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NAC/MEA Conjugate: A New Potent Antioxidant Which Increases the GSH Level in Various Cell Lines

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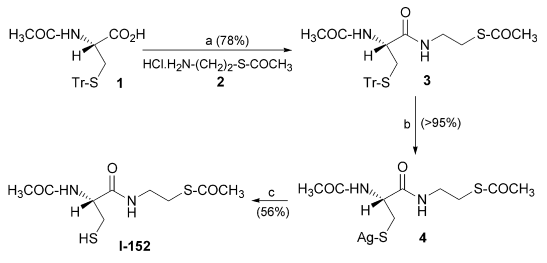
Abstract—I-152 is a prodrug of NAC and MEA with potent pro-GSH effects in human macrophages, astrocytes and lymphocytes. This molecule could be of interest in HIV infection in respect to its antioxidant and anti-HIV activities, but also in other diseases to counteract oxidative stress, that is, inflammation, cardiovascular diseases, and neurodegenerative diseases.

It is now well established that antioxidants could be valuable in various clinical situations in which oxidative stress plays a role in pathological disorders (e.g., viral diseases, cardiovascular diseases, inflammation, cancer, neurological diseases, septic shock, etc.).[1–12] Hence, our interest in this study was in the design of new potent antioxidant molecules. In this respect, we would like to report herewith on an NAC/MEA[13] conjugate compound, **I-152**, which seems to be a highly potent antioxidant by increasing the glutathione (GSH) content in various cell lines. This research is in continuation of our previous work in the area of radical scavengers and other pro-GSH compounds.[14–16]

Glutathione, or L-γ-glutamyl-L-cysteinylglycine, is an anionic tripeptide, widely distributed in animals. It is involved in various cell functions such as the detoxification of xenobiotics, the immune response, inflammatory processes and the biosynthesis of proteins and DNA, and it plays an important role in cell defense against oxidative stress. GSH is an intrinsic efficient scavenger of nitric oxide and peroxinitrite, but it is also the substrate of GSH peroxidases that inactivate peroxides, the substrate of GSH-dehydrogenase that regenerates antioxidant molecules such as ascorbate, and the substrate of GSH-*S*-transferase that detoxifies endogenous and exogenous compounds.[17]

The synthesis of **I-152** is outlined in Scheme 1 and was performed starting from commercially available *N*-acetyl-*S*-trityl-L-cysteine **1** and *S*-acetylcysteamine hydrochloride **2**.[18]

The pseudo-dipeptide **3** was obtained by formation in situ of the mixed anhydride using **1**, isobutyl chloroformate (IBC) and *N*-methylmorpholine (NMM). This anhydride was then added to *N*-hydroxysuccinimide (HOSu) to form the OSu active ester which was finally coupled with **2** in the presence of NMM. After treatment of the reaction mixture, the *S*-trityl derivative **3**



Scheme 1. Reagents and conditions: (a) (1) AcOEt, IBC, NMM, −15 °C, 15 min; (2) HOSu, −15 °C, 15 min; (3) **2**, NMM, −15 °C–rt, 12 h; (b) MeOH, $CHCl_3$, mixture of MeOH/$AgNO_3$/pyridine, rt, 12 h; (c) $CHCl_3$, HCl 37%, rt, 2 h.

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was isolated. This crude product was over 95% pure and can be used in the next step without further purification. *S*-Trityl protecting group was then removed, via formation of the corresponding silver sulfide **4**, according to the described method.[19] Compound **I-152** was isolated after recrystallization from ethyl acetate and petroleum ether.[20]

I-152 is a prodrug expected to liberate after metabolization, NAC and MEA, two potential pro-GSH compounds which may act as part of the glutathione (GSH/GSSG) redox system.[21,22] Hence, intracellular total GSH level was measured in primary human macrophage-derived monocytes (MDMs) and other human cell lines (spleen and broncho-alveolar (BAL) macrophages, astrocytes, and lymphocytes). Peripheral blood mononuclear cells (PBMCs) of healthy blood donors were obtained by Ficoll-Hypaque density gradient centrifugation, and monocytes were separated from peripheral blood lymphocytes (PBLs) using countercurrent centrifugal elutriation. Monocytes were then differentiated in MDM during 7 days of culture. Spleens and broncho-alveolar lavages were generously provided by Pr. T. de Revel (Hôpital D'Instruction des Armées Percy, Clamart, France) and M. Humbert (Hôpital Antoine Béclère, Clamart, France), respectively; spleen and BAL macrophages were obtained by adherence as previously described.[23] Embryonic astrocytes were generously provided by Pr. M. Tardieu (Hôpital de Bicêtre, Kremlin Bicêtre, France). All these cells were cultivated in DMEM Glutamax cell culture medium supplemented with 10% heat-inactivated (+56 °C, 45 min) fetal calf serum, 1% triantibiotic mixture (penicillin, streptomycin, and neomycin). After 24 h treatment, cells were lysed and GSH level was determined using Griffith's method (Cayman, Ann Arbor, MI, USA) and protein level using Bradford's method (BioRad, Hercules, CA, USA).

In human MDM, **I-152** increased GSH level in a dose-dependent manner. At 250 μM, GSH content increased by 165% (Fig. 1A), whereas NAC and MEA, alone or in equimolar combination, had no significant effect at the same dose (Fig. 1B). The decrease in GSH content at high **I-152** doses is probably the consequence of a negative feedback of GSH on γ-glutamylcysteinyl synthetase, the first step in GSH synthesis.[24,25] **I-152** is also efficient to increase GSH in human spleen and BAL macrophages, in human astrocytes, major cells also involved in defense against oxidative stress in the central nervous system, and in PBLs susceptible to oxidative stress-induced apoptosis and particularly in human immunodeficiency virus (HIV) infection (Fig. 2).

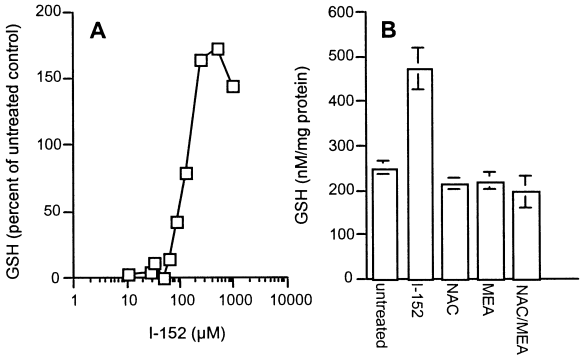


Figure 1. (A) Dose-effect of **I-152** on intracellular GSH level in 24 h treated MDMs; (B) comparison between effects of I-152 and other pro-GSH drugs at 250 μM on intracellular GSH content (GSH dosages 24 h after the initiation of treatment). Experiments were performed in triplicate and results are representative of five independent experiments ($n=15$).

The toxicity of **I-152** was appreciated in vitro (neutral-red uptake) and in vivo. In MDMs, 50% cytotoxic concentrations (CC_{50}) were respectively equal to >5 mM at 24 h of treatment and 635 μM at 12 days.[26] In OF1 mice, the intraperitoneal LD_{50} (30 days) was equal to 700 mg/kg.[27]

HIV infection is associated with systemic and tissular decreases in the GSH content.[28,29] This decreased GSH content may reduce the survival of HIV-infected patients,[30] perhaps by contributing to diverse disorders, that is, CD4+ T cell apoptosis,[24] neuroAIDS[31] and enhancing HIV replication. The anti-HIV effects of pro-GSH compounds such as NAC and L-2-oxo-thiazolidine-4-carboxylic acid confirm the relationships between viral replication and GSH.[32,33] Hence, anti-HIV effects of **I-152** were explored in MDM infected with the reference macrophage-tropic HIV-1/Ba-L strain. In these experimental conditions, **I-152** significantly decreased viral replication in MDMs, with a 50% effective dose (ED_{50}) equal to 3–50 μM regarding the multiplicity of infection (moi, Table 1).

On the basis of such preliminary data, we decided to explore the behavior of **I-152** in cell extracts as a model for intracellular metabolization.

The compound was incubated in total cell extracts of T CD4+ lymphoid CEM-SS cell line and of MDMs. The structure of the metabolites was determined by HPLC/MS coupling and coinjection with authentic synthesized samples. Surprisingly, we observed a rapid transposition of **I-152** (CEM-SS: $T_{1/2}=14$ min, $c=10^{-4}$ M) giving rise

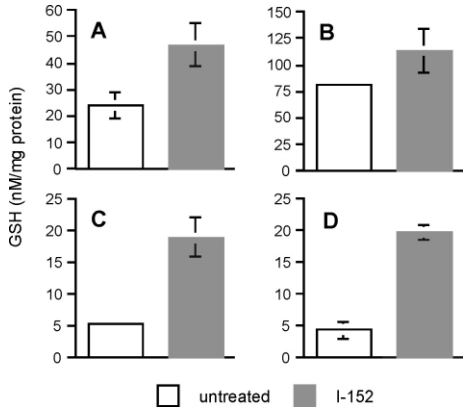
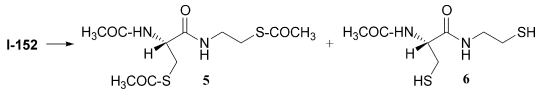


Figure 2. (A) Effects of **I-152** on intracellular GSH level in human spleen macrophages, (B) in human macrophages obtained from broncho-alveolar lavages, (C) in human peripheral blood lymphocytes, (D) in human embryonic astrocytes. Experiments were performed in triplicate and results are representative of at least three independent experiments ($n=9$).

Table 1. Anti-HIV activity of **I-152** in HIV-1/Ba-L-infected MDMs[a]

	Multiplicity of infection (moi)	
	0.001 (μM)	0.01 (μM)
ED_{50}	3	50
ED_{70}	15	90
ED_{90}	75	190

[a]These results are representative of six independent experiments performed in triplicate.



Scheme 2. I-152 transposition in CEM-SS and MDM cell extracts.

to the corresponding fully acetylated derivative **5** and the expected dithiol **6** (Scheme 2). An identical result was observed in MDMs. This transposition is solvent-, temperature-, and concentration-dependent which seems to indicate an intermolecular process.

It is noteworthy that the dithiol derivative **6** may play a crucial role in the **I-152** mode of action as the key moiety of the NAC has been assumed to be related to the thiol function.[34] In addition, we have also shown in a complementary experiment that incubation of **5** in CEM-SS cell extracts gives rise to **I-152** formation ($T_{1/2}=27$ min, $c=10^{-4}$ M) presumably upon esterase activation.

In conclusion, **I-152** is a potent pro-GSH compound as compared to NAC, MEA or other molecules of the same family. These pro-GSH effects are observed in different cell lines suggesting the high potential of this new molecule. The biological interest of **I-152** is confirmed by the significant decrease of HIV replication in MDMs, in absence of cytotoxicity. Altogether, these data suggest that **I-152** could be of great interest in pharmacology but also in cosmetology or other domains involving oxidative stress.

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20. **I-152**: mp 122–124 °C; $[\alpha]_D^{20}=-40°$ (*c* 0.87, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 1.60 (dd, $J=7.6$, 10.3 Hz, 1H, S*H*), 2.07 (s, 3H, $NCOCH_3$), 2.36 (s, 3H, $SCOCH_3$), 2.70 (ddd, $J=6.5$, 10.3, 13.9 Hz, 1H, β *H*a cys), 3.03 (t, $J=6.3$ Hz, 2H, NCH_2CH_2S), 3.06 (ddd, $J=4.3$, 7.6, 13.9 Hz, 1H, β *H*b cys), 3.46 (td, $J=6.0$, 6.3 Hz, 2H, NCH_2CH_2S), 4.59 (ddd, $J=4.3$, 6.5, 7.9 Hz, 1H, α H cys), 6.52 (d, $J=7.9$ Hz, 1H, N*H* cys), 6.75–6.90 (m, 1H, $NHCH_2$); ^{13}C NMR (100.6 MHz, $CHCl_3$) δ 23.2 ($NCOCH_3$), 26.6 (β CH_2 cys), 28.4 (NCH_2CH_2S), 30.6 ($SCOCH_3$), 39.7 (NCH_2CH_2S), 54.1 (α *C*H cys), 169.8 ($NCOCH_3$), 170.4 (*C*O cys), 196.4 ($SCOCH_3$); MS: (FAB$^+$/G-T) *m*/*z* 529 $[2M+H]^+$, 265 $[M+H]^+$. Anal.: $C_9H_{16}N_2O_3S_2$: C, H, N.
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