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Corresponding Author: Dr. Giovanna Rassu, PhD

Corresponding Author's Institution: University of Sassari

First Author: Alessandro Dalpiaz

Order of Authors: Alessandro Dalpiaz ; Marco Fogagnolo ; Luca Ferraro ; Antonio Capuzzo ; Barbara Pavan ; Giovanna Rassu, PhD; Andrea Salis; Paolo Giunchedi; Elisabetta Gavini

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Abstract: Zidovudine (AZT) is an antiretroviral drug substrate of active efflux transporters (AET) that extrude it from the central nervous system (CNS) and macrophages, considered sanctuaries of HIV. The conjugation of AZT with ursodeoxycholic acid is known to produce a prodrug (UDCA-AZT) able to elude the AET systems, evidencing the potential ability of this prodrug to act as a carrier of AZT in the CNS and macrophages. Here, we demonstrate that UDCA-AZT is able to permeate and remain in murine macrophages with an efficiency twenty times higher than AZT. Moreover, we propose the nasal administration of this prodrug in order to induce its CNS uptake. Chitosan chloride based microparticles (CP) were prepared by spray-drying and characterized for size, morphology, density, water uptake and dissolution profile of UDCA-AZT. The CP sample was then nasally administered to rats. All in-vitro and in-vivo measurements were achieved also for a CP parent physical mixture. The CP sample was able to increase the dissolution rate of UDCA-AZT and to reduce water uptake with respect to its parent physical mixture, inducing a better uptake of UDCA-AZT in the cerebrospinal fluid of rats, where the prodrug can act as an AZT carrier in the macrophages.

Suggested Reviewers: Jared Fine
Center for Memory and Aging, Health Partners Institute for Education and Research, Saint Paul
jared.m.fine@healthpartners.com
expert in nose to brain delivery of drug

Marta Roldo
School of Pharmacy and BMS, Portsmouth
marta.roldo@port.ac.uk
expert in drug delivery

Alessio Cardinale
1IRCCS San Raffaele Pisana, Rome, Italy
alessio.cardinale@sanraffaele.it
Expert in biological aspects of brain diseases

Sassari, July 7, 2015

Dear Editor,

please find as attached file the original manuscript entitled “Influence of chitosan chloride microparticles on the in vivo intranasal uptake of a zidovudine prodrug for brain targeting” by A. Dalpiaz, M. Fogagnolo, L. Ferraro, A. Capuzzo, B. Pavan, G. Rassu, A. Salis, P. Giunchedi, E. Gavini.

In order to study new strategies potentially able to enhance the brain targeting of zidovudine (AZT), this manuscript describes the design and formulation of chitosan based microparticles (CP) as a carrier system for the nasal administration of a prodrug obtained by conjugation with ursodeoxycholic acid. We characterize *in vitro* the CP sample and its parent physical mixture and we demonstrate that their nasal administration to rats allows to obtain the prodrug uptake in their cerebrospinal fluid. We also evidence for the first time that the uptake of UDCA-AZT in murine macrophages is definitely higher than that of AZT. We conclude that CP sample appears as an efficacious nasal formulation for the selective uptake in the central nervous system of zidovudine.

We would be grateful if this manuscript could be considered for the publication in *Antiviral Research.* The submission file has been done according to “Author Information Pack”. Nevertheless, as we did a lot of experimental work, and despite we tried to shorten, the length of the manuscript is 5921 words, excluded the references. We would you like to know if the manuscript is nonetheless worthy to be considered for the submission.

Finally, I confirm that all authors have read and approved this version of the article, and no part of this paper has been published nor is it submitted for publication elsewhere and will not be submitted elsewhere.

With best regards,

Giovanna Rassu, Ph.D.

Highlights

- We evidence for the first time that the uptake of prodrug UDCA-AZT in murine macrophages is higher than that of zidovudine.
- Intravenous administration of zidovudine (AZT) and UDCA-AZT is not efficient in brain targeting.
- Nasal administration of physical mixture/microparticles produces detectable amounts of UDCA−AZT in the cerebrospinal fluid.
- Chitosan nasal microspheres show the best performance in UDCA-AZT uptake in cerebrospinal fluid.
- This formulation can induce the AZT transport in macrophages located in the subarachnoid spaces of cerebrospinal fluid.

Nasal chitosan microparticles to target zidovudine prodrug to brain HIV sanctuaries

Alessandro Dalpiaz [a], Marco Fogagnolo [a], Luca Ferraro [b], Antonio Capuzzo [b], Barbara Pavan [b], Giovanna Rassu [c,*], Andrea Salis [c], Paolo Giunchedi [c], Elisabetta Gavini [c]

[a]*Department of Chemical and Pharmaceutical Sciences, University of Ferrara, via Fossato di Mortara 19, 44121 Ferrara, Italy*

[b]*Department of Life Sciences and Biotechnology, University of Ferrara, via Borsari 46, 44121 Ferrara, Italy*

[c]*Department of Chemistry and Pharmacy, University of Sassari, via Muroni 23/a, 07100 Sassari, Italy*

E-mail addresses: dla@unife.it (A. Dalpiaz), marco.fogagnolo@unife.it (M. Fogagnolo), frl@unife.it (L. Ferraro), antonio.capuzzo@unife.it (A. Capuzzo), pvnbbr@unife.it (B. Pavan), grassu@uniss.it (G. Rassu), asalis@uniss.it (A. Salis), pgiunc@uniss.it (P. Giunchedi), eligav@uniss.it (E. Gavini).

* Corresponding author: Giovanna Rassu, Department of Chemistry and Pharmacy, University of Sassari, via Muroni 23/a, 07100 Sassari, Italy. Tel: +39 079228735; Fax: +39 079228733; E-mail address: grassu@uniss.it

ABSTRACT

Zidovudine (AZT) is an antiretroviral drug substrate of active efflux transporters (AET) that extrude it from the central nervous system (CNS) and macrophages, considered sanctuaries of HIV. The conjugation of AZT with ursodeoxycholic acid is known to produce a prodrug (UDCA-AZT) able to elude the AET systems, evidencing the potential ability of this prodrug to act as a carrier of AZT in the CNS and macrophages. Here, we demonstrate that UDCA-AZT is able to permeate and remain in murine macrophages with an efficiency twenty times higher than AZT. Moreover, we propose the nasal administration of this prodrug in order to induce its CNS uptake. Chitosan chloride based microparticles (CP) were prepared by spray-drying and characterized for size, morphology, density, water uptake and dissolution profile of UDCA-AZT. The CP sample was then nasally administered to rats. All *in-vitro* and *in-vivo* measurements were achieved also for a CP parent physical mixture. The CP sample was able to increase the dissolution rate of UDCA-AZT and to reduce water uptake with respect to its parent physical mixture, inducing a better uptake of UDCA-AZT in the cerebrospinal fluid of rats, where the prodrug can act as an AZT carrier in the macrophages.

Keywords: Zidovudine prodrug, HIV treatment, macrophages, brain targeting, nasal formulation, chitosan microparticles

Chemical compounds studied in this article:

Zidovudine (PubChem CID: 35370)

1. Introduction

The combination antiretroviral therapy (cART) has hugely impacted the management of AIDS, but its efficacy is limited by the poor bioavailability of anti-HIV drugs at viral reservoir sites, such as the central nervous system (CNS) (Cunningham et al., 2000; Kolson and Gonzalez-Scarano, 2000), in particular subarachnoid spaces of cerebrospinal fluid (CSF) containing macrophages, that constitute the only site of HIV replication in the brain (Ghersi-Egea et al., 1996; Cunningham et al., 1997). The lack of drug penetration in these "HIV sanctuaries" is mainly due to the expression on the blood−brain (BBB) and blood−cerebrospinal fluid (BCSFB) barriers of active efflux transporter (AET) (Namanja et al., 2012; Pavan and Dalpiaz, 2011) that induce, *in-vivo*, an asymmetric transport of anti-HIV drugs across these barriers, where the rate of drug efflux from CNS to blood is greater than its influx rate (Wang and Sawchuk, 1995).

We recently proved that the conjugation of zidovudine (AZT, an antiretroviral drug used in cART protocols - De Clercq, 2009) with the bile acid ursodeoxycholic acid (UDCA) allows to obtain a prodrug (UDCA−AZT) able to elude the AET systems (Dalpiaz et al., 2012). We have therefore proposed that this prodrug, when taken up in the CNS, should not be extruded in the bloodstream, being able to elude the AET systems. Accordingly, we have demonstrated that the nasal administration of solid lipid microparticles loaded with the UDCA-AZT prodrug allows to obtain its uptake in the cerebrospinal fluid (CSF) of rats (Dalpiaz et al., 2014). It is currently known that nasal administration constitutes a potentially efficacious way in order to obtain the brain uptake of neuroactive agents (Illum, 2000; Vyas et al., 2005; Fine et al, 2014, 2015). Indeed, the drugs deposited on the olfactory epithelium of the nose can have a direct access to the CNS, in particular the CSF via a transcellular transport through olfactory epithelia cells. The drugs absorbed in the CSF can then diffuse into the interstitial fluid (ISF), from where they can penetrate the brain parenchyma (Illum, 2000, 2004; Thorne and Frey, 2001). Moreover, the drugs deposited on the olfactory epithelium can be transported into the brain parenchyma by olfactory neurons or

trigeminal nerves that reach the nasal cavity (Finger et al., 1990; Illum, 2000; Johnson et al., 2010). Finally, the nasally administered drugs can be absorbed in the systemic circulation from the respiratory epithelium (Cho et al, 2014), then they can reach the CNS if they are able to cross the BBB (Illum, 2000).

In general, appropriate strategies are required in order to improve the brain delivery of drugs, such as the addition of penetration enhancers and mucoadhesive materials to formulations, or the preparation of micro and nanoparticulate delivery systems (Casettari and Illum, 2014; Dalpiaz et al., 2008; Horvát et al., 2009; Mistry et al., 2009; Rassu et al., 2015a). In this regards, chitosan is a polysaccharide derived from alkaline deacetylation of chitin, used in different formulations for nose to brain delivery of drugs (Casettari and Illum, 2014) due to its biocompatibility, nontoxicity and high charge density conferring it mucoadhesive properties (Bernkop-Schnürch and Dünnhaupt, 2012; Sinha et al, 2004). Chitosan is poorly soluble in water at physiologic pH values, but it forms salts with inorganic or organic acids, such as hydrochloride and glutamic acid, that are soluble in water and can have better characteristics than chitosan itself, such as mucoadhesiveness and penetration enhancement ability of neuroactive agents in the CNS (Dalpiaz et al., 2008; Gavini et al., 2011; Maestrelli et al., 2004). Chitosan is also characterized by the ability of reversibly open tight junctions, with potential increase of transcellular transport of drugs across the olfactory mucosa (Durhia et al, 2010). Nasal formulation obtained in the presence of water show drawbacks, such as risk of chemical and physical instability and microbiological growth. Also, the residence time of the liquid formulation in the nasal cavity is short as the liquid is often rinsed into the gastrointestinal tract (GIT) or out (Kublik and Vidgren, 1998). These disadvantages can be overcome by using powder-based formulations (Marttin et al., 1997; Rassu et al., 2015b).

The purpose of the present work was firstly to demonstrate that UDCA-AZT can permeate and remain in macrophages more efficiently than its parent drug AZT, then the preparation of microspheres based on chitosan chloride in order to increase the nose-to-brain delivery of UDCA-AZT by using a particulate formulation suitable for nasal administration. Characterization of

microparticles was performed *in-vitro* and *in-vivo*. All *in-vitro* and *in-vivo* measurements performed for the loaded microparticles were achieved also for a parent physical mixture of chitosan chloride and UDCA-AZT, in order to verify the efficacy of the microparticulate system on the brain delivery of the prodrug.

2. Materials and methods

2.1. Materials

The prodrug UDCA−AZT was synthesized as previously described (Dalpiaz et al., 2012). Chitosan chloride (Protasan UP CL 113, molecular weight and degree of deacetylation of 160000 g/mol and 83%, respectively) was purchased from FMC BioPolymer AS (Drammen, Norway). Dow Corning 345, blend of polydimethylcyclosiloxane, was obtained from Dow Corning (Brussels, Belgium). AZT, 7-n-propylxanthine (7n-PX), bovine serum albumin (BSA) were obtained from Sigma Aldrich Italy (Milan, Italy). Methanol, acetonitrile, ethyl acetate and water were high performance liquid chromatography (HPLC) grade from Sigma Aldrich Italy. Monopowder P® insufflators were furnished by Valois Dispray (Mezzovico, Switzerland). Male Wistar rats were purchased from Harlan SRC (Milan, Italy). All other reagents and solvents were of analytical grade (Sigma-Aldrich).

2.2. Uptake of AZT and UDCA-AZT in macrophages

The murine macrophage cell line J774A.1 was obtained from the American tissue Type Culture Collection (LGC Standards, Milan, Italy). J774A.1 macrophages were grown as adherently cultured cells in Dulbecco's modified Eagle's medium + Glutamax supplemented with 10% fetal bovine

serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C. All cell culture reagents were provided by Invitrogen (Life Technologies, Milan, Italy). Method is reported in detail in Supplementary material. Briefly, J774A.1 cells were seeded in 12-well culture plates in a total of 5×10^5 cells and, when semi-confluent, exposed to the solutions of 100 μM AZT and 100 μM UDCA-AZT in growth medium for 30 min. At the end of the treatment, cells were washed and lysed. Cell lysates were dried under nitrogen stream, resuspended in methanol and centrifuged to remove cell debris. The supernatant (10 μl) was used to measure the levels of the test substrates by HPLC analysis. All the values obtained by experiments with J774A.1 cells are the mean of three independent experiments.

2.3. Preparation of loaded and unloaded chitosan microspheres

Chitosan microspheres containing UDCA−AZT (named CP) were prepared by spray-drying method. Chitosan chloride (400 mg) was dissolved in water (15 mL), whereas UDCA−AZT (100 mg) was dissolved in methanol (35 ml). Drug solution was dispersed into chitosan one (solid concentration: 1% w/v of UDCA−AZT and chitosan). Due to the very low solubility of drug, a fine suspension was obtained and spray-dried using a mini spray-dryer equipped with a high performance cyclone (Büchi B-191, Büchi Labortechnik AG, Switzerland) and with a 0.7 mm two-fluid nozzle. The following standard operating conditions were utilized: inlet and outlet temperature, 100 °C and 73 °C; spray flow rate, 500 L/h; pump setting, 8% (2.00 mL/min); aspirator setting, 90%. Aqueous solutions of chitosan chloride (0.8 % w/v) were sprayed in the same conditions to obtain drug empty microspheres (CH). Microparticles were stored in a desiccator at room temperature.

2.4. HPLC analysis

The quantification of the prodrug UDCA−AZT and its hydrolysis product, AZT was performed by HPLC. The chromatographic apparatus consisted of a modular system (model LC-10 AD VD pump and model SPD-10A VP variable wavelength UV−vis detector; Shimadzu, Kyoto, Japan) and an injection valve with 20 μL sample loop (model 7725; Rheodyne, IDEX, Torrance, CA, USA). Separations were performed at room temperature on a 5 μm Hypersil BDS C-18 column (150 mm × 4.6 mm i.d; Alltech Italia Srl, Milan, Italy), equipped with a guard column. Data acquisition and processing were accomplished with a personal computer using CLASS-VP Software, version 7.2.1 (Shimadzu Italia, Milan, Italy). The detector was set at 260 nm. The mobile phase consisted of a mixture of water and methanol regulated by a gradient profile programmed as follows: isocratic elution with 20% (v/v) MeOH in H_2O for 10 min; then a 1-min linear gradient to 75% (v/v) MeOH in H_2O; the mobile phase composition was finally maintained at 75% MeOH for 10 min. After each cycle the column was conditioned with 20% (v/v) MeOH in H_2O for 10 min. The flow rate was 1 mL/min. The xanthine derivative 7n-PX was employed as internal standard for the analysis of rat blood (see below). The retention times for 7n-PX, AZT and the prodrug UDCA−AZT were 6.5, 8.4, and 19.6 min, respectively. The HPLC assay of UDCA-AZT alone was performed isocratically with 80% (v/v) MeOH in H_2O. In this case the retention time of UDCA-AZT was 4.8 min. The chromatographic precision was evaluated (see Supplementary material).

2.5. Characterization of microspheres

Microspheres prepared were characterized in terms of yield of production, drug content and encapsulation efficiency, particle size and particle size distribution, particle morphology, true density and water uptake capacity.

2.5.1. The yields of production

The yields of production (YP) were calculated as a percent weight of microspheres obtained

with respect to the initial amounts of UDCA−AZT and chitosan employed for preparation of feed suspension.

2.5.2. UDCA−AZT content in CP

The UDCA-AZT content in the microparticulate powders was determined. The microparticles (about 0.95 mg) were accurately weighed and dissolved in 3 ml of water to whom 300 µl of 0.2% H_3PO_4 was added; the final volume of the solution was adjusted to 10 mL with methanol. Then, 10 µL of filtered solutions (0.45 µm) were injected into the HPLC system for UDCA-AZT assay. The drug loading and entrapment efficiency were calculated according to equations 1 and 2 (Rassu et al., 2014; see Supplementary material).

All the values obtained are the mean of four independent experiments.

2.5.3. Particle size measurement

Size and size distribution of microspheres were analyzed by the light diffraction method using a Coulter LS 100Q (Beckman Coulter Particle Characterization, Miami, FL). A sample (2 mg) of unloaded and drug-loaded microspheres was suspended in silicon oil, sonicated for about 3 s and analyzed. The results reported are the averages of the triplicate averages. As comparison, test was also performed on the physical mixture containing UDCA−AZT and chitosan chloride (87:13 w/w) (MIX) prepared by geometrical dilution with the use of agate and mortar pestle, as well as on chitosan chloride and UDCA-AZT alone, before and after grinding.

2.5.4. Particle morphology

Shape and surface characteristics of powders were studied by scanning electron microscopy (VP-SEM; Zeiss EVO40XVP, Arese, Milan, Italy). The samples were placed on double-sided tape that had previously been secured on aluminum stubs and then analyzed at 18 kV acceleration voltage after gold sputtering, under argon atmosphere.

2.5.5. True Density

True density of microspheres was measured by helium pycnometry (Micromeritics Accupyc II 1340 Analysis system, Peschiera Borromeo, Italy) at 21 °C (Gavini et al., 2012). The density (ρ) of the powder was determined in triplicate for each batch.

2.5.6. Determination of water uptake capacity

The investigation of water uptake capability of CP and CH was carried out with a modified Enslin apparatus as already described by Rassu et al., 2009. Briefly, the dried microspheres were spread uniformly on the paper filter, previously soaked with phosphate buffer pH 6.5. Volume of fluid absorbed from microspheres during the time (0 to 60 min) was recorded. The values (mean of at least 3 experiments) were expressed as µl of fluid absorbed per gram of microspheres during the time. As comparison, the test was also performed using MIX.

2.6. In-vitro release and dissolution studies

The release tests were performed by using the flow-through dissolution method (European Pharmacopoeia, Apparatus 4), using a modified glass cell useful for organic solvent (described in Supplementary material). CP formulation (20 mg) was placed into the glass cell. The dissolution medium, thermostated at 37±0.1 °C was introduced through the bottom of the cell to obtain a suitable continuous flow. Methanol-water blend (70-30 v/v) was used as dissolution medium. The test solution (10 µL) was analyzed by HPLC and the amount of UDCA−AZT released from the microspheres was calculated as a percentage of the initial amount of UDCA−AZT incorporated in the microspheres prior to the dissolution test. As comparison, the test was also performed using MIX. All experiments were performed in at least three replicates.

2.7. In-vivo UDCA-AZT administration and quantification

Male Wistar rats (200–250 g) anaesthetized during the experimental period, received a femoral intravenous infusion of 0.1 mg/mL UDCA-AZT dissolved in a medium constituted by 20% (v/v) DMSO and 80% (v/v) physiologic solution, with a rate of 0.2 mL/min for 10 min. At the end of infusion and at fixed time points, blood samples (100 μL) were collected and CSF samples (50 μL) were withdrawn by cysternal puncture method described by van den Berg et al. (2002) requiring a single needle stick and allows the collection of serial (40–50 μL) CSF samples which are virtually blood-free (Dalpiaz et al., 2014). A total volume of about 150 μL CSF was collected during the experimental session. Four rats were employed for femoral intravenous infusions. CSF samples (10 μL) were immediately injected into HPLC system for UDCA-AZT detection. The blood samples were hemolysed immediately after their collection with 500 μL of ice cold water, then 50 μL of 10% sulfosalicylic acid and 100 μL of internal standard (30 μM 7n-PX) were added. The samples were extracted twice with 1 mL of water saturated ethyl acetate, and, after centrifugation, the organic layer was reduced to dryness under a nitrogen stream. Two hundred microliters of a water-methanol mixture (70:30 v/v) were added, and, after centrifugation, 10 μL was injected into the HPLC system.

The precision of the analytical method was determined (see Supplementary material). The *in-vivo* half-life of AZT in the blood was calculated by nonlinear regression (exponential decay) of concentration values in the time range within 3 hours after infusion and confirmed by linear regression of the log concentration values *versus* time.

Nasal administration of UDCA-AZT was performed on anaesthetized rats laid on their backs, following two main procedures. The first one consisted of the introduction of 50 μL of an aqueous suspension of UDCA-AZT (2 mg/mL) in each nostril of rats as previously described (Dalpiaz et al., 2014). After the administration, blood (100 μL) and CSF samples (50 μL) were collected at fixed

time points, and they were analyzed with the same procedures described above. Four rats were employed for nasal administration of UDCA-AZT suspension.

The second procedure was based on the insufflations of CP microparticles to each nostril of anaesthetized rats by single dose Monopowder P® insufflators (Valois Dispray SA, Mezzovico, Switzerland). These devices were constituted by a pump, a nasal adapter and a solid formulation reservoir (Sacchetti et al., 2002). The insufflators were loaded with about 0.8 mg UDCA-AZT-loaded microparticles (corresponding to about 100 μg of UDCA-AZT), or with about 0.8 mg the corresponding physical mixture (MIX), and the rats received this amount to each nostril. The amount of powder emitted during administration was obtained by the difference in the insufflator weight before and after each insufflation. After the administration, blood (100 μL) and CSF samples (50 μL) were collected at fixed time points, and they were analyzed with the same procedures described above. Four rats were employed for nasal administration of the microparticulate powder.

All *in-vivo* experiments were performed in accordance with the guidelines issued by the Italian Ministry of Health (D.L. 116/92 and D.L. 111/94-B), the Declaration of Helsinki, and the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health (Bethesda, Maryland). The protocol of all the *in-vivo* experiments has been approved by Local Ethics Committee (University of Ferrara, Ferrara, Italy). Any effort has been done to reduce the number of the animals and their suffering.

The area under concentration curves of UDCA-AZT in the CSF (AUC, μg mL^{-1} min) were calculated by the trapezoidal method. All the calculations were performed by using the computer program Graph Pad Prism (GraphPad Software Incorporated, La Jolla, CA, USA).

2.8. Statistical analysis

Statistical comparisons of the amounts of water uptake of the powder samples or the UDCA-

AZT AUC values obtained in the CSF of rats were made by Student's t test or one way ANOVA (GraphPad Prism). $P<0.05$ was considered statistically significant.

3. Results

3.1. Uptake of AZT and UDCA-AZT in macrophages

The incubation of 100 μM zidovudine or its prodrug with the macrophages for 30 min allowed to detect, in 10^6 cells, 0.054±0.007 moles of AZT and 1.12±0.16 moles of UDCA-AZT, as reported in Fig. 1. These data evidence the ability the prodrug to permeate and remain in the macrophages with an efficiency twenty times higher than that of its parent drug ($P<0.001$). The ability of UDCA-AZT to elude the AET systems (Dalpiaz et al., 2012), appears therefore useful in order to induce its uptake in the macrophages. According to the data reported in Fig. 1 the presence of the prodrug in the CSF should allow its permeation and permanence in the macrophages, constituting therefore an efficient carrier for AZT.

3.2. Characterization of microspheres

3.2.1. The yields of production

Spray drying technique proved to be suitable for preparing loaded and unloaded chitosan microparticles: in fact they have been obtained with good yields of production between 61.7% (CP) and 64.7% (CH).

3.2.2. UDCA−AZT content in CP

The amount of encapsulated UDCA−AZT in CP sample was found to be 13±0.3% (w/w),

showing that chitosan, characterized by hydrophilic properties, was able to encapsulate the lipophilic prodrug with a good encapsulation efficiency (64.9±1.5%).

3.2.3. Particle size measurement

Table 1 reports the volume-surface diameters (d_{vs}) obtained for the loaded (CP) and unloaded microparticles (CH), for MIX and, finally, for chitosan chloride and UDCA-AZT before and after grinding by agate and mortar and pestle. The loading of UDCA−AZT into chitosan microparticles determined an increase of particle size: in fact CP microspheres had d_{vs} of 3.59±0.1 μm compared with 2.32±0.01 μm of CH ($P<0.0001$). Furthermore, the drug presence resulted in a change of particle size distribution: loaded microparticles showed a larger curve than that of drug free microspheres (Fig. 2 up). In particular, CH showed a Gaussian curve while CP curve appeared leptokurtic, right skewed and wide for the presence of small shoulders.

MIX had d_{vs} of 6.11±0.022 μm, significantly higher than microspheres ($P<0.001$) and UDCA-AZT alone, but significant lower than chitosan chloride alone, both analyzed after grinding. The grinding of drug and polymer in agate mortar determined a reduction of particles size and a shifting of size distributions to low values (Fig. 2 down). In fact, the d_{vs} of chitosan chloride changed from 19.98±0.24 μm to 12.67±0.819 μm because of aggregates break. More pronounced was the size reduction in case of UDCA-AZT crystals: the d_{vs} decreased from 23.33±0.06 μm to 5.12±0.018 μm after grinding.

3.2.4. Particle morphology

Fig. 3A reports the SEM image of MIX obtained by geometric dilution with the use of agate mortar and pestle. This picture evidenced not only particles characterized by round morphology and relatively smooth surface, but also the presence of fragments characterized by irregular shape. As a term of comparison, Figs 3B and 3C report the SEM images of chitosan chloride and UDCA-AZT, respectively, grinded by agate mortar and pestle. Fig. 3B showed particles characterized by round

morphology and smooth surface. These morphologic characteristic did not appear significantly different from those of chitosan chloride before grinding, as evidenced by its SEM image reported in Fig. 3E. The SEM picture of grinded UDCA-AZT, Fig. 3C, showed fragments characterized by irregular shape (a detail is evidenced in the SEM image reported in Fig. 3D) with high aptitude to aggregate. These morphologic characteristic appeared significantly different from those of the prodrug UDCA-AZT before grinding, as evidenced in Fig. 3F, where fragments of irregular shape are shown to be characterized by diameters at least one order of magnitude higher than those of grinded UDCA-AZT particles. Taking into account these aspects, we can conclude that the SEM image of the physical mixture of chitosan chloride and UDCA-AZT (Fig. 3A) allows us to discriminate its two components: in particular, the particles with round morphology can be attributed to chitosan chloride, whereas the fragments of irregular shape can be attributed to UDCA-AZT.

Fig. 4A and 4B report SEM images of CP, constituted by the loaded UDCA-AZT microparticles based on chitosan chloride. The pictures evidence the presence of spherical particles characterized by smooth surface and several fragments characterized by irregular shape and high porosity, similar to spongy balls showing a some degree of aggregation. As a term of comparison, Fig. 4C reports the SEM image of the unloaded microparticles (CH), evidencing their round shape with smooth surfaces. Taking into account these aspects we can hypothesize that the spherical particles evidenced in images 3A and 3B are constituted by unloaded chitosan chloride, whereas the spongy balls have been obtained by the interaction between chitosan chloride and UDCA-AZT, giving rise to the highly porous structures.

3.2.5. True density

True density of microspheres was 1.48±0.02 g/cm^3 that is comparable with ρ of nasal microspheres (Gavini et al., 2012).

3.2.6. Determination of water uptake capacity

Fig. 5 shows the water uptake of both loaded and unloaded formulations. Free drug formulations absorbed a greater amount of water than loaded microparticles. Drug loading decreased the water absorption capability of chitosan microspheres ($P<0.05$). UDCA−AZT and chitosan chloride physical mixture showed also water uptake capability larger than CP but lower than CH from 15 to 60 min ($P<0.05$).

3.3. In-vitro release and dissolution studies

The dissolution and release studies of UDCA−AZT were performed in a MeOH/H_2O (70:30, v/v) mixture. The employment of methanol as cosolvent was necessary to increase the low solubility of the prodrug in water (0.0030±0.0001 mg/ml; Dalpiaz et al., 2012), thus ensuring sink conditions.

Fig. 6 reports the release profile of UDCA−AZT from the loaded CP sample. The release pattern is compared with that of UDCA-AZT dissolution from its physical mixture with chitosan chloride. The dissolution rate of the prodrug included in MIX appeared significantly lower than that of UDCA-AZT loaded in the CP sample. Indeed, after two hours of incubation the 38.4±1.6% of the prodrug loaded in the dissolution apparatus appeared dissolved, whereas in the case of CP loaded microparticles the amount of UDCA-AZT dissolved was 86.2±3.4%. As a consequence the dissolution half-life of UDCA-AZT loaded in CP sample appeared of about 15 min, whereas for the prodrug in the physical mixture (MIX) the dissolution half-life was higher than two hours. These profiles suggest that the employment of the CP sample as nasal formulation should be appropriate, being able to release in relatively short time the prodrug UDCA-AZT, allowing its potential fast absorption following the administration of the microparticles.

3.4. In-vivo UDCA-AZT administration

Taking into account that the CP microparticles were characterized not only by a satisfactory encapsulation efficiency but also by their ability to induce a relatively fast dissolution of UDCA−AZT, we have tested these microparticles for nasal administration of the prodrug, in order to verify its potential uptake in the CNS. The nasal administration of UDCA−AZT was performed with the employment of three different formulations: (i) a suspension in water of the raw UDCA-AZT powder, (ii) the powder constituted by loaded CP microparticles and (iii) as term of comparison, the parent physical mixture of chitosan chloride and UDCA-AZT (87:13 w/w). The results have been compared with those obtained by the intravenous infusion of UDCA−AZT to rats.

3.4.1. Intravenous administration of UDCA−AZT

The prodrug was undetectable in rat blood samples following its intravenous infusion. This result is in agreement with the very fast hydrolysis of UDCA−AZT in rat blood at 37 °C (Dalpiaz et al., 2012). Important amounts of AZT were therefore detected following the intravenous administration of the prodrug, as evidenced in Fig. 7. In particular, after infusion of 0.200 mg of UDCA−AZT to rats, the AZT concentration in the bloodstream was 4.50±0.31 μg/mL and decreased during time with an apparent first order kinetic confirmed by the linearity of the semilogarithmic plot reported in the inset of Fig. 8 ($n = 9$, $r = 0.990$, $P<0.0001$), and a half-life of 60.4±3.8 min. These data are in good agreement with those obtained by previous studies on *in-vivo* UDCA-AZT pharmacokinetics (Dalpiaz et al., 2014). No AZT or UDCA−AZT amounts were detected in the CSF within 180 min after intravenous administration of UDCA−AZT.

3.4.2. Nasal Administration of UDCA−AZT

The nasal administration of pure UDCA-AZT as water suspension did not allow to obtain detectable amounts of AZT or prodrug in blood or in CSF, respectively, within 180 min after the administration, as previously evidenced (Dalpiaz et al., 2014). On the contrary, the nasal

administration of the powders constituted by MIX or by the CP microparticles (0.8 mg, about 100 μg of UDCA−AZT to each nostril) produced detectable amounts of UDCA−AZT in the CSF of the rats, as reported in Fig. 8. In particular, the UDCA−AZT concentrations in the CSF were 0.64±0.12 μg/mL and 1.96±0.29 μg/mL at 30 and 60 min after nasal administration of the mixture respectively. The UDCA−AZT concentration then decreased, reaching the value of 0.60 μg/mL at 180 min.

The nasal administration of the same amount of CP microparticles allowed to increase the rate uptake of the prodrug in the CSF of rats. Indeed, the UDCA−AZT concentrations in the CSF were 2.30±0.09 μg/mL and 2.99±0.31 μg/mL at 30 and 60 min after nasal administration of the CP sample, respectively. The UDCA−AZT concentration then decreased, reaching the value of 1.12 μg/mL at 180 min. No AZT amounts were detected in the bloodstream and in CSF within 180 min after nasal administration of the powders constituted by MIX or the CP microparticles.

The area under concentration (AUC) curve values obtained for UDCA−AZT in the CSF, following the nasal administration of MIX and CP microparticles (Fig. 8), were 201.39±13.51 μg mL^{-1} min and 354.4±13.3 μg mL^{-1} min, respectively. The ratio between AUC of CP sample and AUC of the mixture was 1.76, indicating that the nasal formulation constituted by CP microparticles allowed an uptake of UDCA−AZT in the CSF the 76% higher than that obtained by the administration of the parent mixture.

4. Discussion

Although antiretroviral nucleoside derivatives are largely used in AIDS treatment for their efficacy at peripheral level, a total eradication of HIV from the body is currently hard to be obtained. Indeed, the antiretroviral drugs are not able to reach the central nervous system (CNS), (Kaufmann and Cooper, 2000; Strazielle, 2003) that is instead easily reached by HIV through infected monocytes (Davis et al., 1992; Gray et al., 1996) that differentiate into macrophages and

microglia in the brain (Rausch and Stover, 2001). The CNS and the macrophages constitute, therefore, sanctuaries of HIV, where drug resistance is induced and from which the periphery can be reinfected (Cunningham et al., 2000; Kolson and Gonzalez-Scarano, 2000). These phenomena are mainly attributed to the expression of active efflux transporters (AET) on the membranes of the macrophages (Chaudhary et al., 1992; Neyfakh et al., 1989) and the blood brain barrier (BBB) cells (Namanja et al., 2012; Pavan and Dalpiaz 2011), whose activity causes the lack of penetration of antiretroviral drugs in the HIV sanctuaries.

The AZT activity in the CNS appears necessary not only in the brain tissue, but also in the CSF subarachnoid spaces containing macrophages, that constitute the only site of HIV replication in the brain (Ghersi-Egea et al., 1996; Cunningham et al., 1997). It should therefore be important to obtain formulations able to target AZT in the CSF, but it should also as much important to induce its ability to elude the AET systems, in order to avoid its extrusion from the CNS and from the macrophages. In this regards we know that the prodrug UDCA-AZT, obtained by the conjugation of AZT with the ursodeoxycholic acid (UDCA), is able to elude the AET systems (Dalpiaz et al., 2012). Moreover, the results reported in this paper evidence for the first time that the prodrug UDCA-AZT is definitely more efficient than AZT in permeating and remaining into murine macrophage-like J774A.1 cell line, commonly used as model system for studying the internalization process of macrophages **(**Wang et al., 2012**).** It is to remark that the prodrug UDCA-AZT is quickly hydrolyzed in whole blood, so an intravenous administration cannot be suitable for its permeation across the BBB. Indeed, following this type of administration we have detected only AZT amounts, showing concentration values decreasing during time with a half-life of about 1 h. No AZT and UDCA−AZT amounts were detected in the CSF of rats after intravenous administration. On the other hand, the nasal administration of this prodrug appears promising in order to obtain its uptake in the CNS and, in particular, in CSF. We have indeed demonstrated that the nasal administration of chitosan based microparticles can induce a selective uptake of neuroactive agents in the CSF of rats, probably by promoting drug permeation across olfactory nasal mucosa (Dalpiaz et al., 2008; Gavini

et al., 2011; Rassu et al., 2015a). Very recently, the poor water solubility of UDCA-AZT encouraged us to encapsulate this prodrug in solid lipid microparticles (Dalpiaz et al., 2014). In particular, the nasal administration of UDCA-AZT loaded in stearic acid based microparticles induced a selective uptake of the prodrug in the CSF of rats, with amounts up to about 0.4 µg/ml within 60 min after the administration (Dalpiaz et al., 2014). These amounts were sensibly increased (up to about 1.50 µg/mL within 120 min after the administration) when the same quantity of microparticles were nasally administered as a water suspension in the presence of chitosan chloride (Dalpiaz et al., 2014). This result was attributed to the good mucoadhesive properties of chitosan (Dalpiaz et al., 2008) and to its ability to transiently open the tight junctions in the epithelial membranes (Illum et al., 1994; Dodane et al, 1999).

Nasal formulation obtained in the presence of water can be related to risk of chemical and physical instability and microbiological growth. Moreover, in the nasal cavity the liquid formulations are often rinsed into the gastrointestinal tract (GIT) or out, so their residence time is generally short (Kublik and Vidgren, 1998). These disadvantages can be overcome by using powder-based formulations (Marttin et al., 1997; Rassu et al., 2015b). For this reason we have prepared microspheres based on chitosan chloride in order to increase the nose-to-brain delivery of UDCA-AZT. The efficacy of the microparticulate system on the brain delivery of the prodrug was verified by comparing the properties of microparticles with those of their parent physical mixture, that was obtained by geometrical dilution of the components with the use of agate mortar and pestle. The grinding process induced a decrease of the d_{vs} diameters of chitosan chloride and UDCA-AZT particles. The chitosan chloride particles were characterized by round morphology and a relatively smooth surface before and after grinding, whereas UDCA-AZT appeared as fragments with irregular shape. These two different morphological characteristic were distinctly recognized in the SEM picture of the physical mixture, where small irregular fragments of UDCA-AZT appeared on the surface of larger and round chitosan chloride microspheres.

The spray-drying process allowed to obtain unloaded microparticles of chitosan chloride (CH) with the "classical" round morphology and small d_{vs} diameter. The loaded microparticles (CP) evidenced a slight increase of size and a morphology characterized by spherical particles mixed with fragments of irregular shape and high porosity, similar to spongy balls showing a some degree of aggregation. It can be deduced that the spherical particles are attributable to pure chitosan chloride, that during the spray drying process has not interacted with UDCA-AZT, whereas the "spongy balls" were obtained by a combination of the prodrug with polymer, inducing the formation of the highly porous structures. The totally different degree of water solubility between UDCA-AZT and chitosan can justify these characteristics of the CP formulation. The description of CP's morphology appears in agreement with the dissolution and water uptake profiles. Indeed, we have observed that the dissolution rate of UDCA-AZT from the CP sample was sensibly higher than that from the parent physical mixture. This phenomenon can be attributable to the highly porous structures observed in CP sample, that allows a faster dissolution of UDCA-AZT with respect to its fragments included in MIX. Moreover, as previously demonstrated by Gavini et al., 2011, water soluble chitosans, such as salts, are more able to completely amorphize poorly soluble drugs into the polymer matrix compared to chitosan base; as consequence, the improvement of the dissolution rate of poorly soluble drugs and their bioavailability occur.

Moreover, we have observed that the water uptake rate of CH sample was significantly reduced if chitosan chloride was mixed with UDCA-AZT, but a drastic decrease was observed in the case of CP sample. The presence of UDCA-AZT, a very poor water soluble molecule, in the physical mixture can explain its reduction of water uptake with respect to the CH sample, but the drastic reduction registered for CP microparticles can be attributed to their porous structures, where the presence of the prodrug and chitosan chloride together contributed to dramatically reduce the water uptake aptitude of the polymer.

The CP sample appeared therefore as a valuable formulation for the nasal administration of UDCA-AZT: in particular, we have hypothesized that the size and density of the microparticles

should induce their deposition on nasal mucosa, that should not be dehydrated by their presence, being poor the water uptake of this sample. Moreover, the ability of the loaded microparticles to increase the dissolution rate of UDCA-AZT suggested their potential aptitude to induce the prodrug permeation across nasal mucosa, a phenomenon probably potentiated by the chitosan ability to transiently open their tight junctions (Illum et al., 2015).

The nasal administration to rats of raw UDCA−AZT did not produced any detectable levels of both UDCA−AZT and AZT in the bloodstream and CSF, whereas the nasal administration of the CP sample and its parent physical mixture allowed to obtain relatively high levels of UDCA-AZT in the CSF of rats, 60 min after their administration. The relative bioavailability attributed to the CP sample was the 176% with respect to its parent physical mixture ($P<0.001$). No AZT amount were detected in the bloodstream of rats after nasal administration of CP sample and its parent physical mixture, confirming the existence of a direct nose−CNS pathway for this prodrug (Dalpiaz et al., 2014). Our results evidence that the role of chitosan in inducing the selective UDCA-AZT uptake in the CSF is potentiated when formulated as microparticulate system by spray-drying. This phenomenon appears clearly illustrated in Fig. 9, reporting a comparison of the AUC values in CSF obtained after the nasal administration to rats of the same dose of UDCA-AZT (200 μg) by using different formulations, i.e. the solid lipid microparticles (SLMs), their water dispersion in the presence of chitosan (SLMs + Ch), previously obtained by Dalpiaz et al., 2014, MIX and CP, here described. In particular CP was able to induce an uptake of UDCA-AZT in the CSF of rats 1.8, 3.2 and 18.6 times higher than its parent physical mixture (MIX), the dispersion of solid lipid particles in the presence of chitosan (SLMs + Ch) and the SLMs in the solid form, respectively.

In conclusion, chitosan microspheres induce, after nasal administration, an efficient uptake of UDCA-AZT in the CSF; thus, the ability of the prodrug to elude the AET systems can avoid its extrusion in the bloodstream and induce the AZT transport in the macrophages located in the subarachnoid spaces of CSF.

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Caption of Figures

Fig. 1. Intracellular levels of AZT and its prodrug UDCA-AZT after incubation (30 min) with J774A.1 murine macrophages. Data are reported as the mean ± S.D. of three independent experiments. *: P<0.001 versus AZT.

Fig. 2. Size distribution of loaded (CP) and unloaded (CH) chitosan microspheres (up) as well as of MIX and, as comparison, of chitosan chloride and UDCA-AZT alone, before and after grinding (down). Particle size distribution was graphically expressed as curves obtained by plotting the volume of particles in percentage *versus* size (μm), shown in logarithmic scale

Fig. 3. Scanning electron microscopy (SEM) micrographs of [A] the mixture of chitosan chloride and UDCA-AZT (87:13 w/w, magnification of 3,610 times); [B] grinded chitosan chloride magnification of 3,140 times); grinded UDCA-AZT with magnification of 512 times [C] and 10,000 times [D]; [E] chitosan chloride before grinding (magnification of 2,740 times); [F] UDCA-AZT before grinding (magnification of 505 times).

Fig. 4. Scanning electron microscopy (SEM) micrographs with magnifications of 11,250 times [A] and 3,620 times [B] of the loaded microparticles (sample CP) and [C] SEM micrograph of unloaded chitosan microparticles (sample CH) with magnification of 2,000 times.

Fig. 5. Water Uptake profiles of loaded (CP) and unloaded (CH) microspheres until 60 min compared with that of physical mixture (MIX, chitosan chloride and UDCA−AZT (87:13 w/w). Data are reported as the mean ± SD of three independent experiments.

Fig. 6. *In-vitro* release of UDCA−AZT from CP microparticles based on chitosan chloride. The

release profile is compared with that of dissolution obtained by the parent physical mixture of chitosan chloride and UDCA-AZT (87:13 w/w) (MIX). Data are reported as the mean±SD of three independent experiments.

Fig. 7. Elimination profile of AZT after 0.200 mg infusion of UDCA−AZT to rats. Data are expressed as the mean±SD of four independent experiments. The elimination followed an apparent first order kinetic, confirmed by the semilogarithmic plot reported in the inset (n =9, r = 0.990, $P<0.0001$). The half-life of AZT was calculated to be 60.4 ± 3.8 min.

Fig. 8. UDCA−AZT concentrations (μg/mL) detected in the CSF after nasal administration of loaded CP microparticles and its parent mixture (MIX) of chitosan chloride and UDCA-AZT. Each dose contained 200 μg of the prodrug. Data are expressed as the mean±SD of four independent experiments.

Fig. 9. A comparison among the AUC values obtained in CSF of rats after the nasal administration of 200 μg of UDCA-AZT by using different formulations, i.e. loaded solid lipid microparticles (SLMs), their water dispersion in the presence of chitosan (SLMs + Ch), the physical mixture of chitosan and prodrug (MIX) and the loaded microparticles based on chitosan chloride (CP). Data refereed to SLMs and SLMs + Ch formulations were previously reported (Dalpiaz et al., 2014). Data are expressed as the mean±SD of four independent experiments.

Table 1.

Volume-surface diameters (d_{vs}, μm) of unloaded (CH) and loaded (CP) microparticles together with d_{vs} values of the parent mixture (MIX) of chitosan chloride and UDA-AZT and its single components before and after grinding by agate mortar and pestle. Data are reported as the mean ± SD of three independent experiments.

Sample	d_{vs} (μm)
CP	3.59 ± 0.14
CH	2.32 ± 0.11
MIX	6.11 ± 0.02
Grinded Chitosan HCl	12.67 ± 0.82
Grinded UDCA-AZT	5.12 ± 0.02
Chitosan chloride before grinding	19.98 ±0.06
UDCA-AZT before grinding	23.33 ± 0.06

Figure 1

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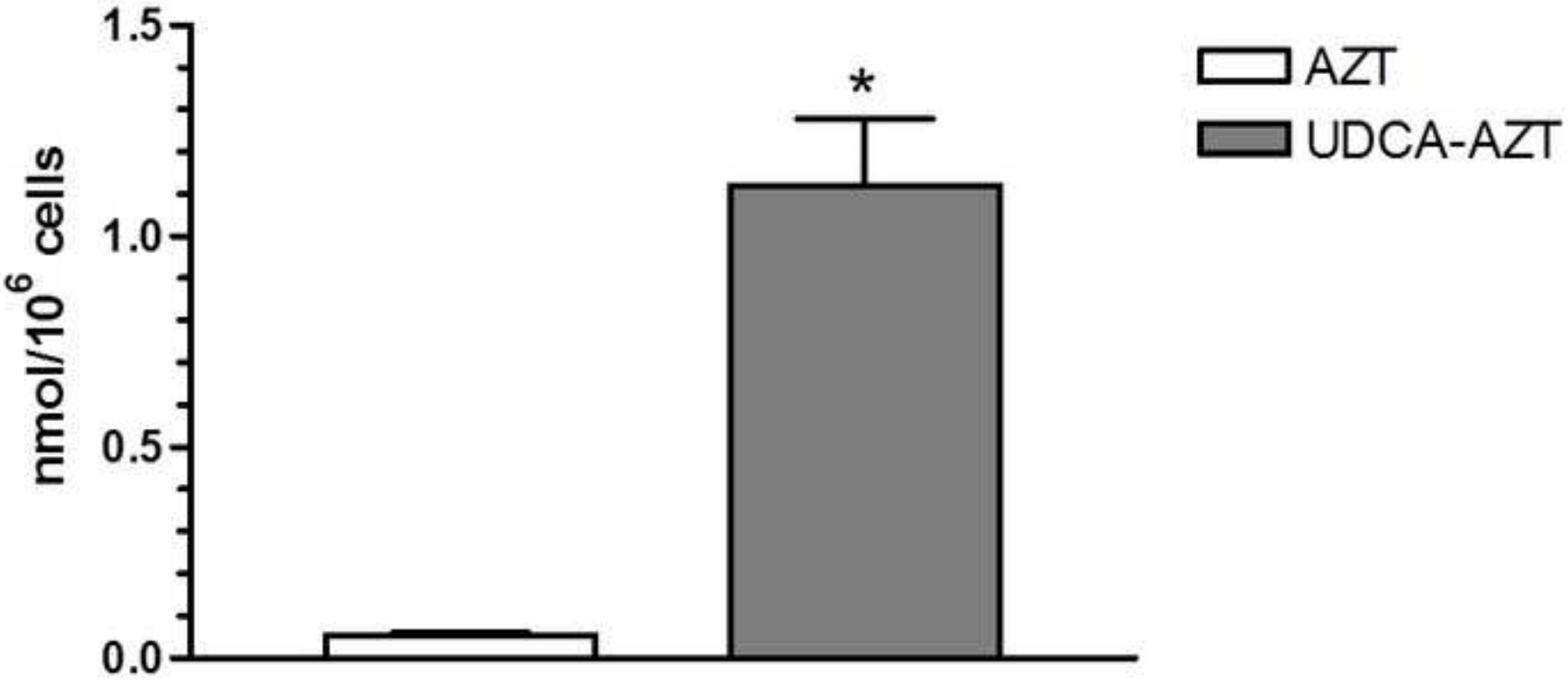

Figure 2

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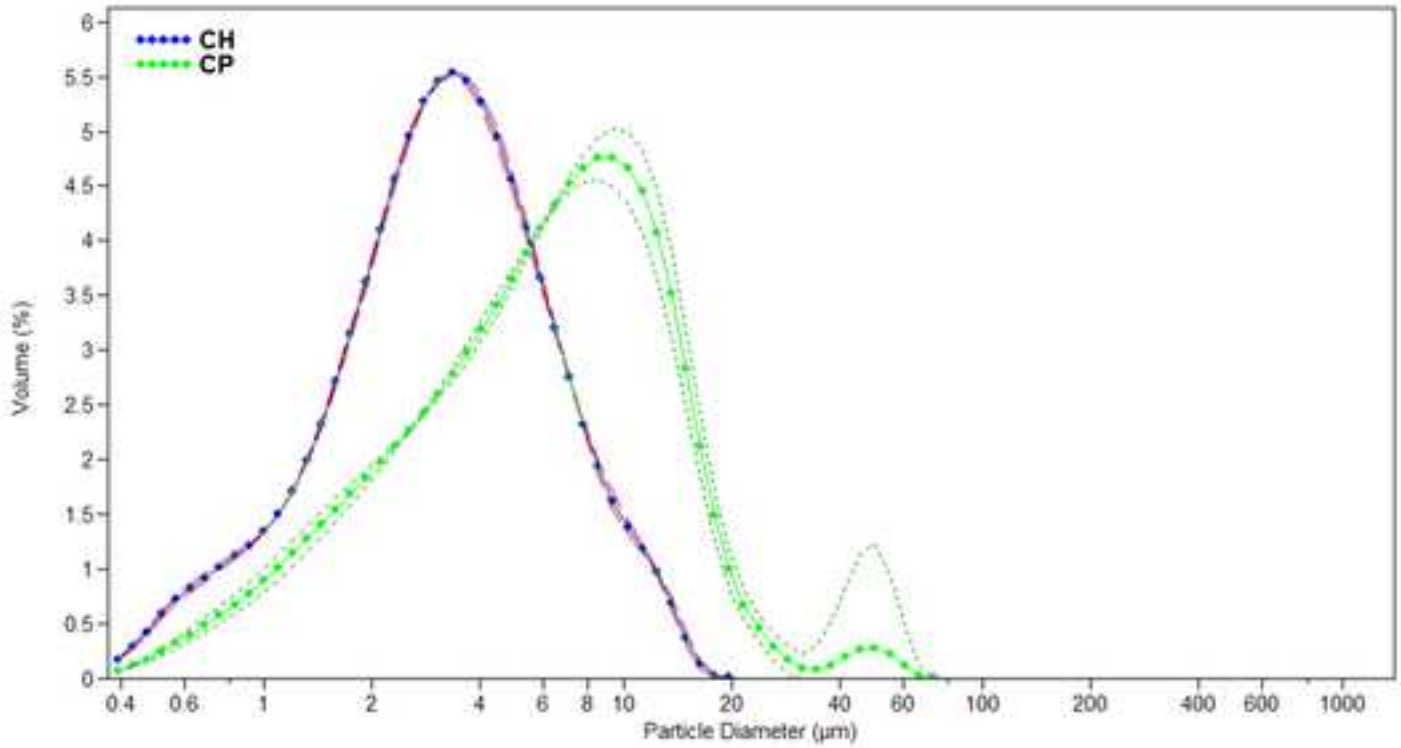

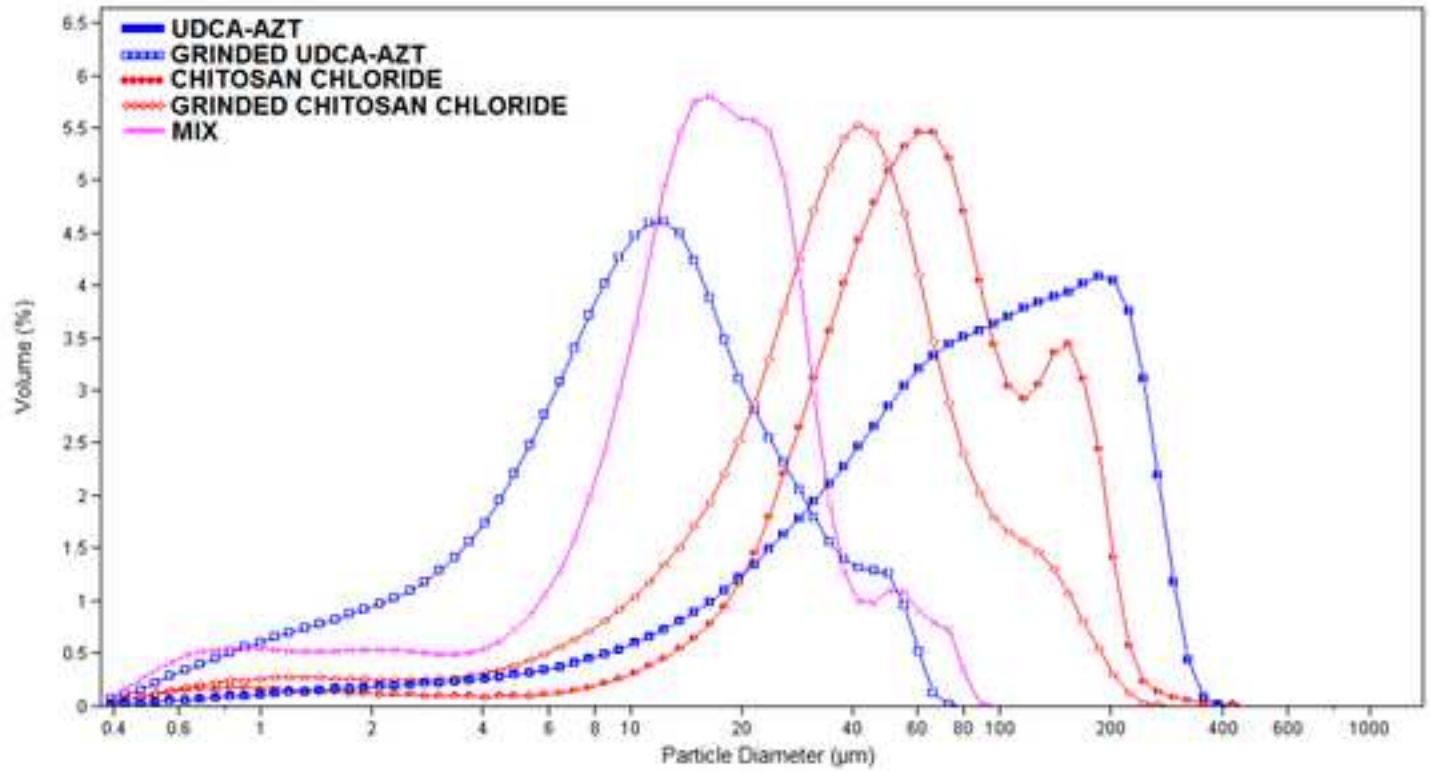

Figure 3

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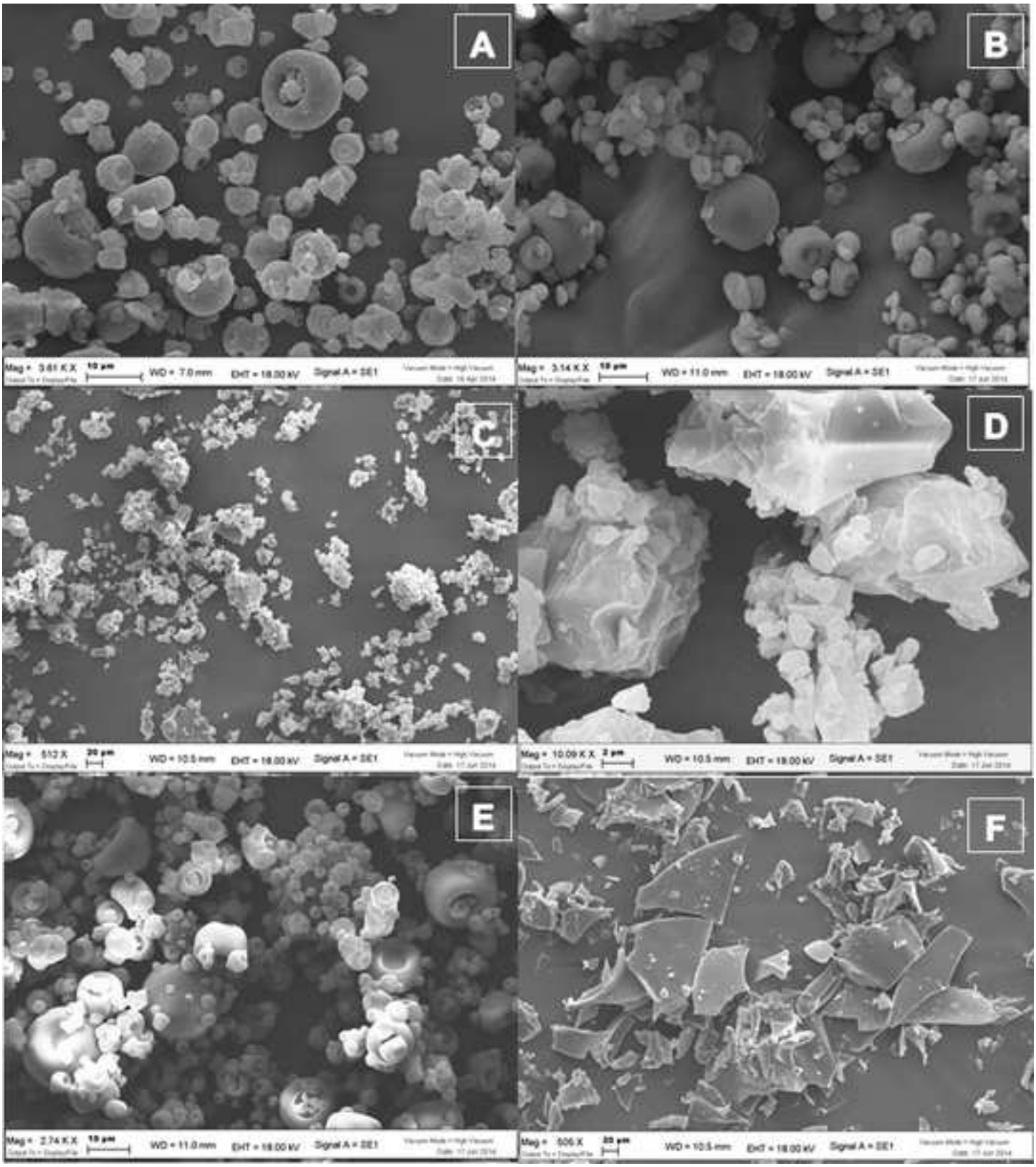

Figure 4

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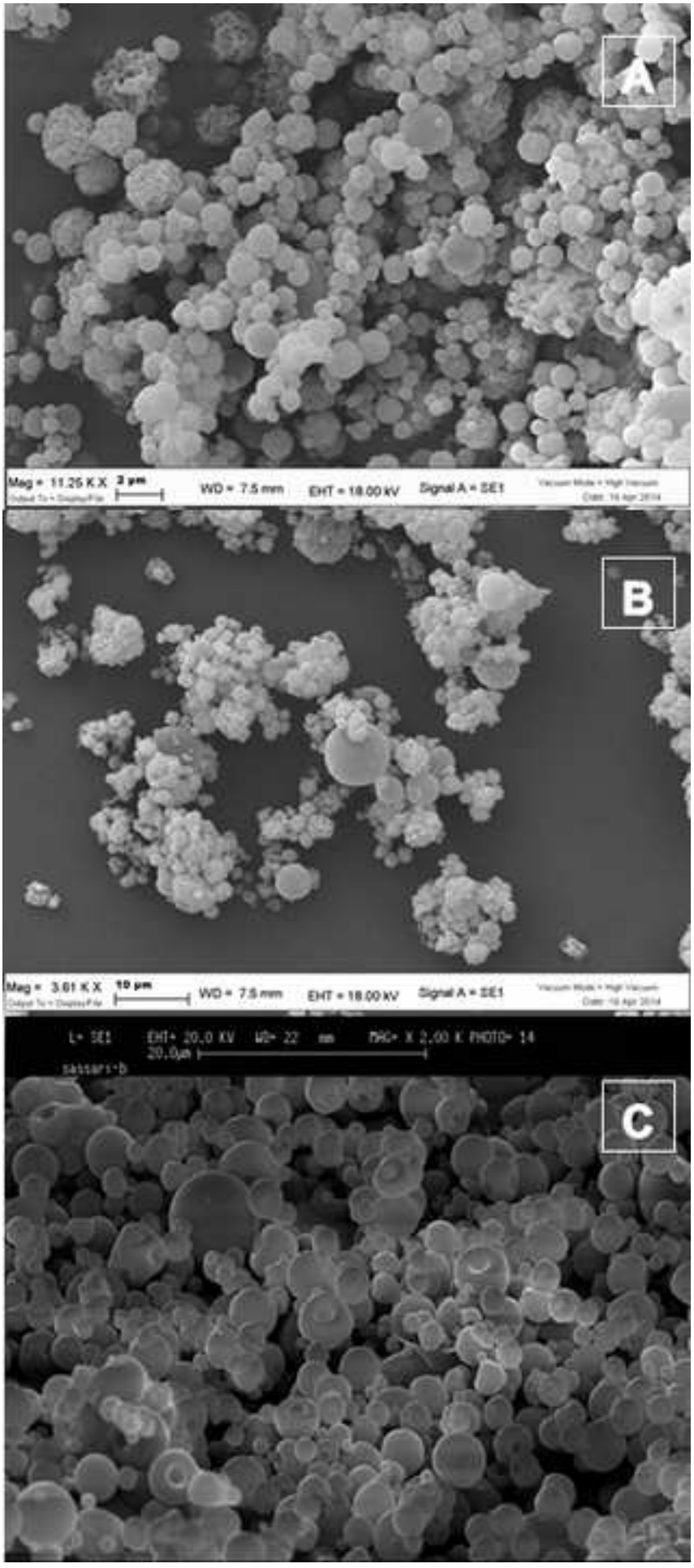

Figure 5

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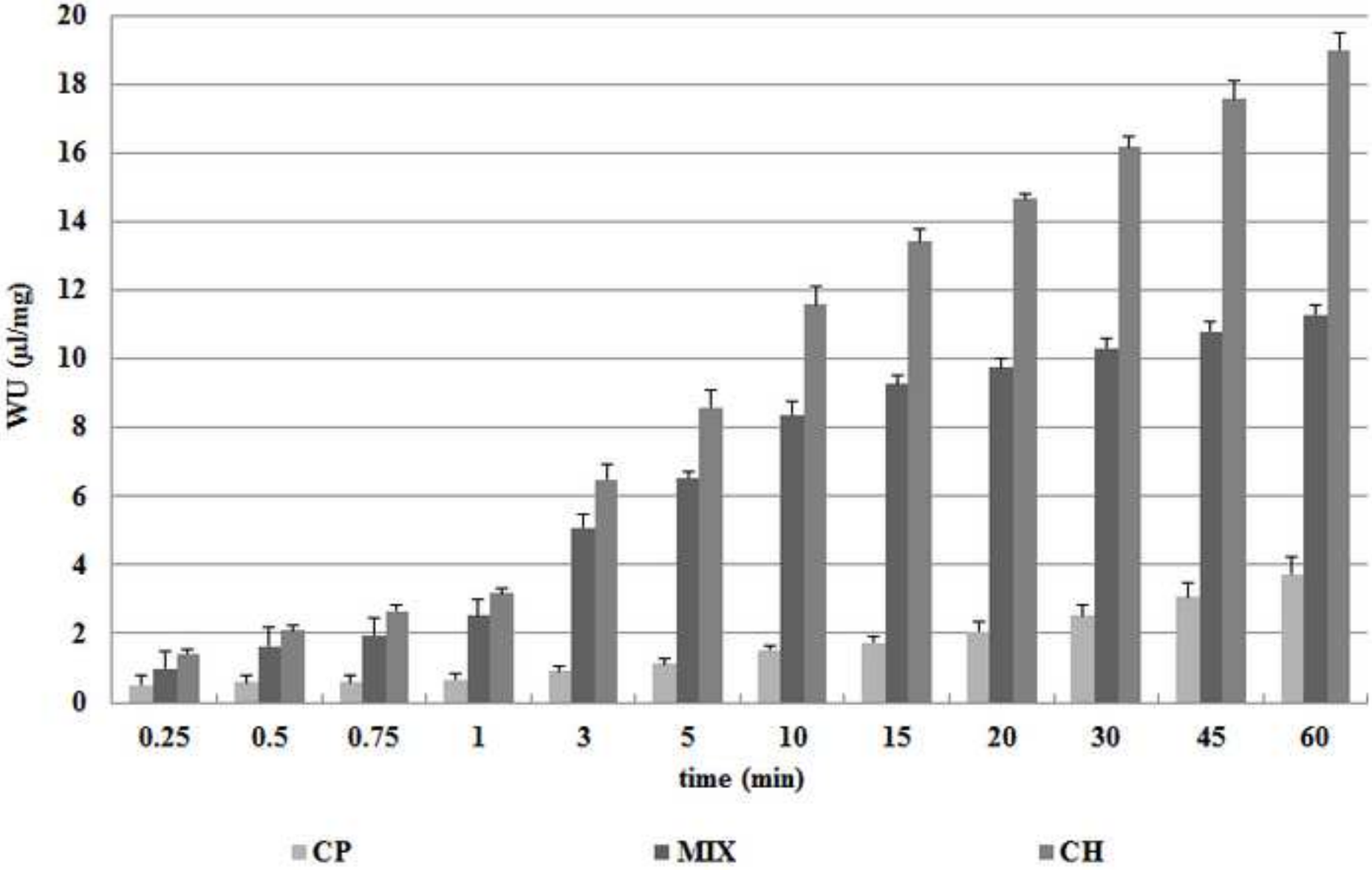

Figure 6

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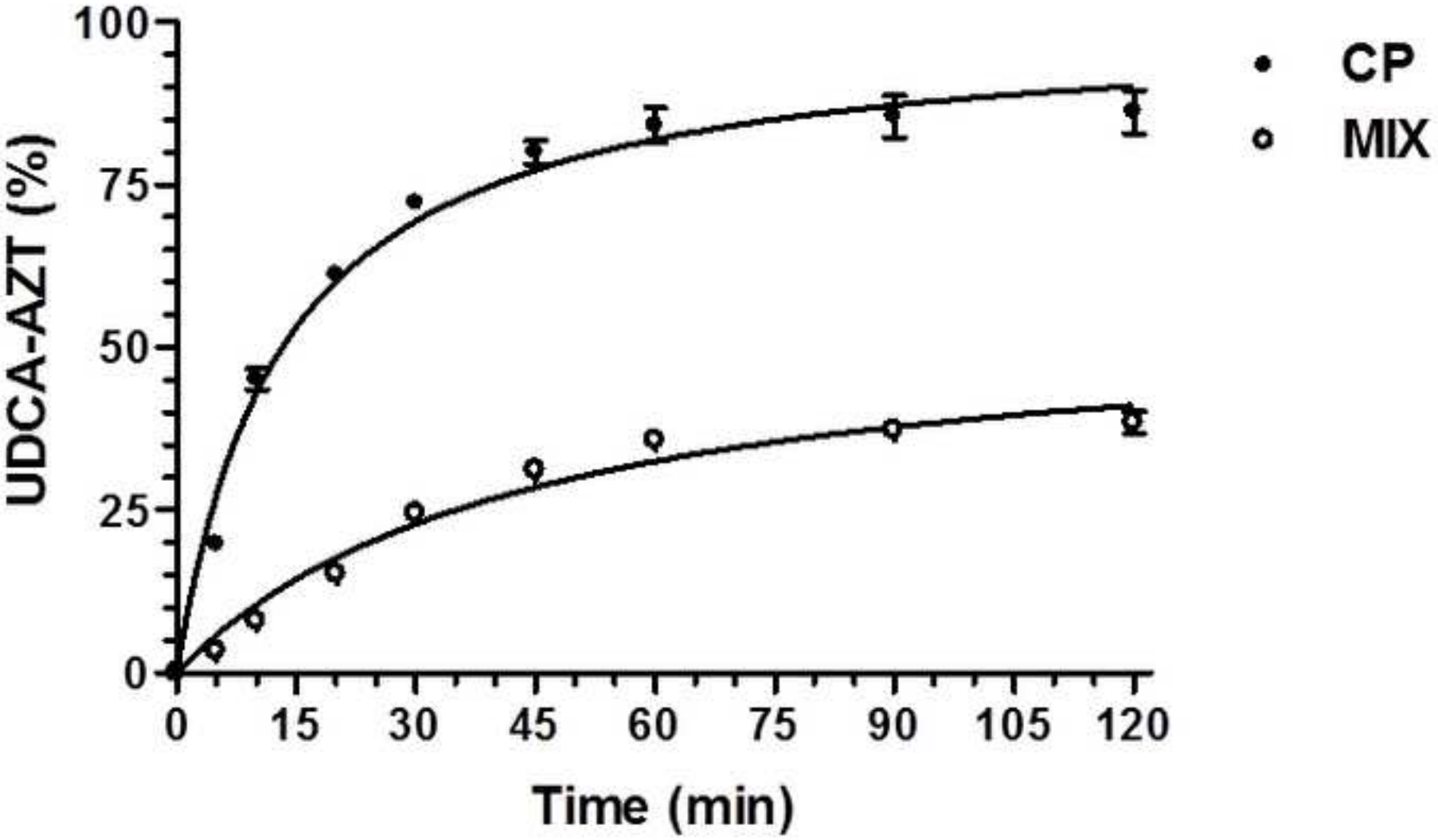

Figure 7

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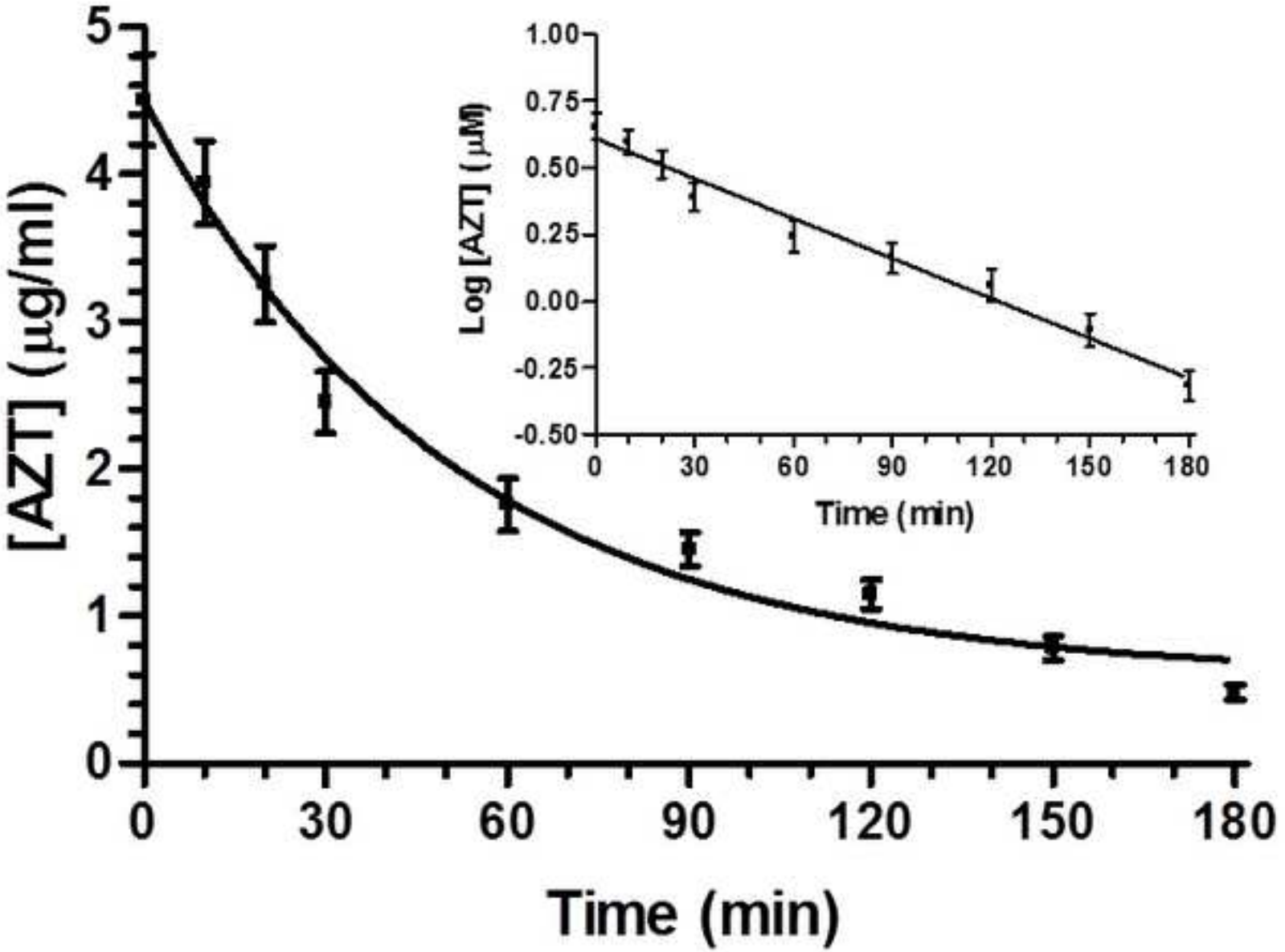

Figure 8

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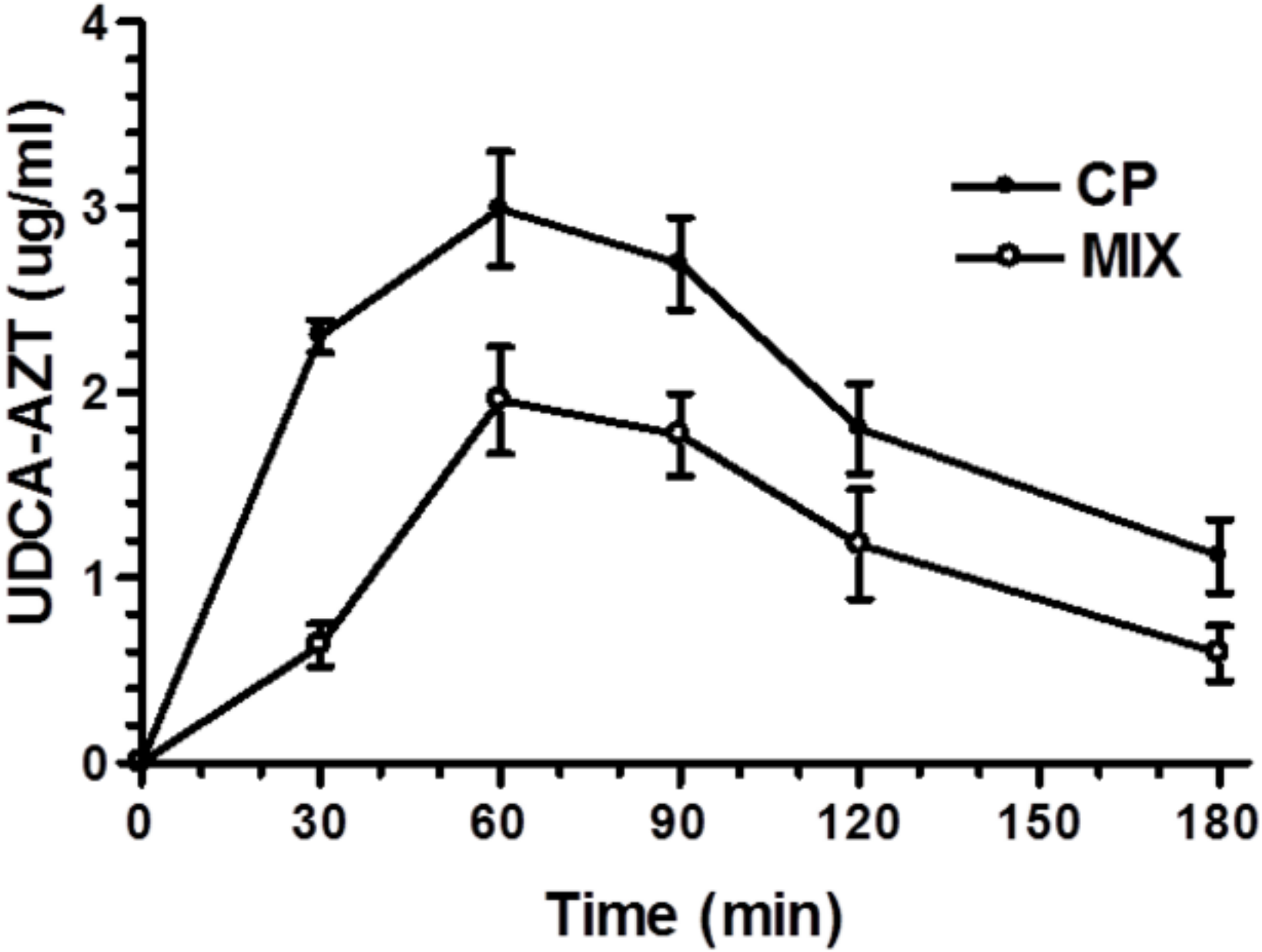

Figure 9

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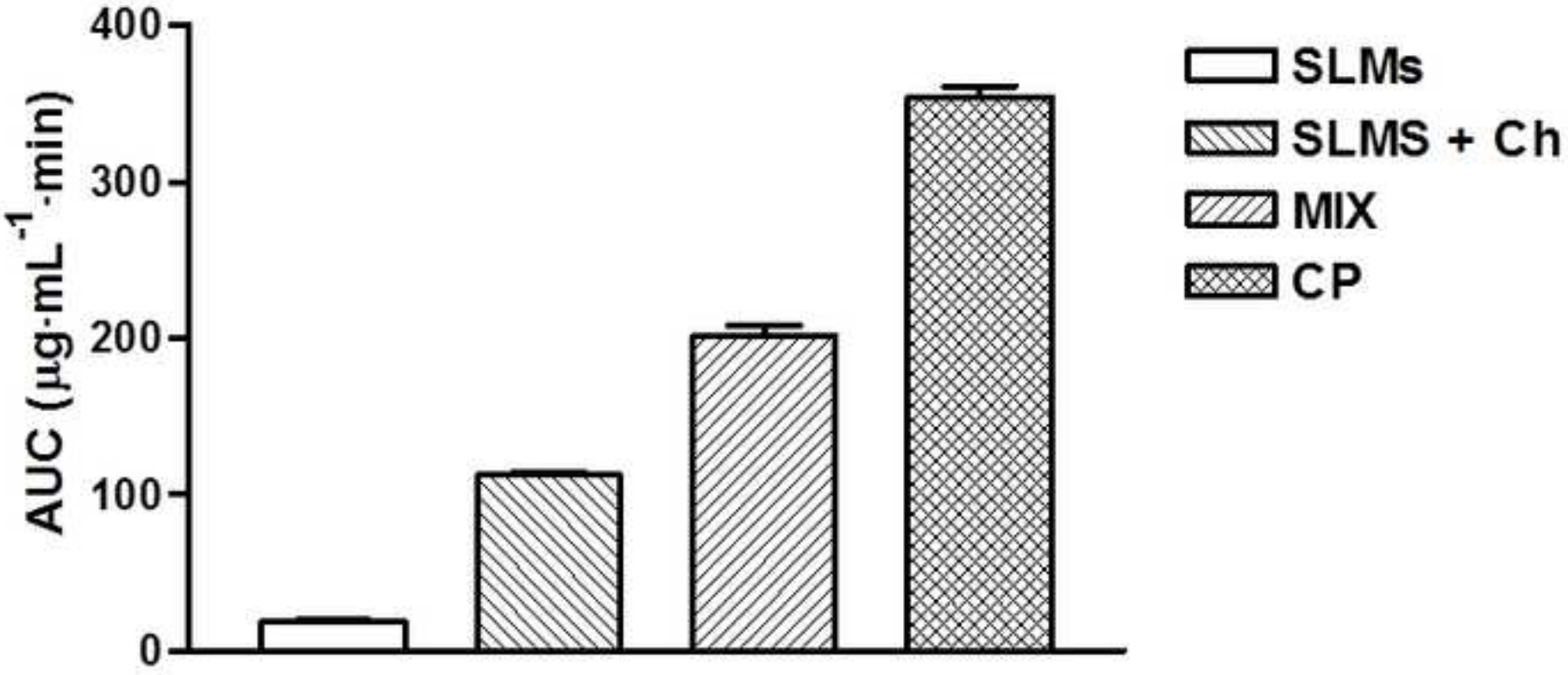

Graphical abstract

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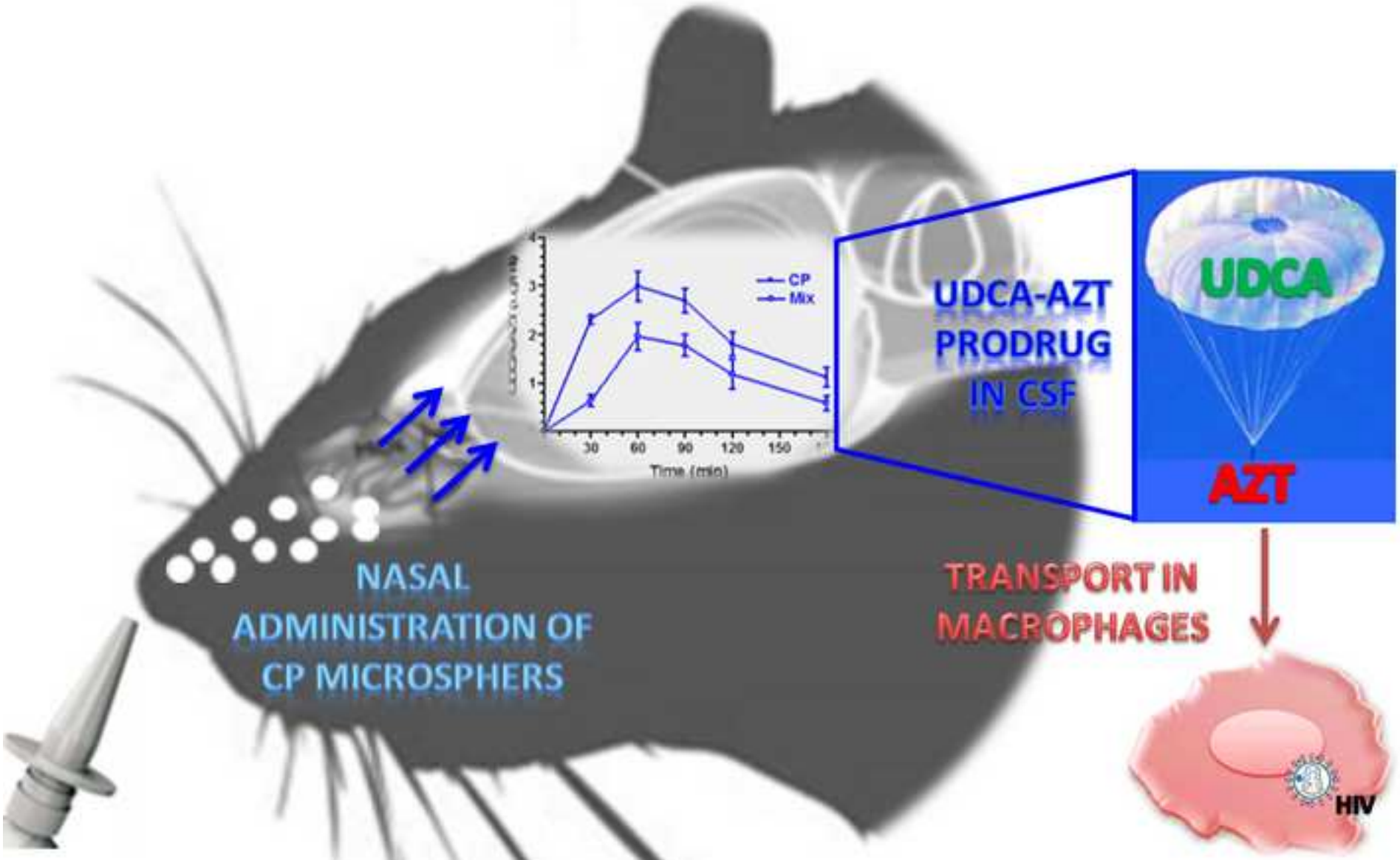

Supplementary material

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