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# Synthesis and Biological Evaluation of New Creatine Fatty Esters Revealed Dodecyl Creatine Ester as a Promising Drug Candidate for the Treatment of the Creatine Transporter Deficiency

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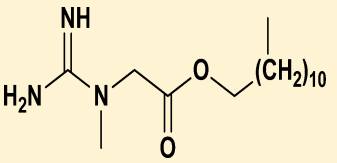
Supporting Information

**ABSTRACT:** The creatine transporter deficiency is a neurological disease caused by impairment of the creatine transporter SLC6A8, resulting in mental retardation associated with a complete absence of creatine within the brain and cellular energy perturbation of neuronal cells. One of the therapeutic hypotheses was to administer lipophilic creatine derivatives which are (1) thought to have better permeability through the cell membrane and (2) would not rely on the activity of SLC6A8 to penetrate the brain. Here, we synthesized creatine fatty esters through original organic chemistry process. A screening on an in vitro rat primary cell-based blood−brain barrier model and on a rat primary neuronal cells model demonstrated interesting properties of these prodrugs to incorporate into endothelial, astroglial, and neuronal cells according to a structure−activity relationship. Dodecyl creatine ester showed then a 20-fold increase in creatine content in pathological human fibroblasts compared with the endogenous creatine content, stating that it could be a promising drug candidate.




## INTRODUCTION

Cerebral creatine deficiency syndromes (CCDS) are a group of inborn errors of creatine biosynthesis and transport through the cellular membranes.[1] These diseases are associated with severe neurologic features: mental retardation, expressive speech and language delay, autistic-like behavior, and epilepsy. They are characterized by a lack of creatine in the brain[1,2] and metabolic disturbances in the nervous system because the creatine is involved in the cellular phosphocreatine energy system.[3] The only way to treat patients is to restore the cerebral creatine pool by bringing creatine into the brain. Nowadays, the treatment of creatine biosynthesis defects has yielded significant clinical improvement.[4] However, successful therapeutic strategies still need to be discovered in order to treat the creatine transporter defect.[2,5]

Some clinical studies based on the use of creatine supplemented by amino acids such as L-arginine and L-glycine showed no improvement of clinical features in long follow-up of patients.[6] The absence of functional creatine transporters at the blood−brain barrier (BBB) may prevent the entry of creatine into the brain, thus affecting the cognitive functions.[7−9] For instance, creatine amino acids and phosphocreatine−Mg complex show neuroprotective activity in in vivo animal models of cerebral stroke, ischemia, or hypoxia.[10−12] In addition, a 9-week treatment with cyclocreatine as treatment in SLC6A8 knockout mice[13] resulted in an increase in phosphocreatine and phosphocyclocreatine $^{31}$P-MRS signals as well as normalization of behavioral test findings.[14]

Creatine esters are believed to play an important role in the restoration of cerebral creatine content. First, they are highly lipophilic and so probably cross biological membranes such as the BBB by passive transport.[15] Second, cellular esterases are able to biotransform these prodrugs and deliver creatine into the cells. Two creatine esters have been considered so far (creatine benzyl ester and creatine ethyl ester). Creatine benzyl ester showed interesting properties and increased the creatine pool in mouse hippocampal slices.[15] The same was observed with creatine ethyl ester.[16] However, a 12-month clinical study in patients did not reveal any neuropsychologic improvement,[17] likely because of a lack of chemical stability: creatine esters are highly susceptible to hydrolysis in the gastric environment and to the effect of plasmatic esterases.[18−20] The esters are thus biotransformed to creatine which has no therapeutic properties.

Herein we present the synthesis of creatine derivatives by means of an original method for preparing creatine fatty esters by carrying out a ring-opening step on diprotected creatinine with a molecule bearing an alcohol functional group. The whole chemical library of creatine fatty esters was screened for their ability to cross the BBB and to be internalized in primary neuronal cells. Moreover, we shed light on the molecular mechanisms underlying translocation processes using human fibroblasts from patients affected by the creatine transporter deficiency.

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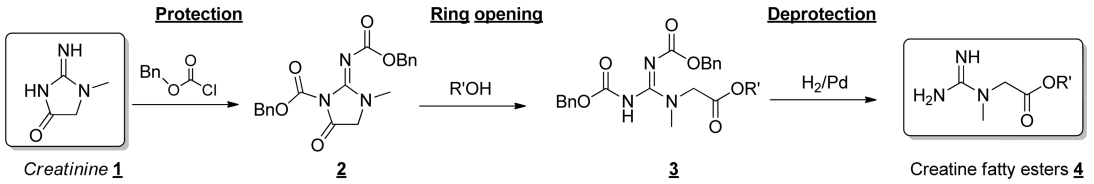


**Figure 1.** Synthesis of creatine fatty ester by ring-opening of protected creatinine by fatty alcohols.

## RESULTS

**Chemistry.** Although several procedures have already been described for the preparation of anhydride (WO 2008/101309) and amide (US 2011/0269986, US 2008/0200705) derivatives of creatine, efficient methods leading to creatine fatty esters are still lacking. To the best of our knowledge classical acid-catalyzed esterification of creatine by alcohols is the only method described so far (US 2005/0049428). Because the alcohol partner is used as solvent in this process, the method is limited to the preparation of small esters like ethyl, propyl and butyl esters of creatine.

We therefore were interested in developing a more general method leading to real fatty esters of creatine. Figure 1 describes our general strategy for a brand new synthesis of creatine fatty esters. The strategy is based on activation of the electrophilicity of the carbonyl moiety of creatinine **1** by double protection of this cyclic guanidine. Indeed, the carbamate derivative of the guanidine moiety should be a good leaving group and therefore should promote the opening of the creatinine ring in presence of nucleophiles such as alcohols. This ring-opening reaction followed by carbamate deprotection would thus generate the desired creatine esters.

*Protection of Creatinine.* First attempts to protect the guanidine moiety of creatinine with $Boc_2O$ gave very poor yields under either aqueous or anhydrous conditions (Figure 1).

Fmoc double protection was also found to be difficult but, fortunately, the reaction with the benzoyl chloroformate yielded 87% of diprotected creatinine **2c** after crystallization in hexane (entry 4, Table 1).

**Table 1. Protection of Creatinine**[a]

| entry | reagent | solvent | base | product | yield (%) |
|---|---|---|---|---|---|
| 1 | $Boc_2O$ (5 equiv) | dioxane/water | NaOH (1 equiv) | **2a** | traces |
| 2 | $Boc_2O$ (6 equiv) | DCM | DIEPA (6 equiv) | **2a** | traces |
| 3 | FmocCl (3 equiv) | DCM | DIEPA (3 equiv) | **2b** | 21 |
| 4 | CBzCl (3 equiv) | DCM | DIEPA (3 equiv) | **2c** | 87 |

[a]Reactions were carried out with 0.1 M reactants at room temperature overnight.

*Nucleophilic Addition of Alcohols to Diprotected Creatinine.* Double CBz-protected creatinine **2c** undergoes spontaneous ring-opening at room temperature once dissolved in methanol to afford creatine methyl ester **3a** (entry 1, Table 2). Heating up to 80 °C is however needed for alcohols bearing longer chain. We were particularly pleased to observe that the reaction is still effective with long, hydrophobic alcohols. The octadecyl creatine ester **3h** was for example successfully obtained using only 2 equiv of stearyl alcohol (entry 9, Table 2). These results represent a significant improvement because such hydrophobic creatine esters were never successfully obtained by other methods.

*Carbamate Deprotection of the Creatine Fatty Esters **3**.* The deprotection of the **CBz** groups of creatine esters **3** was carried out under hydrogen using palladium-supported catalysis (Figure 1). This step was found to be quite delicate because of the polarity of the guanidine part of the molecules which do not facilitate product recovery. After screening of a panel of reaction conditions, palladium over alumina was finally selected and products were successfully recovered after washing the catalyst with methanol. Using these conditions, creatine esters were obtained in quantitative yields except for products **4c** and **4e**, which required purification on reversed phase column. These products were isolated in poor and moderate yields, respectively, because of their relative instability on silica gel (Table 3).

**Pharmacology.** *Stability of Creatine Fatty Esters.* The main pitfall for experimentations with creatine fatty esters is their chemical stability.[18−20] Thus, particular attention has been paid to finding a way to maintain satisfactory stability of our products during the experiments. The degradation of the esters occurs by intramolecular cyclization, leading to biologically inactive creatinine and alcohols. We showed that the rate of degradation is higher with the longer carbon chains. Thus, 60 min of incubation at 37 °C degraded creatine octadecyl ester by 87%, dodecyl ester by 72%, and octyl ester by 21.25% (Table 4). This degradation can be sustained when the creatine esters are in acidic solution.[18−20] In our experimental conditions, the dilution of samples in acetonitrile supplemented by 5% formic acid stopped the undergoing degradation.

*Creatine Fatty Esters Are Not Toxic As Regards of Cell Viability and Do Not Alter Blood−Brain Barrier Integrity.* None of the experimental conditions showed a decrease in the cell viability of brain endothelial cells, glial cells, or neuronal cells compared with a 100% vehicle, suggesting that none of the tested conditions are toxic in terms of cell viability (Table 7). The permeability of Lucifer Yellow (LY $P_{app}$), a paracellular route marker compound, was then evaluated in in vitro cell-based rat BBB model in the presence of the creatine fatty ester. Whatever the creatine fatty ester used in the experiments, the LY $P_{app}$ value was below the limit range of $5 \times 10^{-6}$ cm·s$^{-1}$,[21] suggesting that the creatine fatty esters did not compromise the integrity of the in vitro cell-based rat BBB model and that the cell monolayer is intact.

*Translocation of Creatine Fatty Esters through the in Vitro Cell-Based Rat BBB Model.* Having demonstrated the integrity of the BBB cell monolayer in the presence of different creatine fatty esters (Table 7), we investigated the ability of the creatine fatty esters to reach the brain. This was determined by an in vitro cell-based model of rat BBB.[21] We demonstrated that the creatine esters were able to penetrate into the brain endothelial cells according to a structure−activity relationship (Table 6). After a 60-min incubation of 10 $\mu$g·mL$^{-1}$ of creatine fatty esters,


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**Table 2. Nucleophilic Addition of Alcohols to Compound 2c[a]**

| entry | alcohol | stoichiometry (equiv) | conditions | yield (%) | product |
|---|---|---|---|---|---|
| 1 | $CH_3OH$ | as solvent | 25 °C, 2 h | 92 | **3a** |
| 2 | $CH_3CH_2OH$ | as solvent | 80 °C, 4 h | 72 | **3b** |
| 3 | $CH_3(CH_2)_3OH$ | 10 | 60 °C, 4 h | 60 | **3c** |
| 4 | $CH_3(CH_2)_7OH$ | 4 | 80 °C, 4 h | 40 | **3d** |
| 5 | $CH_3(CH_2)_8OH$ | 8 | 80 °C, 5 h | 25 | **3e** |
| 6 | $CH_3(CH_2)_{11}OH$ | 8 | 80 °C, 7 h | 47 | **3f** |
| 7 | $CH_3(CH_2)_{15}OH$ | 6 | 80 °C, 7 h | 55 | **3g** |
| 8 | $CH_3(CH_2)_{17}OH$ | 1,5 | 80 °C, 16 h | 25 | **3h** |
| 9 | $CH_3(CH_2)_{17}OH$ | 2 | 80 °C, 5 h | 50 | **3h** |

[a]Reactions were carried out with 0.1 M reactants.

**Table 3. Deprotection of the CBz Groups**

| reactant | solvent | product | yield (%) |
|---|---|---|---|
| **3c** | DCM | **4c** | 7 |
| **3d** | DCM/MeOH (1/1) | **4d** | >95 |
| **3e** | DCE | **4e** | 50 |
| **3f** | DCM/MeOH (1/4) | **4f** | >95 |
| **3f** | DCM/MeOH (1/2) | **4f** | >95 |
| **3g** | DCM/MeOH (1/1) | **4g** | >95 |
| **3h** | DCM/MeOH (1/2) | **4h** | >95 |

**Table 4. Degradation of Creatine Esters at 37 °C[a]**

| | creatine ester concentration ($\mu g \cdot mL^{-1}$) | | |
|---|---|---|---|
| | octyl ester | dodecyl ester | octadecyl ester |
| *t*: 0 min | 2.07 ± 0.18 | 6.47 ± 0.60 | 5.74 ± 0.10 |
| *t*: 60 min at 37 °C | 1.63 ± 0.04 | 1.81 ± 0.69 | 0.76 ± 0.07 |
| loss by spontaneous degradation (% initial dosage) | 21.25 | 72 | 87 |

[a]Creatine ester concentration was quantified by HPLC-MS/MS at initial and after a 60 min incubation at 37 °C. The loss of product by spontaneous degradation has been expressed as % of initial dosage.

we found 70.6 ± 7.80 $nmol \cdot mg^{-1}$ of proteins of dodecyl creatine ester in brain endothelial cell lysate, which was 10 times that with octadecyl (6.46 ± 1.42 $nmol \cdot mg^{-1}$) and nonyl (8.14 ± 3.95 $nmol \cdot mg^{-1}$) creatine ester and more than 200 times that with octyl creatine ester (0.33 ± 0.10 $nmol \cdot mg^{-1}$ of proteins). None of the ethyl and butyl creatine ester was detected in BBB endothelial cell lysates. When we looked at the astroglial lysate, we observed that not only had the creatine esters crossed the brain endothelial cells but they had also entered the astroglial cells. About 31.6 ± 13.2 $nmol \cdot mg^{-1}$ proteins and 13.3 ± 4.50 $nmol \cdot mg^{-1}$ proteins of dodecyl and nonyl creatine esters were detected in astroglial lysates, respectively, whereas octyl and octadecyl creatine esters were poorly detectable (0.24 ± 0.04 $nmol \cdot mg^{-1}$ and 0.12 ± 0.07 $nmol \cdot mg^{-1}$ proteins, respectively). Ethyl and butyl creatine esters were undetectable in our experimental conditions.

*Translocation of Creatine Fatty Ester in an in Vitro Primary Neuronal Cell Culture Model.* We next analyzed the translocation of these creatine prodrugs into primary cortical neuronal cells, which are the target cells (Table 6) expressing neuronal cell markers such as microtubule associated protein-2 (MAP2) and neurofilament proteins (data not shown). Intracellular creatine ester content was determined and compared with the residual content in the supernatant after 60 min of incubation. Dodecyl and octadecyl were best able to enter the neurons because we found 257 ± 76.8 $nmol \cdot mg^{-1}$ and 53.9 ± 10.7 $nmol \cdot mg^{-1}$ in the neuronal cell lysate, respectively, corresponding to 58.2 ± 11.3% and 32.2 ± 1.45% of administered dose, respectively. 5.88 ± 0.44 $nmol \cdot mg^{-1}$ proteins (11.4 ± 0.43% of initial dose) of octyl creatine fatty ester were retrieved in the cell lysate, whereas the ethyl creatine ester was poorly detectable in the cell lysate (1.47 ± 0.34 $nmol \cdot mg^{-1}$ proteins equivalent to 0.16 ± 0.01% of the administered dose).

These data indicated that dodecyl creatine ester compound was best incorporated in brain endothelial cells and astroglial cells and was thus able to diffuse through the BBB to the neurons.

**Table 5. Multiple Reaction Monitoring Parameters for the Detection of Creatine, Creatinine, and Creatine Ethyl (4b/C2), Butyl (4c/C4), Octyl (4d/C8), Nonyl (4e/C9), Dodecyl (4f/C12), and Octadecyl (4h/C18) Fatty Esters by HPLC-MS/MS**

| compd/chain length | transition (ES+) | parent ion *m/z* | product ion *m/z* | collision energy (eV) | tube lens |
|---|---|---|---|---|---|
| creatine (CR) | T1 | 132.156 | 90.185 | 10 | 100 |
| creatinine (CRN) | T1 | 114.124 | 86.196 | 10 | 75 |
| | T2 | 114.124 | 44.254 | 10 | 75 |
| **4b**/C2 | T1 | 160.153 | 90.195 | 15 | 20 |
| | T2 | 160.153 | 132.126 | 12 | 20 |
| | T3 | 160.153 | 118.175 | 12 | 20 |
| **4c**/C4 | T1 | 188.197 | 90.175 | 15 | 70 |
| | T2 | 188.197 | 132.144 | 12 | 70 |
| | T3 | 188.197 | 146.166 | 12 | 70 |
| **4d**/C8 | T1 | 244.236 | 90.165 | 20 | 80 |
| | T2 | 244.236 | 132.137 | 15 | 80 |
| | T3 | 244.236 | 202.185 | 15 | 80 |
| **4e**/C9 | T1 | 258.225 | 90.175 | 13 | 100 |
| | T2 | 258.225 | 132.135 | 20 | 100 |
| | T3 | 258.225 | 342.346 | 15 | 100 |
| **4f**/C12 | T1 | 300.285 | 90.125 | 25 | 110 |
| | T2 | 300.285 | 132.146 | 20 | 110 |
| | T3 | 300.285 | 258.195 | 15 | 110 |
| **4h**/C18 | T1 | 384.364 | 90.185 | 35 | 115 |
| | T2 | 384.364 | 132.136 | 25 | 115 |
| | T3 | 384.364 | 342.346 | 20 | 115 |

*Pharmacological Activity of Creatine Fatty Esters.* The pharmacological activity of creatine fatty esters was evaluated in terms of their capacity to restore the creatine pool into the cells. We first attempted to determine the esterase activity (Figure 2) in the pathological model using pediatric patients' fibroblasts,


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Table 6. Structure–Activity Relationships of Ethyl (4b/C2), Butyl (4c/C4), Octyl (4d/C8), Nonyl (4e/C9), Dodecyl (4f/C12), and Octadecyl (4h/C18) Creatine Fatty Esters[a]

| creatine fatty ester | MW | log P | PSA (Å) | concentration (μg/mL) | endothelial cells | | astroglial cells | | neurons | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | cell lysate (nmol/mg) | %TO | cell lysate (nmol/mg) | %TO | supernatant (nmol) | %TO | cell lysate (nmol/mg) |
| **4b**/C2 | 159.2 | −0.72 | 81.15 | 1 | nd | nd | nd | nd | 4.78 ± 0.17 | 108 ± 3.75***[d] | 0.21 ± 0.04[b] |
| | | | | 10 | nd | nd | nd | nd | 44.4 ± 0.88 | 96.0 ± 1.88***[d] | 1.47 ± 0.34[b] |
| **4c**/C4 | 187.2 | 0.25 | 81.15 | 1 | nd | nd | nd | nd | – | – | – |
| | | | | 10 | nd | nd | nd | nd | – | – | – |
| **4d**/C8 | 243.4 | 2.03 | 81.15 | 1 | – | – | – | – | 2.21 ± 0.36 | 98.4 ± 16.0***[d] | 5.88 ± 0.44 |
| | | | | 10 | 0.33 ± 0.10 | 0.18 ± 0.06***[d] | 0.24 ± 0.04 | 0.22 ± 0.03 | 21.3 ± 0.62 | 104 ± 3.06***[d] | 5.88 ± 0.44 |
| **4e**/C9 | 257.4 | 2.47 | 81.15 | 1 | 1.59 ± 0.48 | 5.24 ± 1.55 | 1.44 ± 1.42 | 5.82 ± 1.66 | – | – | – |
| | | | | 10 | 8.14 ± 3.95 | 4.21 ± 1.23***[d] | 13.3 ± 4.50 | 7.83 ± 4.00***[d] | – | – | – |
| **4f**/C12 | 299.5 | 3.81 | 81.15 | 1 | – | – | – | – | 0.18 ± 0.03 | 5.87 ± 1.62 | 18.6 ± 3.58 |
| | | | | 10 | 70.6 ± 7.80 | 15.4 ± 1.70 | 31.6 ± 13.2 | 11.5 ± 4.77 | 1.85 ± 0.24 | 10.5 ± 2.52 | 257 ± 76.8 |
| **4h**/C18 | 383.6 | 5.59 | 81.15 | 1 | – | – | – | – | 0.27 ± 0.06 | 43.1 ± 9.93*[c] | 12.5 ± 1.54 |
| | | | | 10 | 6.46 ± 1.42 | 2.03 ± 0.45***[d] | 0.12 ± 0.07 | 0.06 ± 0.03***[d] | 6.85 ± 1.39 | 80.0 ± 16.3***[d] | 53.9 ± 10.7 |

[a]The physicochemical parameters, including molecular weight (MW), lipophilicity (log P), and polar surface area (PSA), show that the carbonyl chains linkages increase both MW and log P but not PSA. The quantitative determinations in each cell types (e.g., endothelial, astroglial cells, and neurons) are referred to as quantity, expressed in nmol per mg of proteins in the cellular extract or in nmoles in supernatants, and percentage of initial content. nd, not detectable; –, not performed. [b]Values near the lowest quantitation limit. The statistical significance was assessed by an ANOVA followed by a Bonferonni post-test with dodecyl creatine ester as a reference because its uptake into cells is the greatest. [c]$p < 0.05$. [d]$p < 0.0001$. For each compounds, two experiments were performed in triplicates.

which lack a functional SLC6A8 transporter. We noticed that each fibroblast batch was able to convert the 4-nitrophenyl acetate into *p*-nitrophenol, with peak activity reached at 30 min for control K and CTp3 and 80 min for DTp1 and VLp2. This indicates that fibroblasts expressed functional esterases and allowed us to perform a 60 min incubation. We then evaluated the uptake of dodecyl creatine ester (Figure 3) in human fibroblasts. This compound accumulated in human fibroblast lysates at between 100 and 150 nmol·mg$^{-1}$ of proteins in 60 min. The same profile was obtained for the control K (107 ± 8.92 nmol·mg$^{-1}$ of proteins), the child with a functional creatine transporter, and the pathological DTp1 (123 ± 11.2 nmol·mg$^{-1}$ of proteins), VLp2 (149 ± 8.26 nmol·mg$^{-1}$ of proteins), and CTp3 (107 ± 20.5 nmol·mg$^{-1}$ of proteins). This suggests that the dodecyl creatine ester is able to accumulate inside the fibroblasts even if the creatine transporter SLC6A8 is no longer functional.

The therapeutic strategy is based on the conversion of the creatine ester into creatine, so we measured the impact of the treatment with dodecyl creatine ester on the creatine cell content (Figure 4). A 5.8-fold increase in the creatine content was noted in the control K compared with the same experimental conditions without the dodecyl creatine ester treatment. In comparison, the endogenous creatine content detectable in fibroblasts from control K cells was 33.6 ± 1.24 nmol·mg$^{-1}$ of proteins. Considering the endogenous value in these cells, the increase in creatine was 2.6-fold, but the most interesting observations were made on fibroblasts from patients suffering from a SLC6A8 creatine transporter deficiency syndrome. Whereas the creatine loading in the cells in the absence of dodecyl creatine ester was below our lowest quantitation limit of 0.05 μg.mL$^{-1}$, the creatine content increased after a 60 min incubation with dodecyl ester up to 66.1 ± 4.33, 76.1 ± 2.19, and 55.4 ± 7.78 nmol·mg$^{-1}$ of proteins in the DTp1, VLp2, and CTp3 patients, respectively. When considering the initial endogenous creatine content, these values were increased 23.9-, 20.0-, and 21.6-fold, respectively, assuming that the dodecyl creatine ester may have been taken up by cell esterases and converted into creatine to replenish the cell's energy supply.

## DISCUSSION AND CONCLUSION

Cerebral creatine deficiency is a neurological disorder which induces severe neurodevelopmental delays in children. One of the established etiologies is deficiency in creatine transporter because of mutations of the encoding gene.[22] Therapeutic strategies rely on the use of a creatine prodrug, which enters neuronal cells by a passive transport and is converted into creatine. On the basis of the principle that increasing drug lipophilicity will promote the diffusion through the BBB, we aimed to develop creatine esters linking long and fatty carbon chains to the creatine structure. Due to the presence of cellular esterases, these prodrugs could be converted in creatine within the appropriate target cells.

What is first striking when considering the creatine derivatives is the huge number of patents: several synthesis pathways have been described to date, but very few compounds have been reported in the literature. The only one which has been extensively described is the creatine ethyl ester.[12,16] When we first tried to reproduce the published methods, it turned out that they were not convenient for the synthesis of long-chain creatine esters. An original synthesis process was therefore implemented, and a library of nine creatine prodrugs was


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Table 7. In Vitro Evaluation of Toxicity of Creatine Ethyl (4b/C2), Butyl (4c/C4), Octyl (4d/C8), Nonyl (4e/C9), Dodecyl (4f/C12), and Octadecyl (4h/C18) Fatty Esters[a]

| | concentration ($\mu g \cdot mL^{-1}$) | LY $P_{app}$ ($\times 10^{-6}$ cm/s) | MTT endothelial cells (% of control) | MTT astroglial cells (% of control) | LDH neurons (ratio compared to ctrl) |
|---|---|---|---|---|---|
| **4b**/C2 | 1 | 1.54 ± 0.28 | – | – | 0.53 |
| | 10 | 1.13 ± 0.47 | – | 76.9 ± 21.5 | 0.53 |
| **4c**/C4 | 1 | 1.23 ± 0.55 | – | – | 0.54 |
| | 10 | 1.81 ± 0.69 | – | 90.6 ± 9.48 | 0.58 |
| **4d**/C8 | 1 | 0.93 ± 0.33 | – | – | 0.65 |
| | 10 | 2.42 ± 1.26 | 84.6 ± 13.0 | 122 ± 20.4 | 0.64 |
| **4e**/C9 | 1 | 1.49 ± 0.33 | – | – | 0.52 |
| | 10 | 1.18 ± 0.25 | – | 143 ± 33.2 | 0.45 |
| **4f**/C12 | 1 | 0.70 ± 0.05 | – | – | 0.67 |
| | 5/10 | 1.70 ± 0.49 | 87.8 ± 13.0 | 145 ± 19.9 | 0.69 |
| **4h**/C18 | 1 | – | – | – | 0.65 |
| | 10 | – | 95.3 ± 6.21 | 88.3 ± 8.81 | 0.69 |

[a]Three tests assessing the cellular toxicity were performed. The Lucifer Yellow permeability (LY $P_{app}$) showed values less than $5 \times 10^{6}$ $cm \cdot s^{-1}$ for all conditions, indicating that none of them induced BBB disruption. The mitochondrial function was not altered in any conditions as showed by the MTT test, in which the cell viability did not differ of more than 20% from the control, and by the LDH test, in which the ratio compared with the control was below 1, meaning that no conditions induced neurotoxicity. –, test not performed.

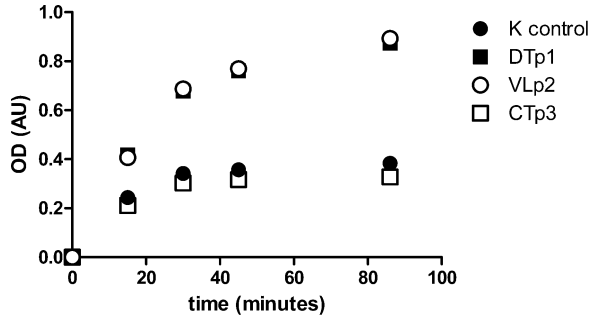


**Figure 2.** Activity of cellular esterases in fibroblasts. The activity of cellular esterases in human fibroblasts is represented by the optical density of *p*-nitrophenol as a function of time for control subject K (black circle) and three patients (DTp1, VLp2, CTp3) with creatine transporter deficiency.

prepared. Only six of these were considered during the pharmacological studies. None of the six compounds decreased cell viability or increased BBB permeability, and so they were usable for further investigations on their brain penetrability and diffusion, using the in vitro BBB model. This revealed a structure–activity relationship: the longer the carbon chain is the greater the entry in both brain endothelial and astroglial cells. The compound showing the most interesting properties was the dodecyl creatine ester, whereas the shortest carbon chains (ethyl to octyl) displayed limited diffusion through cell membranes. The octadecyl creatine ester was detected in brain endothelial cells but not in astroglial cells, indicating that it was probably stuck into the endothelial phospholipid bilayer. In the BBB model, the detection of the creatine ester did not correlate with an increase in intracellular creatine content but with an increase in the creatine signal in the blood compartment. This observation suggested two hypotheses. First, in the BBB model, the creatine transporter is functional and it seems to mediate the efflux of creatine from blood to brain and from brain to blood. Second, the creatine content of healthy cells is very high: 55.5 ± 26.9 and 173.4 ± 38.8 $\mu$M in endothelial cells and astroglial cells, respectively. This compared with 16.2 ± 1.42 $\mu$M of exogenous creatine provided by the 5% serum in cell culture media and indicates that these cells may rely on a creatine biosynthesis in addition to the uptake from the

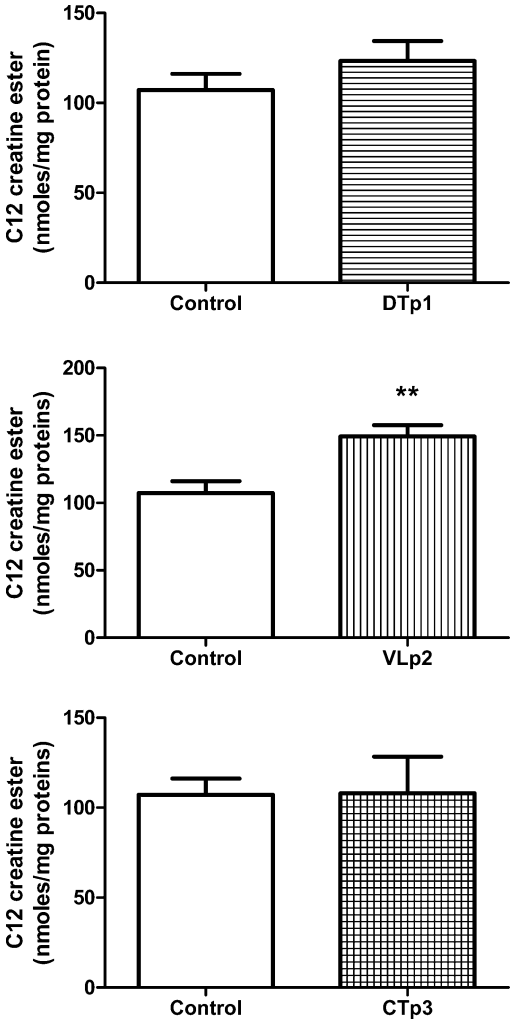


**Figure 3.** Quantitative determination of dodecyl creatine ester. Dodecyl creatine ester is expressed as nmol of ester per mg of proteins in the cellular extract in the fibroblasts of three patients compared with the control subject. Student's *t* test was used to determine significant differences between control and patient, ** stands for $p < 0.005$.


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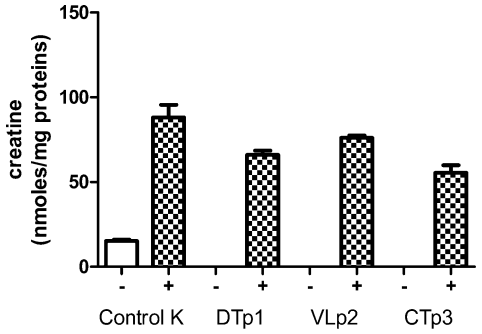


**Figure 4.** Quantitative determination of creatine. The amount of creatine is expressed as nmol of ester per mg of proteins in the cellular extract in the fibroblasts of the control subject and three patients in the presence (+) or absence (−) of dodecyl creatine ester.

supernatant. Creatine esters taken up in this way may be turned into the desired creatine, but it remains unclear whether creatine metabolism can be saturated. In this hypothesis, excess creatine could be released in the blood compartment.

Interestingly, the same profile was obtained in a primary neuronal model. The dodecyl and octadecyl creatine esters penetrated cells well, unlike the ethyl creatine ester, which was scarcely detectable in the cell lysate. Penetration of the octyl creatine ester was intermediate.

These experiments show that the incorporation of creatine fatty esters in endothelial, astroglial, and neuronal cells depends on the length of the carbonyl chain. We sought a new therapeutic strategy for creatine transporter deficiency, using experiments with the pathological model. Human fibroblasts from patients with cerebral creatine deficiency caused by a transporter deficiency were used to investigate whether the dodecyl creatine ester could enter the cells even if it could not be taken up by the SLC6A8 transporter. Creatine content in the fibroblasts was increased, especially in the pathological fibroblasts when treated by dodecyl creatine ester. This demonstrated that the dodecyl can be acted up by cellular esterases, which we demonstrated are active in these cells, and replenishes the creatine content in these cells. Interestingly, the ethyl creatine ester was not able to penetrate the cells and so did not increase creatine content. This suggests that on the basis of published observations,[16,17] ethyl creatine ester is not an effective drugable compound because of its poor cell membrane permeability. In contrast, dodecyl creatine ester is a good candidate for development as a treatment option in patients with creatine deficiency transporter.

However, further investigations are needed for the protection of dodecyl creatine ester chemical structure from the degradation by plasma esterases. A vehicle approach such as the use of Lipid NanoCapsules (LNC) would be a suitable way to cross the BBB and deliver dodecyl creatine ester into the parenchyma target cells. Besides a nanovector approach, further strategy such as the improvement of the chemical template, e.g., exploiting the chemical properties of the cationic guanidine to induce the self-assembly of dodecyl creatine ester could be also considered. To treat the creatine transporter deficiency, the challenge will be to protect the dodecyl creatine ester from the degradation in biological fluids, deliver it through the BBB, and improve neuronal creatine and phosphocreatine to restore the neuronal functionality.

## EXPERIMENTAL SECTION

**Chemistry.** All the reagents were from Aldrich (Steinheim, Germany). TLC was performed on Merck F 254 plates using the specified solvent system. Analytical and preparative LC-MS were performed on a Waters Autopurify system. The elution was done on a reversed phase column (Waters XBridge C18 100 mm × 4.6 mm, particles of 3.5 $\mu$m particles) with three methods depending on the polarity of the different compounds: the time T is in min.

**Generic:**

T0: 95/5 $H_2O$/ACN (+1/1000 $HCO_2H$)
T8: 100% ACN + 1/1000 $HCO_2H$
T13 min: 100% ACN + 1/1000 $HCO_2H$

**Eluent 1:**

T0: 95/5 $H_2O$/ACN (+1/1000 $HCO_2H$)
T5: 100% ACN + 1/1000 $HCO_2H$
T13 min: 100% ACN + 1/1000 $HCO_2H$

**Eluent 2:**

T0: 95/5 $H_2O$/ACN (+1/1000 $HCO_2H$)
T2: 100% ACN + 1/1000 $HCO_2H$
T13 min: 100% ACN + 1/1000 $HCO_2H$

All the compounds were identified by LC-MS and NMR spectra (Bruker UltraShield 400 MHz for proton and 100 MHz for carbon 13). Most purifications were performed on Combiflash Teledyne Isco on silica columns with UV detection. Additional analyses were done when possible (IR on Perkin-Elmer 2000 FT-IR, melting points on Büchi melting point B-545). Proton NMR assignments were based on the integration on the singulet of the *N*-methyl group (3H). The $\alpha$ methylene of the carboxy group also gave a singlet, which was used to calculate the NMR purity of the compounds. The analytical method LC-MS was used to determine purity confirming ≥95% purity for all compounds.

*General Procedure for Synthesis of (E)-tert-Butyl 2-((tert-butoxycarbonyl)imino)-3-methyl-5-oxoimidazolidine-1-carboxylate (**2c**, Supporting Information Figure 1).* Benzoylchloroformate (4.2 mL, 3 equiv) was added to a solution of di-isopropyl ethylamine (5.2 mL, 3 equiv) with creatinine (1.124 g, 1 equiv) in 100 mL of anhydrous dichloromethane under nitrogen. Benzoylchloroformate was added dropwise in an ice bath. The mixture was stirred for 30 min in the ice bath and overnight at room temperature.

The reaction is checked by TLC (silica, heptane/ethyl acetate) and LC-MS. The reaction medium was extracted by addition of dichloromethane and water. The dichloromethane phase was washed three times with water and dried with magnesium sulfate.

The dichloromethane solution was concentrated by evaporation under vacuum and allowed to crystallize. Crude ($Z_2$)-creatinine was obtained (3.25 g, 87%). The ($Z_2$)-creatinine was purified on silica gel (heptane/ethyl acetate gradient) for structure determination. The crude product was used for the esterification step.

*(E)-tert-Butyl 2-((tert-Butoxycarbonyl)imino)-3-methyl-5-oxoimidazolidine-1carboxylate (**2c**).* Chemical formula: $C_{20}H_{19}N_3O_5$. MW: 381 g·mol$^{-1}$. Appearance: amber colored solid. $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$: 3.07 (s, 3H), 4.01 (s, 2H), 5.02 (s, 2H), 5.09 (s, 2H) and 7.34 (m, 10H). $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$: 31.5, 51.4, 67.8, 70.2, 127.0, 133.7, 136.1, 147.2, 151.7, 159.5, 155.9. FTIR (KBr) $\nu$ max: 3361, 2976, 2667, 1800, 1744, 1669, 1623, 1453, 1384, 1318, 1222, 1135, 1072, 981, 902, 871, 799, 728, 695 cm$^{-1}$. MS (ES+): *m*/*z* 382.3. mp: 124 °C. $R_f$: 0.25 (5:5, heptane/ethyl acetate, revealed by UV 254 nm).

*General Procedure for Synthesis of ($CBz)_2$-Creatine Fatty Esters.* Compound **2c** was mixed with excess fatty alcohol without solvent in a pill box according to Table 2 and heated with stirring at 80 °C for 3−5 h until the mixture became a solution (depending on the melting point of the fatty alcohol). The reaction mixture was analyzed by LC-MS analysis and purified on the CombiFlash apparatus.

*($CBz)_2$-Butyl 2-(2,3-Bis((benzyloxy)carbonyl)-1methylguanidino)-acetate (**3c**, Supporting Information Figure 2).* Chemical formula: $C_{24}H_{29}N_3O_6$. MW: 455 g·mol$^{-1}$. Appearance: colorless oil. $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$: 0.95 (t, 3H), 1.36 (q, 2H), 1.6 (q, 2H), 3.12 (s,


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3H), 4.14 (m, 4H), 5.14 (m, 4H), 7.36 (m, 10H). MS (ES+): $m/z$ 456.19. $R_f$: 0.75 (5:5, heptane/ethyl acetate, revealed by UV 254 nm). Purification: Combiflash on silica; solvent, heptane/ethyl acetate; gradient, 100/0 → 80/20.

*$(CBz)_2$-Octyl 2-(2,3-Bis((benzyloxy)carbonyl)-1methylguanidino)-acetate (**3d**, Supporting Information Figure 3).* Chemical formula: $C_{28}H_{37}N_3O_6$. MW: 511 g·mol$^{-1}$. Appearance: yellow oil. $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$: 0.87 (t, 3H), 1.26 (m, 10H), 1.62 (q, 2H), 3.13 (s, 3H), 4.14 (m, 2H), 4.19 (s, 2H), 5.14 (s, 2H), 5.19 (s, 2H) 7.33 (m, 10H). $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$: 14.0, 14.1, 20.9, 22.5, 25.7, 28.4, 29.0, 31.6, 38.8, 60.3, 65.5, 67.7, 128.1, 128.2, 128.3, 128.4, 128.5, 156.6, 168.2, 171.0. MS (ES+): $m/z$ 512.2. $R_f$: 0.7 (5:5, heptane/ethyl acetate, revealed by UV 254 nm). Purification: Combiflash on silica; solvent, heptane/ethyl acetate; gradient, 100/0 → 80/20.

*$(CBz)_2$-Nonyl 2-(2,3-Bis((benzyloxy)carbonyl)-1methylguanidino)acetate (**3e**, Supporting Information Figure 4).* Chemical formula: $C_{29}H_{39}N_3O_6$. MW: 525 g·mol$^{-1}$. Appearance: yellow oil. $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$: 0.87 (t, 3H), 1.25 (m, 14H), 1.61 (q, 2H), 3.12 (s, 3H), 3.92 (s, 2H), 4.16 (m, 2H), 5.14 (s, 2H), 5.16 (s, 2H) 7.36 (m, 10H). MS (ES+): $m/z$ 526.11. $R_f$: 0.7 (5:5, heptane/ethyl acetate, revealed by UV 254 nm) Purification: Combiflash on silica; solvent, heptane/ethyl acetate; gradient, 100/0 → 80/20.

*$(CBz)_2$-Dodecyl 2-(2,3-Bis((benzyloxy)carbonyl)-1methylguanidino)acetate (**3f**, Supporting Information Figure 5).* Chemical formula: $C_{32}H_{45}N_3O_6$. MW: 567 g·mol$^{-1}$. Appearance: yellow oil. $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$: 0.88 (t, 3H), 1.25 (m, 18H), 1.62 (q, 2H), 3.07 (s, 3H), 4.16 (m, 4H), 5.14 (s, 2H), 5.19 (s, 2H) 7.35 (m, 10H). $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$: 8.0, 8.5, 14.0, 22.6, 25.7, 28.4, 29.1, 38.9, 45.7, 52.9, 53.3, 63.3, 65.5, 67.7, 126.9, 128.0, 128.4, 128.5, 156.5, 168.1. MS (ES+): $m/z$ 568.3. $R_f$: 0.45 (5:5, heptane/ethyl acetate, revealed by UV 254 nm). Purification: Combiflash on silica; solvent, heptane/ethyl acetate; gradient, 100/0 → 80/20.

*$(CBz)_2$-Octadecyl 2-(2,3-Bis((benzyloxy)carbonyl)-1-methylguanidino)acetate (**3h**, Supporting Information Figure 6).* Chemical formula: $C_{38}H_{57}N_3O_6$. MW: 651 g·mol$^{-1}$. Appearance: yellow oil. $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$: 0.88 (t, $J$ = 8.0 Hz, 3H), 1.26 (m, 30H), 1.629 (t, $J$ = 8.0 Hz, 2H), 3.13 (s, 3H), 4.14 (t, $J$ = 8.0 Hz, 2H), 4.19 (s, 2H), 5.15 (s, 2H), 5.20 (s, 2H), 7.33 (m, 10H). MS (ES+): $m/z$ 652.0. $R_f$: 0.17 (5:5, heptane/ethyl acetate, revealed by UV 254 nm). Purification: Combiflash on silica; solvent, heptane/ethyl acetate; gradient, 95/05 → 80/20.

*General Procedure for Synthesis of Creatine Fatty Esters.* First, 10 mg·mL$^{-1}$ pure $(CBz)_2$ creatine fatty ester was dissolved in anhydrous dichloromethane/methanol solution (1/2) or acetonitrile/methanol (1/1) under nitrogen. Then 5% $Pd/Al_2O_3$ was added and the reaction mixture was degassed under vacuum, frozen, and purged three times with hydrogen. The medium was allowed to reach room temperature and react with vigorous stirring.

The reaction was monitored by TLC (silica, heptane/ethyl acetate) and LC-MS. When the reaction was complete, generally after 3 h, the creatine fatty ester solution was filtered (0.5 $\mu$m filter).

*Butyl 2-(1-Methylguanidino)acetate (**4c**, Supporting Information Figure 8).* Chemical formula: $C_8H_{17}N_3O_2$. MW: 187 g·mol$^{-1}$. Appearance: colorless oil. $^1$H NMR ($D_2O$, 400 MHz) $\delta$: 0.9 (t, $J$ = 7 Hz, 3H), 1.37 (m, $J$ = 7 Hz, 2H), 1.65 (m, $J$ = 7 Hz, 2H), 3.07 (s, 3H), 4.24 (m, 4H). MS (ES+): $m/z$: 188.4.

*Octyl 2-(1-Methylguanidino) Acetate (**4d**, Supporting Information Figure 9).* Chemical formula: $C_{12}H_{25}N_3O_2$. MW: 244 g·mol$^{-1}$. Appearance: thick white solid. $^1$H NMR (MeOD, 400 MHz) $\delta$: 0.90 (t, $J$ = 5.2 Hz, 3H), 1.31 (m, 12H), 3.04 (s, 3H), 3.30 (t, $J$ = 8.0 Hz, 2H), 3.93 (s, 2H). $^{13}$C NMR (MeOD, 100 MHz) $\delta$: 12.9, 22.2, 25.5, 29.4, 29.4, 29.5, 31.4, 36.5, 55.8, 66.8, 169.9, 186.1. MS (ES+): $m/z$: 244.1. FTIR $\nu$ max: 3455, 2953, 2849, 2085, 1636, 1493, 1396, 1363, 1249, 1110, 1064, 1028, 898, 823.

*Nonyl 2-(1-Methylguanidino) Acetate (**4e**, Supporting Information Figure 10).* Chemical formula: $C_{13}H_{27}N_3O_2$. MW: 258 g·mol$^{-1}$. Appearance: thick white solid. $^1$H NMR (MeOD, 400 MHz) $\delta$: 1.09 (t, 3H), 1.49 (m, 14H), 1.86 (m, 2 H), 3.25 (s, 3H), 4.38 (t, 2H), 4.42 (s, 2H). MS (ES+): $m/z$: 258.80.

*Dodecyl 2-(1-Methylguanidino)acetate (**4f**, Supporting Information Figure 11).* Chemical formula: $C_{16}H_{32}N_3O_2$. MW: 300 g·mol$^{-1}$. Appearance: thick white solid. $^1$H NMR (MeOD, 400 MHz) $\delta$: 0.90 (t, $J$ = 6.8 Hz, 3$H$), 1.29 (m, 16H), 1.67 (m, 4H), 3.06 (s, 3H), 4.18 (t, $J$ = 6.8 Hz, 2H), 4.22 (s, 2H). $^{13}$C NMR (MeOD, 100 MHz) $\delta$: 12.9, 22.2, 25.4, 28.1, 28.8, 28.9, 29.1, 29.1, 29.2, 29.2, 29.5, 31.5, 32.1, 36.3, 51.0, 61.5, 65.5, 77.4, 77.7, 78.1, 167.8. MS (ES+): $m/z$ 300.8. FTIR (KBr) $\nu$ max: 2921, 2852, 1743, 1669, 1592, 1498, 1466, 1418, 1328, 1220, 1116, 681, 608 cm$^{-1}$. mp: 101.4 °C.

*Octadecyl 2-(1-Methylguanidino)acetate (**4h**, Supporting Information Figure 12).* Chemical formula: $C_{22}H_{45}N_3O_2$. MW: 383 g·mol$^{-1}$. Appearance: white solid. $^1$H NMR (MeOD, 400 MHz) $\delta$: 0.86 (t, $J$ = 8 Hz, 3H), 1.26 (m, 30H), (t, $J$ = 7.2 Hz, 2H), 3.03 (s, 3H), 4.12 (t, $J$ = 8 Hz, 2H), 4.31 (s, 2H). $^{13}$C NMR (MeOD, 100 MHz) $\delta$: 14.0, 22.6, 25.7, 29.1, 29.3, 29.4, 29.4, 29.5, 29.6, 29.6, 31.8, 32.7, 63.0, 114.9, 127.9, 128.2. MS (ES+): $m/z$ 384.4. FTIR (KBr) $\nu$ max: 3415, 2958, 2918, 2850, 2397, 2303, 1742, 1588, 1468, 1263, 1209, 1163, 1071, 967 cm$^{-1}$. mp: 55.5 °C

*General Procedure for Synthesis of Creatine Ethyl Ester.* Pure thionyl chloride (2.5 mL) was added through a condenser under nitrogen to a solution of creatine (1.23g −9.38 mmol) in dried ethanol (80 mL) according to US 2005/0049428.The solution was refluxed for 1 h and then allowed to cool to room temperature. Creatine ethyl ester hydrochloride was recovered by crystallization in a 50% yield.

*Ethyl 2-(1-Methylguanidino)acetate HCl (**4b**, Supporting Information Figure 7).* Chemical formula: $C_6H_{13}N_3O_2$. MW: 159 g·mol$^{-1}$. Appearance: crystal white needles. $^1$H NMR ($D_2O$, 400 MHz) $\delta$: 1.26 (t, $J$ = 7 Hz, 3H), 3.05 (s, 3H), 4.25 (q, $J$ = 7 Hz, 2H), 4.24 (s, 2H). MS (ES+): $m/z$: 160.

**Pharmacology.** *In Vitro Primary Cell-Based Rat Blood–Brain Barrier Model.* We first tested the capacity of the creatine fatty esters to cross the in vitro BBB model, which consisted of a coculture of rat primary endothelial and astroglial cells. Primary rat astroglial cells were seeded at a density of $2 \times 10^4$ cells/well in 1500 $\mu$L on a 12-well plate. The astroglial culture medium was a mixture of $\alpha$-MEM/F-12 (Life Technologies) supplemented with 5% FBS (Lonza), 1% human serum (Sigma Aldrich), 1% penicillin/streptomycin/neomycin (Life Technologies), and 0.4% FGF (Millipore). Then 24 h later, Transwell inserts (Costar; pore size, 0.4 $\mu$m; diameter, 12 mm; surface, 1.12 cm$^2$) were placed inside the wells and primary rat endothelial cells were plated out on the upper layer at a density of $8 \times 10^4$ cells/insert in 500 $\mu$L of EBM-2 basal medium (Lonza) supplemented with the EGM-2MV kit (Lonza). The chambers containing endothelial cells and astroglial cells were considered as the apical and basolateral compartments, respectively. The plates were incubated at 37 °C in an atmosphere containing 5% $CO_2$, and the BBB model formed confluent monolayers within 12 days.[21,23]

After 12 days, the integrity of this BBB model was assessed (see below), and the creatine fatty esters were screened. The apical and basolateral media were replaced by specific transport buffer (150 mM NaCl, 5.2 mM KCl, 2.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 6 mM $NaHCO_3$, 2.8 mM glucose, and 5 mM Hepes) in which the creatine fatty esters were dissolved at 1 and 10 $\mu$g·mL$^{-1}$. After 60 min of incubation, supernatants were diluted 5-fold in acetonitrile/5% formic acid and cells were scrapped in a mixture 20% water/76% acetonitrile/4% formic acid. An HPLC-MS/MS was then performed to detect the creatine fatty esters in each compartment and in endothelial and astroglial cell lysates. The creatine fatty esters which were concerned in this experiment were **4b**, **4c**, **4d**, **4e**, **4f**, and **4h**.

The integrity of the cell-based BBB models was demonstrated by measuring the flux of [$^{14}$C]-sucrose, [$^3$H]-vinblastine, and [$^3$H]-propranolol (Perkin-Elmer) through the monolayer. Transwells with rat endothelial cells monolayers were transferred to new 12-well plates. A specific transport buffer was added: 500 $\mu$L to the apical compartment and 1500 $\mu$L to the basolateral compartment. After 60 min of incubation at 37 °C of 0.1 $\mu$Ci/mL of [$^{14}$C]-labeled sucrose, 1 $\mu$Ci/mL of [$^3$H]-propranolol in the apical compartment, and 0.1 $\mu$Ci/mL of [$^3$H]-vinblastine in the apical and basolateral compartment,


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supernatants from both apical and basolateral compartments were collected. The amount of tracer that passed through the endothelial monolayer was determined by scintillation counting and the permeability $P_{app}$ of each compound was assessed using the formula (A):

$$P_{app}X_{A\to B} = \frac{[X_{basolateral}] \times V_B}{T \times S \times [X_0]} \quad (A)$$

$X$ is the compound for which the permeability is assessed, $[X_{basolateral}]$ the concentration of the compound $X$ in the basolateral compartment at the end of the incubation, $V_B$ the total volume of the basolateral compartment (1.5 mL), $T$ the time of the incubation, $S$ the transwell surface area, and $[X_0]$ the concentration of compound $X$ at $T_0$.

Validated BBB models have sucrose permeability below $8 \times 10^{-6}$ cm·s$^{-1}$, propranolol permeability above $16 \times 10^{-6}$ cm·s$^{-1}$, and vinblastine permeability ratio above 2.

The absence of toxicity of the creatine fatty esters on the endothelial cells viability was estimated by an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma Aldrich) cell viability test on endothelial monolayers. The endothelial culture medium was replaced by a solution of 10 $\mu$g·mL$^{-1}$ of creatine fatty ester in the transport buffer. The cells were incubated for 75 min at 37 °C in an atmosphere containing 5% $CO_2$. The solution was then removed, and 1 mg·mL$^{-1}$ MTT in PBS was added for an overnight incubation. MTT was reduced by metabolically active cells to insoluble purple formazan dye crystals. DMSO (Sigma Aldrich) was then added to the wells, solubilizing the crystals so the absorbance could be read using a spectrophotometer at wavelengths of 550 and 650 nm. Results are expressed as $[OD_{(550\ nm)} - OD_{(650\ nm)}]$, percentage of control.

The Lucifer Yellow (LY, Sigma Aldrich) permeability test was used to study the effect of creatine fatty esters on BBB integrity. LY was diluted in transport buffer to a final concentration of 100 $\mu$M and added to the apical compartment during creatine ester incubation. Fluorescence leakage was determined for LY with 485 nm excitation and 530 nm emission using a fluorescence plate reader. The LY permeability (LY $P_{app}$) was then assessed using formula (A). An LY $P_{app}$ value below $5.10^{-6}$ cm·s$^{-1}$ indicates that the creatine ester did not damage BBB integrity.

*In Vitro Rat Primary Neuronal Model.* Primary cortical neurons were prepared from fetal (E18) rat cortices according to the 86/609 European directive. The pregnant female was anesthetized with isoflurane, and the embryos were extracted by laparotomy and placed in a Petri dish containing HBSS supplemented with 5 mM glucose and 10 mM Hepes, pH 7.4. The brain was removed from the skull, and the cortices were dissected. Two enzymatic dissociations were performed with 0.25% trypsine−EDTA (Life Technologies) without and then with DNase (Sigma Aldrich). Cortical cells were then seeded at a density of $10^5$ cells/cm$^2$ onto poly-D-lysine (Sigma)-coated 24-well plates in a medium consisting of DMEM (Life Technologies) supplemented with 10% horse serum (Life Technologies) first. Then 2 h later, the medium was replaced by Neurobasal containing B27 1X (Life Technologies), 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Sigma Aldrich), and 10 U/mL penicillin/streptomycin/neomycin (Life Technologies). At culture day 4, 3 $\mu$M of cytosine-$\beta$-D-arabinose was added. Neuronal cells express typical markers such as MAP2 and neurofilament (data not shown).

The tests with creatine esters were performed at 10 DIV in HBSS at 1 and 10 $\mu$g·mL$^{-1}$. After 1 h at 37 °C and under 5% $CO_2$, the supernatant was diluted 5-fold in acetonitrile/5% formic acid and cells were scrapped in 20% water/76% acetonitrile/4% formic acid. An HPLC-MS/MS was then performed to detect the creatine fatty esters in the cell lysates.

A lactate deshydrogenase (LDH) test was performed to assess the absence of neuronal death. First, 70 $\mu$L of LDH lysis solution was added to each well after the 60 min experiment and was incubated at 37 °C under 5% $CO_2$ for 45 min. Then 50 $\mu$L was removed for testing and 100 $\mu$L of LDH assay mixture was added for 30 min at room temperature. Then 10 $\mu$L of 1N HCl was finally added to the wells and the absorbance was read using a spectrophotometer at wavelengths of 490 and 690 nm. Results are expressed as $[DO_{(490\ nm)} - DO_{(690\ nm)}]$, ratio to the control. A ratio equal to or less than 1 indicates an absence of neuronal cell toxicity.

*Human Primary Fibroblasts Cell Culture.* Human fibroblasts were obtained from skin biopsies through a gift of the Centre de Référence des Maladies Héréditaires du Métabolisme at the Necker Hospital in Paris. Three patients with cerebral creatine deficiency caused by lack of the creatine transporter functionality and 1 control were studied. All of the mutations were previously described in Valayannopoulos et al., 2013:[24] p.Asn336del c.1006_1008delAAC (patient 1, DTp1) and p.(Gly499del) c.1497_1500delGAG (patient 2, VLp2) as P3 and P4, respectively, or in Valayannopoulos et al., 2012:[25] p.(G414del) c.1221_1223delTTC (patient 3, CTp3) as P2. The fibroblasts were plated out at $3 \times 10^3$ cells per well in 6-well plates in a DMEM medium (Life Technologies) supplemented with 10% fetal bovine serum (Lonza), 1% penicillin/streptomycin/neomycin (Life Technologies), 1% sodium pyruvate (Sigma Aldrich), and 1% L-glutamine (Life Technologies). They were cultured for 6 days by replacing the medium every 2−3 days. The incubation of creatine esters consisted in replacing the medium by HBSS (Life Technologies) in which the compound was diluted to 10 $\mu$g·mL$^{-1}$. After 1 h at 37 °C, 5% $CO_2$, the supernatant was diluted 5-fold in acetonitrile/5% formic acid and cells were scrapped in 20% water/76% acetonitrile/4% formic acid. An HPLC-MS/MS was then performed to detect the creatine fatty esters in the cell lysates.

*Esterase Activity Determination.* Esterase activity in fibroblast cells was determined by incubating 20 $\mu$g·mL$^{-1}$ of cell proteins with 350 $\mu$M of 4-nitrophenyl acetate (Sigma Aldrich) in deionized water. Conversion of the 4-nitrophenyl acetate by esterases leads to *p*-nitrophenol, which can be monitored by absorbance spectroscopy at 405 nm.

*HPLC-MS/MS Analysis.* Liquid chromatography (Shimadzu HPLC system LC 20AD) with a 2.0 mm × 150 mm Uptisphere Diol HPLC column (UP6OH, Interchim) was used for elution of creatine esters. The mobile phase was isocratic at 40/60 (detection of creatine, ethyl and butyl creatine esters) or 20/80 (detection of octyl, nonyl, dodecyl, and octadecyl creatine esters) A/B, where solvent A was $H_2O$ containing 0.1% formic acid and solvent B was acetonitrile containing 0.1% formic acid; the flow rate was 0.4 mL/min. Analyte (10 $\mu$L) was injected onto the column placed in an oven at 40 °C. The total run time was 6 min.

Detection was done by tandem mass spectrometry (Finnigan TSQ Quantum Discovery with Xcalibur and LC Quan softwares, Thermo) in positive electrospray mode. Spray voltage was 3.0 kV, and sheath and auxiliary gas pressures were 50 and 20 (arbitrary units), respectively. The in-source CID energy was fixed at 12 V, and capillary temperature was 350 °C. Tube lens and collision energy values were optimized for each compound and are summarized in Table 5. Multiple reaction monitoring was used for the detection of the ion transitions (Table 5). The standard curves showed linearity for creatine over a range of 0.05−10 $\mu$g·mL$^{-1}$ and of 0.01−5 $\mu$g·mL$^{-1}$ for ethyl, butyl, octyl, nonyl, dodecyl, and octadecyl creatine esters. Creatine fatty esters and creatine concentrations and amounts were determined in each compartment and in endothelial cells, astroglial cells, and fibroblasts lysates. The amount of creatine fatty esters and the amount of creatine were standardized to the amount of protein in each lysate.

Each experiment was performed in triplicate.

**Statistical Analysis.** Statistical analysis was performed using the Prism 5.01 program (GraphPad Software, Inc., San Diego SA). Statistical comparisons were made using a two-tailed unpaired *t* test and a variance analysis one-way ANOVA followed by a Bonferroni post-test. Changes were considered statistically significant at $p < 0.05$.

## ■ ASSOCIATED CONTENT

### Supporting Information

$^1$H NMR spectra of selected compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS USED

Boc, *tert*-butoxy carbonyle; CBz, Z = benzyl carbamate = benzyloxy carbonyl; Fmoc, fluorenylmethoxycarbonyl; FTIR, Fourier transform infrared spectroscopy; KBr, potassium bichromate; mp, melting point; NMR, nuclear magnetic resonance; $P_{app}$, permeability; TLC, Thin layer chromatography

## REFERENCES


(1) Stockler, S.; Schutz, P. W.; Salomons, G. S. Cerebral creatine deficiency syndromes: clinical aspects, treatment and pathophysiology. *Subcell. Biochem.* **2007**, *46*, 149−166.

(2) Schulze, A. Creatine deficiency syndromes. *Mol. Cell. Biochem.* **2003**, *244*, 143−150.

(3) Wyss, M.; Kaddurah-Daouk, R. Creatine and creatinine metabolism. *Physiol. Rev.* **2000**, *80*, 1107−1213.

(4) Stockler, S.; Hanefeld, F.; Frahm, J. Creatine replacement therapy in guanidinoacetate methyltransferase deficiency, a novel inborn error of metabolism. *Lancet* **1996**, *348*, 789−790.

(5) Poo-Arguelles, P.; Arias, A.; Vilaseca, M. A.; Ribes, A.; Artuch, R.; Sans-Fito, A.; Moreno, A.; Jakobs, C.; Salomons, G. X-Linked creatine transporter deficiency in two patients with severe mental retardation and autism. *J. Inherit. Metab. Dis.* **2006**, *29*, 220−223.

(6) van de Kamp, J. M.; Pouwels, P. J.; Aarsen, F. K.; ten Hoopen, L. W.; Knol, D. L.; de Klerk, J. B.; de Coo, I. F.; Huijmans, J. G.; Jakobs, C.; van der Knaap, M. S.; Salomons, G. S.; Mancini, G. M. Long-term follow-up and treatment in nine boys with X-linked creatine transporter defect. *J. Inherit. Metab. Dis.* **2012**, *35*, 141−149.

(7) Cecil, K. M.; Salomons, G. S.; Ball, W. S., Jr.; Wong, B.; Chuck, G.; Verhoeven, N. M.; Jakobs, C.; DeGrauw, T. J. Irreversible brain creatine deficiency with elevated serum and urine creatine: a creatine transporter defect? *Ann. Neurol.* **2001**, *49*, 401−404.

(8) Ohtsuki, S.; Tachikawa, M.; Takanaga, H.; Shimizu, H.; Watanabe, M.; Hosoya, K.; Terasaki, T. The blood−brain barrier creatine transporter is a major pathway for supplying creatine to the brain. *J. Cereb. Blood Flow. Metab.* **2002**, *22*, 1327−1335.

(9) deGrauw, T. J.; Cecil, K. M.; Byars, A. W.; Salomons, G. S.; Ball, W. S.; Jakobs, C. The clinical syndrome of creatine transporter deficiency. *Mol. Cell. Biochem.* **2003**, *244*, 45−48.

(10) Perasso, L.; Lunardi, G. L.; Risso, F.; Pohvozcheva, A. V.; Leko, M. V.; Gandolfo, C.; Florio, T.; Cupello, A.; Burov, S. V.; Balestrino, M. Protective effects of some creatine derivatives in brain tissue anoxia. *Neurochem. Res.* **2008**, *33*, 765−775.

(11) Perasso, L.; Adriano, E.; Ruggeri, P.; Burov, S. V.; Gandolfo, C.; Balestrino, M. In vivo neuroprotection by a creatine-derived compound: phosphocreatine-Mg-complex acetate. *Brain Res.* **2009**, *1285*, 158−163.

(12) Burov, S.; Leko, M.; Dorosh, M.; Dobrodumov, A.; Veselkina, O. Creatinyl amino acids: new hybrid compounds with neuroprotective activity. *J. Pept. Sci.* **2011**, *17*, 620−626.

(13) Skelton, M. R.; Schaefer, T. L.; Graham, D. L.; Degrauw, T. J.; Clark, J. F.; Williams, M. T.; Vorhees, C. V. Creatine transporter (CrT; Slc6a8) knockout mice as a model of human CrT deficiency. *PLoS One* **2011**, *6*, e16187.

(14) Kurosawa, Y.; Degrauw, T. J.; Lindquist, D. M.; Blanco, V. M.; Pyne-Geithman, G. J.; Daikoku, T.; Chambers, J. B.; Benoit, S. C.; Clark, J. F. Cyclocreatine treatment improves cognition in mice with creatine transporter deficiency. *J. Clin. Invest.* **2012**, *122*, 2837−2846.

(15) Lunardi, G.; Parodi, A.; Perasso, L.; Pohvozcheva, A. V.; Scarrone, S.; Adriano, E.; Florio, T.; Gandolfo, C.; Cupello, A.; Burov, S. V.; Balestrino, M. The creatine transporter mediates the uptake of creatine by brain tissue, but not the uptake of two creatine-derived compounds. *Neuroscience* **2006**, *142*, 991−997.

(16) Adriano, E.; Garbati, P.; Damonte, G.; Salis, A.; Armirotti, A.; Balestrino, M. Searching for a therapy of creatine transporter deficiency: some effects of creatine ethyl ester in brain slices in vitro. *Neuroscience* **2011**, *199*, 386−393.

(17) Fons, C.; Arias, A.; Sempere, A.; Poo, P.; Pineda, M.; Mas, A.; Lopez-Sala, A.; Garcia-Villoria, J.; Vilaseca, M. A.; Ozaez, L.; Lluch, M.; Artuch, R.; Campistol, J.; Ribes, A. Response to creatine analogs in fibroblasts and patients with creatine transporter deficiency. *Mol. Genet. Metab.* **2010**, *99*, 296−299.

(18) Katseres, N. S.; Reading, D. W.; Shayya, L.; DiCesare, J. C.; Purser, G. H. Non-enzymatic hydrolysis of creatine ethyl ester. *Biochem. Biophys. Res. Commun.* **2009**, *386*, 363−367.

(19) Katseres, N. S.; Reading, D. W.; Shayya, L.; DiCesare, J.; Purser, G. H. Hydrolysis of creatine ethyl ester. 237th National Meeting of the American Chemical Society, Salt Lake City, Utah, March 22−26, 2009, 350.

(20) Giese, M. W.; Lecher, C. S. Non-enzymatic cyclization of creatine ethyl ester to creatinine. *Biochem. Biophys. Res. Commun.* **2009**, *388*, 252−255.

(21) Lacombe, O.; Videau, O.; Chevillon, D.; Guyot, A. C.; Contreras, C.; Blondel, S.; Nicolas, L.; Ghettas, A.; Benech, H.; Thevenot, E.; Pruvost, A.; Bolze, S.; Krzaczkowski, L.; Prevost, C.; Mabondzo, A. In vitro primary human and animal cell-based blood−brain barrier models as a screening tool in drug discovery. *Mol. Pharmaceutics* **2011**, *8*, 651−663.

(22) Salomons, G. S.; van Dooren, S. J.; Verhoeven, N. M.; Cecil, K. M.; Ball, W. S.; Degrauw, T. J.; Jakobs, C. X-linked creatine-transporter gene (SLC6A8) defect: a new creatine-deficiency syndrome. *Am. J. Hum. Genet.* **2001**, *68*, 1497−500.

(23) Brun, E.; Carriere, M.; Mabondzo, A. In vitro evidence of dysregulation of blood−brain barrier function after acute and repeated/long-term exposure to TiO(2) nanoparticles. *Biomaterials* **2012**, *33*, 886−896.

(24) Valayannopoulos, V.; Bakouh, N.; Mazzuca, M.; Nonnenmacher, L.; Hubert, L.; Makaci, F. L.; Chabli, A.; Salomons, G. S.; Mellot-Draznieks, C.; Brule, E.; de Lonlay, P.; Toulhoat, H.; Munnich, A.; Planelles, G.; de Keyzer, Y. Functional and electrophysiological characterization of four non-truncating mutations responsible for creatine transporter (SLC6A8) deficiency syndrome. *J. Inherit. Metab. Dis.* **2012**, *36*, 103−112.

(25) Valayannopoulos, V.; Boddaert, N.; Chabli, A.; Barbier, V.; Desguerre, I.; Philippe, A.; Afenjar, A.; Mazzuca, M.; Cheillan, D.; Munnich, A.; de Keyzer, Y.; Jakobs, C.; Salomons, G. S.; de Lonlay, P. Treatment by oral creatine, L-arginine and L-glycine in six severely affected patients with creatine transporter defect. *J. Inherit. Metab. Dis.* **2012**, *35*, 151−157.