L-Lysine Pro-Prodrug Containing *trans*-Ferulic Acid for 5-Amino Salicylic Acid Colon Delivery: Synthesis, Characterization and *in Vitro* Antioxidant Activity Evaluation

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In the present work, we report the synthesis of a new 5-amino salicylic acid (5-ASA) pro-prodrug, useful in Crohn disease treatment, and the evaluation of its antioxidant activity. Using as pharmacological carrier L-lysine amino acid and taking advantage of its intrinsic chemical reactivity, due to the presence of two amino groups, placed on the chiral center and in ε-position, we inserted *trans*-ferulic acid in ε-position, through amidation reaction, esterified with methanol the carboxylic group and, finally, submitted the free amino group to diazotation with 5-ASA, principal drug for inflammatory bowel diseases (IBD) care. All intermediates of synthesis and the final product (derivative A) were characterized with usual spectroscopic techniques, as FT-IR, GC/MS and ^{1}H-MNR. Finally, the derivative A antioxidant activity in inhibiting the lipid peroxidation, in rat-liver microsomal membranes, induced *in vitro* by two different sources of free radicals, 2,2′-azobis (2-amidinopropane) (AAPH) and *tert*-butyl hydroperoxide (*tert*-BOOH), was evaluated. Our pro-prodrug could be successfully applied in pharmaceutical field both as prodrug of 5-ASA than as carrier of *trans*-ferulic acid.

Key words colon specific; 5-amino salicylic acid; pro-prodrug; L-lysine; *trans*-ferulic acid

Creation of pharmaceutical forms able to selectively release therapeutic agent, is of great interest to reduce collateral effects and increase therapeutic efficacy. In particular, inflammatory bowel diseases (IBD), characterized of chronic inflammation and unknown etiology, expressed in Crohn disease and ulcerative colitis, needs pharmaceutical forms poorly absorbed in gastric mucosa and in superior portions of intestinal part to protect active substance and to arrive in intact form into the colon. For this reason, it has recourse to prodrugs, pH-based polymers, hydrogels, multi-layered systems with time-dependent degradation and bacteria-degradated polymers.[1)]

Actually, pharmaceutical approach to IBD is with salicylates and corticosteroids which may induce gastric mucosa irritation and a lot of adverse reactions, moreover, some of them cannot be assumed by oral administration because of its sensibility to gastric juice's enzymes.[2)] In particular, sulfasalazine, composed by 5-amino salicylic acid (5-ASA) (or mesalazine) and sulfapyridine, is used as standard therapy in IBD. The active moiety is 5-ASA, whereas sulfapyridine acts as a carrier that protects active substance, but it is the most responsible of adverse effects. For this reason the idea to change sulfapyridine with L-lysine amino acid, an absolutely non toxic carrier, reducing to minimum the collaterals effects.

5-ASA is rapidly absorbed in the small intestine before its entering in the colon, then is necessary its administration in modified release systems or as colon specific pro-prodrug, and is a highly potent scavenger of reactive oxygen species (ROS) and was shown to reduce lipid peroxidation in Crohn's disease and ulcerative colitis.[3)] In fact, oxidative stress has been recognized as one of the key elements of tissue injury in IBD.[4)] So, in order to improve 5-ASA antioxidant action, we also linked to L-lysine *trans*-ferulic acid (Fig. 1), which

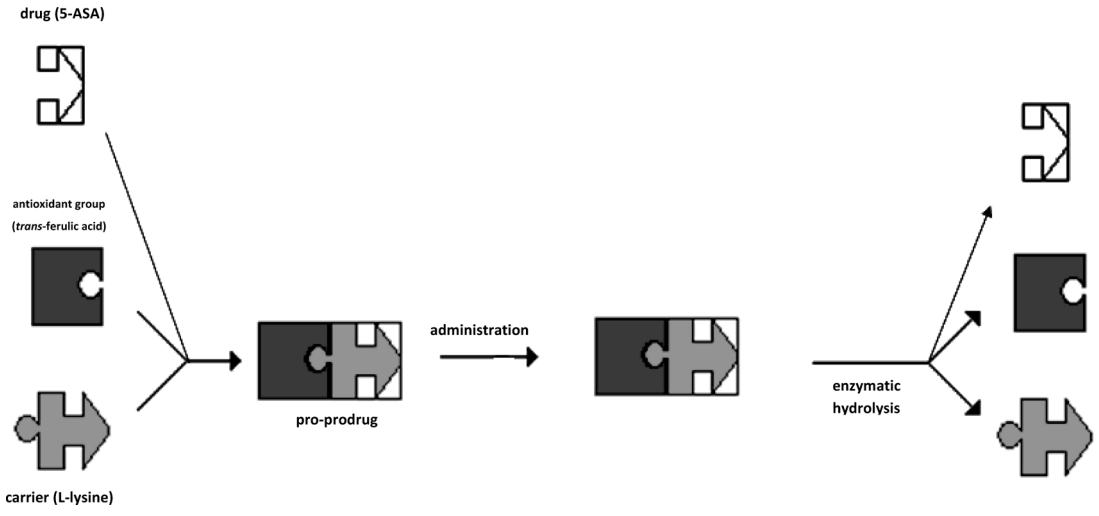

Fig. 1. Pro-Prodrug Composition and *in Vivo* Degradation

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maintains its strong antioxidant activity even if covalently bonded, as demonstrated in our previous works.[5—7)]

The pro-prodrug idea is due to the presence of two amino groups in L-lysine, bonded respectively to chiral center of amino acid and to methyl group in the ε-position. Exploiting this intrinsic chemical reactivity of L-lysine, we bonded in ε-position ferulic acid, esterified with methanol the carboxylic group and, finally, submitted the free amino group to diazotation with 5-ASA, released in the colon by azoreductase enzymes action. All intermediates of synthesis and the final product (derivative A) were characterized with usual spectroscopic techniques, as FT-IR, GC-MS and ^{1}H-MNR.

The pro-prodrug antioxidant activity in inhibiting the lipid peroxidation in rat-liver microsomal membranes induced *in vitro* by 2,2′-azobis(2-amidinopropane) (AAPH), which exogenously produces peroxyl radicals by thermal decomposition, and *tert*-butyl hydroperoxide (*tert*-BOOH), which endogenously produces alkoxyl radicals by Fenton reactions, was also evaluated.

The synthesis of pro-prodrug (derivative A) was effected in the following way: 2.26 g (16 mmol) of L-lysine were dissolved in 50 ml of dry chloroform and added, under stirring, to *trans*-ferulic acid (3 g, 16 mmol) previously treated with 1.864 ml of thyonil chloride (0.015 mmol) in chloroform. Reaction mixture was refluxed at 80 °C for 2 h and then carried on for more than 24 h to room temperature. To obtain a complete hydrolysis, 50 ml of distilled water were added to reaction mixture and 3 extractions with chloroform were conduced. Organic extracts are left one night on dry sodium sulfate which was removed though filtration. Raw product was purified through chromatographic column and we obtained derivative **2** (Chart 1), analyzed by FT-IR, GC/MS and ^{1}H-NMR (Table 1). Yield 67%.

0.250 g (0.776 mmol) of derivative **2** were added to 1.61 ml (0.040 mmol) of cool methanol containing 0.028 ml ($3.855 \cdot 10^{-4}$ mmol) of thyonil chloride. Reaction was given to 60—70 °C and left to reflux for 7 h under stirring. After 1 h, 0.008 ml of thyonil chloride ($1.102 \cdot 10^{-4}$ mmol) (30% in excess) were added to reaction mixture. To remove dimethyl sulfite excess, raw product was treated with 20 ml (0.192 mmol) of ethylic ether at 0 °C. Derivative **3** (Chart 1), purified by recystallization from methanol (yield 72%), was characterized through FT-IR, GC/MS and ^{1}H-NMR techniques (Table 1) and then submitted to diazotation.

0.173 g (0.514 mmol) of **3** were dissolved in 30 ml of water, then 0.032 ml ($1.035 \cdot 10^{-3}$ mmol) of hydrochloric acid were slowly added at 0—5 °C. A sodium nitrite aqueous solution (0.007 g, 0.101 mmol of $NaNO_2$ in 0.050 ml of distilled water) was added to obtain acid conditions that were kept with subsequent insertions of 0.011 ml ($3.559 \cdot 10^{-4}$ mmol) of hydrochloric acid to stabilize salt and to minimize secondary reactions. To avoid diazonium salt hydrolysis (intermediate **4**, Chart 1), reaction temperature is monitored at 0—5 °C.

Finally, 0.141 g (1.021 mmol) of salicylic acid were dissolved in a sodium hydroxide aqueous solution (0.082 g, 2.05 mmol of NaOH in 15 ml of distilled water) and solution temperature was maintained to 5 °C. Diazonium salt was slowly added in alkaline conditions. Reaction is kept to reflux for 24 h and reaction water was removed through lyophilization. The residue was loaded on a silica gel open column and eluted with $CHCl_3$/MeOH (75 : 25). The final product **5** (derivative A, yield 79%) was initially characterized with FT-IR, GC/MS and ^{1}H-NMR (Table 1) and then,

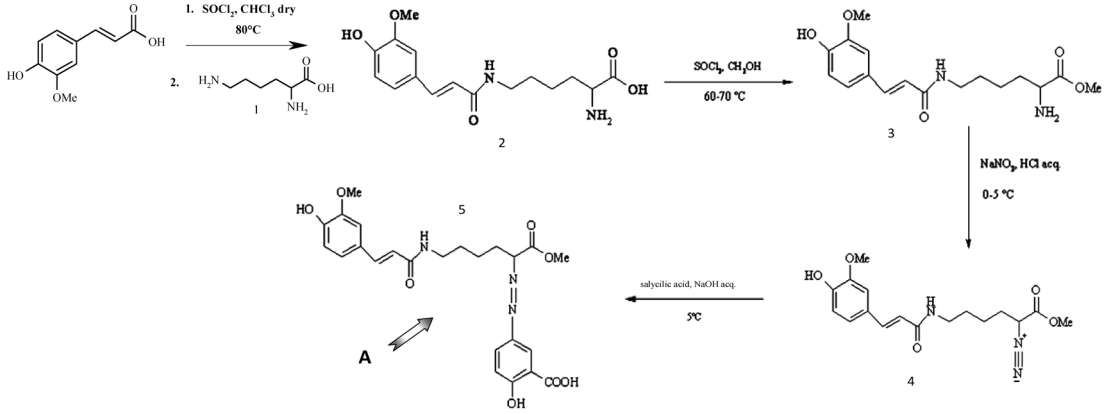

Chart 1. Synthetic Route for Derivative A Preparation

Table 1. GC-MS, FT-IR and ^{1}H-NMR Data

Compound	*m/z*	Wavenumber ν (cm^{-1})	Chemical shift (δ) ppm
2	222 (100%), 177 (71%), 77 (6%)	1711 (–CONH)	7.75 (1H, sb), 7.27 (2H, m), 6.85 (1H, d), 6.60 (1H, sb), 3.90 (3H, s), 3.36 (1H, m), 2.78 (2H, t), 1.12—1.72 (6H, m)
3	208 (100%), 177 (61%), 145 (38%), 77 (9%)	1735 (–$COOCH_3$)	7.58 (1H, d), 7.13 (2H, m), 6.80 (1H, d), 6.33 (1H, d), 3.90 (3H, s), 3.80 (3H, s), 3.36 (1H, m), 2.78 (2H, t), 1.12—1.72 (6H, m)
5	285 (100%), 177 (65%), 77 (6%)	1742 (–$COOCH_3$)	7.86 (1H, m), 7.83 (1H, m), 7.28 (2H, m), 6.78 (2H, m), 6.57 (1H, d), 6.19 (1H, d), 3.81 (3H, s), 3.36 (3H, s), 3.13 (1H, m), 2.74 (2H, m), 1.11—1.55 (6H, m)

GC-MS spectra were performed by using Hewlett Packard GC-MS 5972. NMR spectra were acquired on Bruker VM-300 ACP using CD_3OD as solvent. FT-IR spectra were measured on a Jasco 4200 using KBr disks.

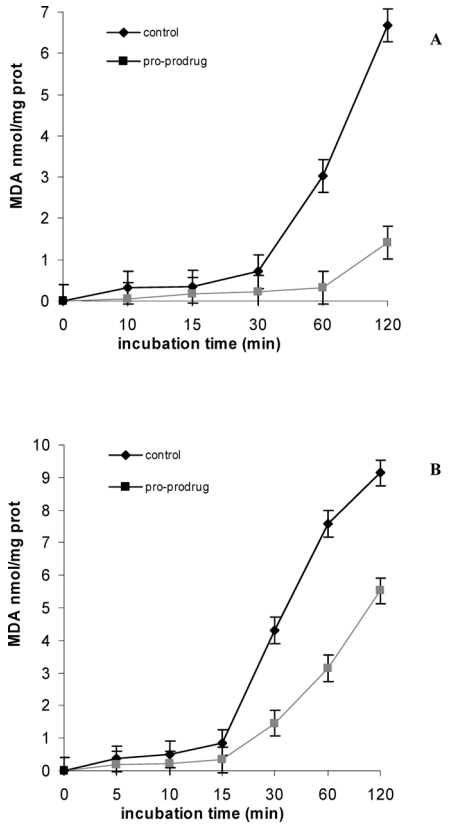

Fig. 2. Effects of Pro-Prodrug on MDA Production Induced by (A) *tert*-BOOH and (B) AAPH in Rat-Liver Microsomal Membranes

The microsomal membranes were incubated with $0.25 \cdot 10^{-3}$ M *tert*-BOOH or $25 \cdot 10^{-3}$ M AAPH at 37 °C under air in the dark. The results represent means ± S.E.M. of six separate experiments.

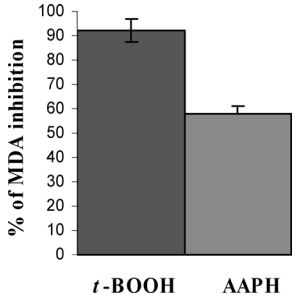

Fig. 3. Percentage of Inhibition of *tert*-BOOH- and AAPH-Induced MDA Formation in the Presence of Pro-Prodrug in Rat-Liver Microsomal Membranes after 30 min of Incubation

The microsomal membranes were incubated with $0.25 \cdot 10^{-3}$ M *tert*-BOOH or $25 \cdot 10^{-3}$ M AAPH at 37 °C under air in the dark. The results represent the mean ± S.E.M. of six separate experiments.

submitted to evaluation of its antioxidant activity in inhibiting the lipid peroxidation in rat-liver microsomal membranes, during 120 min of incubation, induced *in vitro* by two different sources of free radicals including AAPH and *tert*-BOOH.[8]

The effects of pro-prodrug on the lipid peroxidation were time-dependent and brought as malondialdehyde (MDA) nmol/mg proteins (Fig. 2). Derivative A was a stronger antioxidant in protecting the membranes from *tert*-BOOH- than from AAPH-induced lipid peroxidation, showing in either case higher efficiency at 30 min of incubation (Fig. 3) and the preservation of antioxidant activity up to 2 h confirming results found previously.

In conclusion, the aim of our work is to target 5-ASA to the colon in a specific way and reduce to minimum the collaterals effects, changing sulfapyridine,[9] which is mostly responsible of adverse effects, with L-lysine amino acid, an absolutely non toxic carrier. With this approach and in order to obtain a pro-prodrug with increased antioxidant properties, we successfully bonded *trans*-ferulic acid to the L-lysine ε-amino group obtaining a hydrophilizing promoiety which was, subsequently, derivatized with 5-ASA, attaining derivative A, susceptible to cleavage by enzymes secreted by the gastrointestinal tract bacterial microflora. Increased hydrophilicity of our pro-prodrug should allow 5-ASA colon specific release. In fact, literature data suggest that following pro-prodrug oral administration, 5-ASA absorption in the stomach and small intestines is decreased because of the promoiety polar nature. Therefore, greater levels of the drug, in the form of the pro-prodrug, can reach the colon. Within the colon, the bacterially derived enzymes catalyze the conversion of the pro-prodrug to the more lipophilic drug which is now available for absorption through the colonic membrane.[10] From the earlier discussion, it is quite natural to presume that the main objective of this work was to synthesize safe pro-prodrug of 5-ASA that could be free from all the adverse effects, successfully applied in pharmaceutical field also as prodrug of *trans*-ferulic acid, and whose *in vivo* release will be tested in the future. In this work we effected the lipid peroxidation assay for antioxidant activity and the results suggested that our pro-prodrug possesses an excellent antioxidant activity.

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