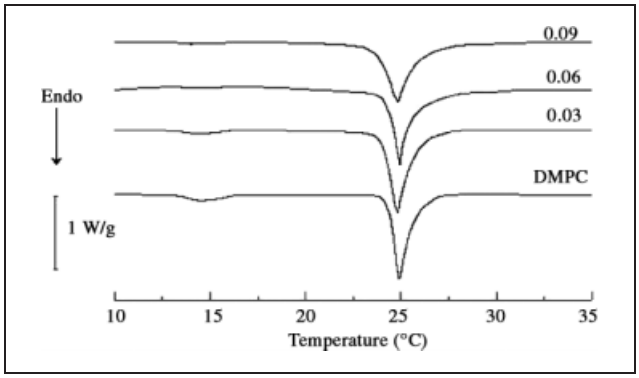


Fig. 1: DSC thermograms of DMPC MLVs containing different molar fractions of TP5

No attempt was made in the present study to detect the site(s) of hydrolytic cleavage of the conjugates, but it is presumable that a progressive fragmentation of the peptide chain occurred, besides than the break of the Tyr-LAA linkage. By the facts, the chromatographic peak corresponding to TP5 was always registered in the HPLC analysis of the conjugates along the stability tests, but it was associated to many other minor peaks, most probably corresponding to the lower peptide fragments generated by the hydrolysis of TP5 itself.

From these preliminary data, lipophilic conjugates of TP5 with LAAs appear as an interesting approach to increase the stability of the drug in biological media, while obtaining in the same time a slow-release of the active peptide.

In the DSC experiments, the mode and depth of the interaction of these lipophilic conjugates with an anisotropic biomembrane model, consisting of DMPC multilamellar liposomes, as expected were linked to the type of LAA modifier. The pure peptide showed only a marginal influence on DMPC bilayers structure and organisation, as the limited changes in the main phase transition temperature (Tm) and the associated enthalpy variations (ΔH) indicated (Fig. 1). Such a behaviour is due to the polar nature of the drug and its small molecular size, that limit the interactions with the phospholipid structures to the external (interlamellar) polar choline head groups.

The increasing lipophilicity of conjugates **2–4** progressively enhanced their ability of penetrating and altering DMPC bilayers packing (Figs. 2–4). The effects on Tm and ΔH are dependent on the molar fraction of drug encapsulated in the liposomes during their preparation, with

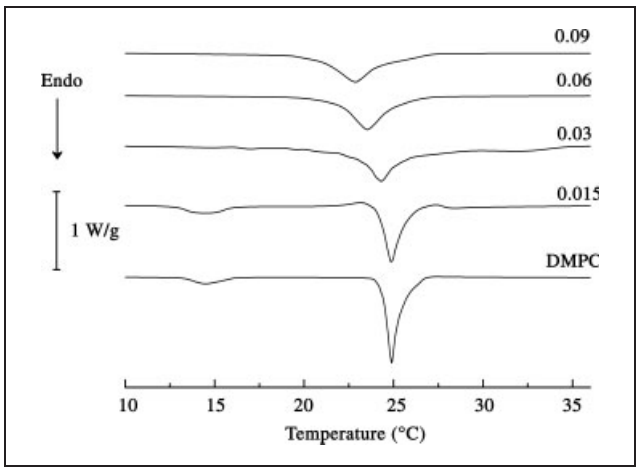


Fig. 2: DSC thermograms of DMPC MLVs containing different molar fractions of the conjugate **2**

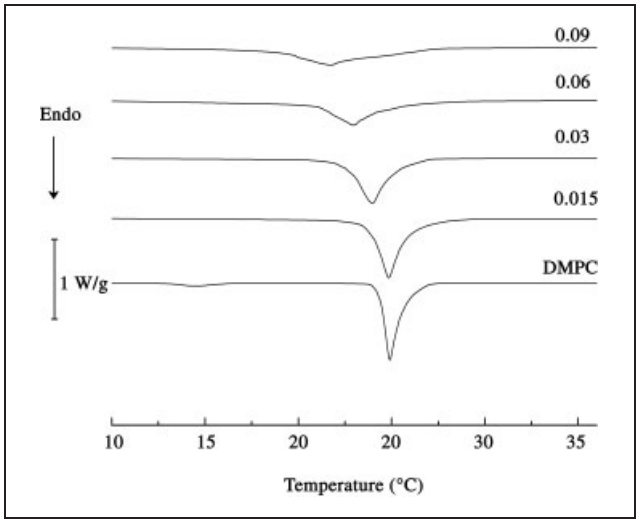


Fig. 3: DSC thermograms of DMPC MLVs containing different molar fractions of the conjugate **3**

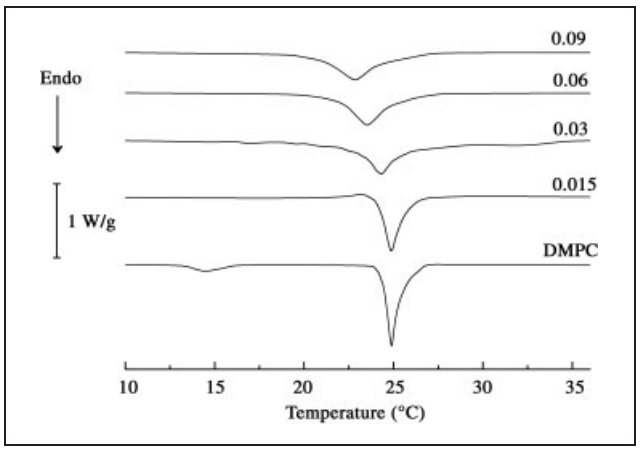


Fig. 4: DSC thermograms of DMPC MLVs containing different molar fractions of the LCP conjugate **4**

preliminary signs of phase segregation in the DSC curves only at the higher molar fractions of the more lipophilic compounds **3** and **4**. This latter phenomenon denoted the tendency of the tested compounds to accumulate in separate drug-rich domains within the phospholipid vesicles (Castelli et al. 2005; Pignatello et al. 2006b). The presence of conjugates **2–4** mainly affected the enthalpy changes rather then Tm values of DMPC liposomes; this further confirms the localisation of such lipophilic compounds within the DMPC acyl chains in the vesicle bilayers.

The increase in lipophilicity induced by LAA conjugation in a drug molecule can enhance its passive penetration through cell membranes (Pignatello et al. 2001, 2006a, 2006b, 2007); thus, the observed stronger interaction of TP5-LAA conjugates with the biomembrane model can also be predictive of a better biological activity. The preliminary *in vitro* biological tests showed that the different conjugation of TP5 with the LAA moieties did not suppress the growth inhibitory activity against a human lymphoblastoid cell line (CCRF-CEM cells) (Fig. 5).

Although this biological model is not completely indicative of the clinical potentiality of TP5, it however allowed a comparison of the relative activity between the native peptide and the prepared conjugates. Blood tumour cell lines have often been used for preliminary testing of thymus peptides (Baker et al. 1988; Panico et al. 1991, 1992, 1993) and, even recently, TP5 has been shown to inhibit

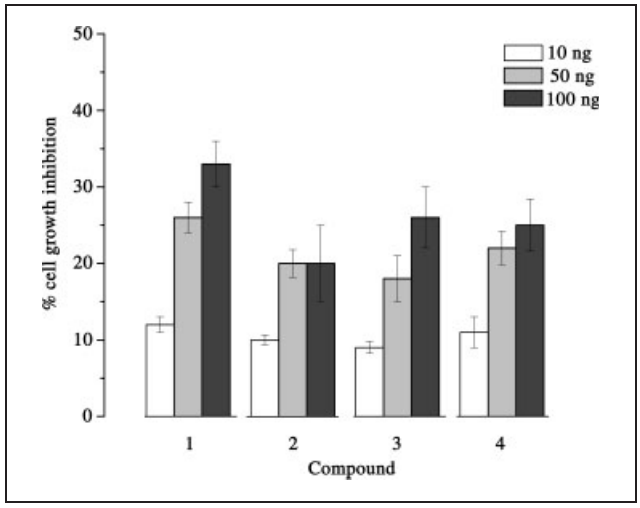


Fig. 5: *In vitro* CCRF-CEM cell growth inhibition by TP5 (**1**) and conjugates **2**–**4**, after 48 h of incubation. Concentration of each compound was expressed as ng-equivalents of TP5/ml

proliferation and colony formation of a human promyelocyte leukaemia cell line (Fan et al. 2006).

In conclusion, we have applied to the immunoactive pentapeptide TP5 the conjugation with LAAs, both in a linear and dendrimeric form. The conjugates showed enhanced lipophilicity and enzymatic stability with respect to TP5, and these properties affected their degree of interaction with a phospholipid biomembrane model. The conjugation with LAA promoieties did not suppress the *in vitro* tumour cell growth inhibitory activity of the drug. Although further biological investigations would be required, these data substantiate our chemical strategy for improving the stability and bioavailability of this peptide drug.

3. Experimental

3.1. Materials and analytical techniques

TP5 was kindly gifted from Cilag S.p.A., Cologno Monzese (Italy); purity was greater than 99% (HPLC). Foetal calf serum (FSC, Sigma), 1,2-L-α-dimiristoylphosphatidylcholine (DMPC, Fluka), O-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-diisopropylethylamine (DIEA) (Aldrich) were purchased from Sigma-Aldrich Chimica Srl (Milan, Italy). Solid phase synthesis procedures were carried out using Novabiochem (Nottingham, UK) MBHA-resin and Boc-protected amino acids. N-Boc-2-aminotetradecanoic acid was prepared as described previously (Gibbons et al. 1990). *N*,*N*-dimethylformamide (DMF) was distilled and kept dry over molecular sieves until use; all other solvents and reactants were reagent grade or better and were used without further purification.

^{1}H NMR spectra were obtained in $CDCl_3$ or in 1 : 1 (v/v) $CDCl_3$/MeOD with a Brüker AM500 instrument at 500 MHz; chemical shifts are reported in ppm downfield from TMS as the internal reference. Fast-atom bombardment ionisation (FAB) mass spectra were recorded with a VG Analytical ZAB-SE instrument, using a 20-kV Cs^+ ion bombardment, with 2 μl of appropriate matrix (either 3-nitrobenzyl alcohol or thioglycerol/glycerol/TFA). A NaI methanol solution was added to produce nitrated species when no protonated molecular ion was observed. Laser Mass spectrometry was carried out on a VG Tofspec instrument, using sinapinic acid as the matrix. TLC was performed on Merck $F_{254+366}$ silica gel aluminium backed plates; spots were detected by UV light, exposure to iodine vapours or pouring into a 10% sulphuric acid ethanol solution. Column chromatography was carried out on Merck *dry* silica gel (230–400 mesh), with the eluent systems reported. Elemental analysis was carried out on a Carlo Erba mod. 1106 analyser; samples were kept *in vacuo* for 24 h over P_2O_5 before analysis; found values are within ± 0.4% of theoretical ones.

3.2. Synthesis of conjugates 2 and 3

The MBHA-resin (0.5 g, 0.025 mmol) was washed with dry DMF. A four-fold excess of Boc-amino acids (0.1 mmol), activated immediately before the conjugation by treatment with HBTU (0.1 mmol in DMF) and DIEA (0.2 mmol), were added stepwise with a 10-min contact with the resin un-

der mechanical shaking. The Boc-protecting moiety was then removed with 100% TFA and a following washing with DMF. The procedure steps were repeated for the different amino acids or LAA residues to give the linear conjugates **2** and **3**.
The crude resin was then added of an equal amount of *p*-cresol and about 1 ml/100 mg resin of HF and kept at 0 °C for 90 min. At the end the mixture was washed with ethyl acetate and the solution was dried, dissolved in glacial acetic acid and freeze-dried for about 24 h, to give the final crude product with an average 30% yield. The latter was purified by semi-preparative HPLC (see below). Collected fractions were monitored by MS analysis to select those containing the desired conjugates:
TP5-[C14] conjugate **2**: MW: 904.84; FAB-MS (m/z, %): 905 $[M+1]^+$ (23), 627 (100).
TP5-[$(C14)_2$] conjugate **3**: MW: 1130.76; Laser-MS (m/z, %): 1131 $[M+1]^+$ (100).

3.3. Synthesis of the LCP-TP5 conjugate (4)

Compound **4** was synthesised similarly, adding the reactants according to the following order:
1. MBHA-resin (0.5 mmol), 2. Boc-LAA (4 equivalents), 3. Boc-LAA (4 equivalents), 4. Boc-Lys (Boc-OH) (4 equivalents), 5. Boc-Lys Boc(Boc-OH) (8 equivalents), 6–10. Boc-Tyr (2-Br-Z), Boc-Val(OH), Boc-Asp (OBzl), Boc-Lys (Cl-Z), Boc-Arg (Tos) (16 equivalents each).
The resin was then treated as above described and the crude product was lyophilised and purified by semi-preparative HPLC (see below), to isolate the wished LCP conjugate **4** with a final yield of 65%. MW: 3503.36; Laser-MS: 3504 m/z $[M+1]^+$ (100%).

3.4. Conjugate analysis and purification

The purity of the prepared conjugates was assessed by an RP-HPLC method. The system consisted of a Waters 616/600S twin pump system equipped with a 717plus automatic sampler and a 486 tuneable UV detector monitored by a Millennium software package. A Vydac C_4 column (4.6 × 150 mm, 5 μm ODS) was employed, linked to a Beckman Ultrasphere C_8 pre-column, at a constant flow rate of 1.2 ml min^{-1}. Wavelength was set at 310 nm in both cases, sensitivity at 1.0 AUFS. The mobile phase consisted of water (A) and acetonitrile (B); both solvents contained 0.1% (v/v) TFA and were filtered under vacuum through a 0.22 μm polyamide filter, mixed and degassed by a helium flow before use. The solvent gradient ranged linearly from 0% to 100% solvent B in 10 min and then decreased to the initial conditions in 10 min. Test samples were dissolved in 20% aqueous acetic acid and filtered through 0.45-μm polyamide filters before injection.
Purification of crude reaction products was also afforded by semi-preparative RP-HPLC on a Vydac C_8 column (8 × 150 mm, 5 μm ODS), at a constant flow rate of 5 ml min^{-1}. Separation was achieved with the same solvents as above, using a linear gradient between 0 and 60% solvent B in 150 min, staying at these conditions for 30 min, and then decreasing steadily to 0% solvent B in 30 min. The gradient was effected by two microprocessor-controlled Gilson 302 single-piston pumps. Eluates were detected by a Holochrome UV-Vis detector set at 300 nm and chromatograms were recorded using a LKB 2210 single-channel recorder. The identity of compounds in the collected fractions was confirmed by FAB-MS analysis.

3.5. Stability evaluation

TP5 or conjugates **2** or **4** (about 2 mg) were dissolved in 100 μl DMSO and added to 1.9 ml FCS. The mixture was incubated at 37 °C and, at selected times, 50 μl aliquots were withdrawn and proteins precipitated by addition of 200 μl of ice-cold methanol. The mixture was centrifuged and the supernatant diluted with 100 μl water and submitted to analytical HPLC (see above).

3.6. Differential Scanning Calorimetry (DSC) experiments

DMPC multilamellar liposomes (MLV) were prepared by the classical thin-film method. Five milligrams DMPC were used to prepare 1 ml of liposome suspension. The TP5 conjugates were mixed to the phospholipid in different molar fractions (0.015 to 0.09) before the formation of the film and its re-hydration with water. The water soluble TP5 was instead dissolved in the water volume used to re-hydrate the phospholipid film, in the required amounts to reach the same final molar fractions. Empty (pure) DMPC vesicles were prepared as reference under the same conditions but without any active.
The phospholipid suspensions were kept at 40 °C for 2 min and then vortex-mixed for 2 min; the entire cycle was repeated four times. After MLV formation, the suspension was kept at about 40 °C for 2 h and then at room temperature for another 2 h, to ensure an equilibrium between the aqueous and lipid phases.
Forty μl of each liposome suspension were then sealed into an aluminium crucible and subjected to the DSC experiment, using a Mettler DSC12E differential scanning calorimeter, equipped with a Haake DS-G thermocryostat. Indium was used to calibrate the temperature scale and transition enthalpy. A crucible containing 40 μL of water was used as a reference. Sample and reference pans, placed in the DSC cell, were equilibrated at 5 °C for 15 min. The heating scanning rate was 2 °C min^{-1} in a 5–40 °C range. Main phase transition temperature (Tm) and the associated enthalpy changes (ΔH) were calculated from the peak areas by the instrument software (Mettler TA89E, version 2.0). Each sample was subjected to three heating and cooling cycles in triplicate experiments. The data coming from the first scan of each set of runs were always discarded in order to avoid mixing artefacts among suspension components.

3.7. Cell growth inhibition assay

The human T-lymphoblastic leukemia cell line CCRF-CEM was grown at 37 °C, in a 5% CO_2 atmosphere, in RPMI 1640 (HyClone) supplemented with 10% FCS, glutamine (2 mM) and antibiotics. Cells were plated in plastic 24-well plates at a concentration of 1×10^5 cells/ml/well and test compounds were concomitantly added in a concentration range of 10–100 ng TP5-equivalents/ml. Dilutions were made using the culture medium from 1 mM stock solutions of the drugs in DMSO. Controls were treated with an equivalent amount of solvent, diluted as above, to assess the absence of toxicity due to the solvent. After 48 h of incubation, viable cells were counted with a haemocytometer by the trypan blue exclusion assay. Each experiment was repeated three times in duplicate.

[1] Part of this work was presented at the 4th World Meeting on Pharmaceutics Biopharmaceutics and Pharmaceutical Technology, Florence, 8–11 April 2002

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