INTRODUCTION
Membrane proteins are targets of prime interest to the pharmacological field largely because of their major role as receptors or drug transporters. Furthermore, the importance of membrane transporters in the pharmacokinetics and tissue distribution (bioavailability) of many therapeutics becomes recently evident, as well as the remarkable action of efflux pumps in the development of drug resistance.

The application of the classical high-resolution 2-DE (two dimensional gel electrophoresis) separation technique and mass spectrometry methods for identification of proteins showed to be a very successful tactic to study proteins expressed in a system. While the characterization of abundant and soluble proteins through such proteomics approaches continues revealing new proteins, membrane proteins remain extremely difficult to analyze in this way. In particular, proteins embedded in the lipid bilayer, like transporters possessing variable number of transmembrane spans (TMSs) are underrepresented or non present in the 2D gels. The difficulties that hamper or even prevent the characterization reside in their very intrinsic properties: extreme hydrophobicity and poor solubility.

Here, we standardized a protocol for extraction of proteins from membrane preparations from the murine macrophages cell line J774 and their analysis by 2-DE and MS. And, we evaluate the efficiency of the nonionic detergent ASB14 to solubilize proteins with MRP1 (multidrug transporter) and 12TMs (Glut1, glucose transporter) by Western Blot.

MATERIALS AND METHODS
Cell lines. J774 macrophages, a murine cell line were maintained at 37°C in 5% CO₂ atmosphere, in DPM medium supplemented by 10% fetal calf serum. Cultures were rotated at a density of 0.2 × 10⁶ cells/10 cm². Cells were harvested and washed 48 hrs after when they reached confluence.

Extraction Buffer. The extraction buffer was composed of a solution (pH 7.3-7.4) containing 100 mM KCl, 1 mM MgCl₂, 20 mM Tris-HCl, 1 mM PMSF, 1 mM sodium orthovanadate, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml pepstatin A, layered on top of a 50% sucrose solution and ultracentrifuged in a Beckman SW40 rotor at 280,000 x g for 2 hrs at 4 °C.

Protein samples were ultracentrifuged in a Type 50 Ti rotor at 100,000 x g for 30 min at 4 °C. Supernatant and pellet were resuspended in 7 M urea, 2 M thiourea, 2.5% CHAPS, 5% ASB14, 10 mM Tris-HCl, pH 8.0.

Western Blot. Proteins were separated on 7.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Membranes were blocked with 3% milk in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) at 4 °C overnight. Subsequently, membranes were washed three times in TBST and incubated with primary antibodies (rabbit polyclonal anti-MRP1, Merck, 1:1000 dilution in TBST containing 5% milk, 1 h at room temperature). After washing with TBST, membranes were incubated with a secondary antibody (goat polyclonal anti-rabbit, Sigma, 1:1000 dilution) for 1 h at room temperature. Membranes were washed with TBST and developed with the chemiluminescent method (Pierce).

RESULTS AND DISCUSSION
To gain insight into potential protein targets of pharmacological interest using a classic proteomics approach we first separated the cellular membranes and then treated them with the nonionic detergent ASB14 to extract the proteins and prepare suitable samples for 2DE separation and MS analysis. In a typical experiment following the Protocol I, 10 mg of total proteins would render after the preparation and treatment of membranes (Materials and Methods) a 10% of proteins recovered by ultracentrifugation as a soluble supernatant and a 6% remained as an insoluble pellet. To further analyze the proteins remaining in the ASB14-pellet, the pellet was resuspended in 7 M urea and 1.2% CHAPS, ultracentrifuged and 2% of proteins were collected from this supernatant (Fig. 1).

The efficiency of the solubilization by the ASB14-treatment was evaluated by detecting membrane transporters showing more than ten transmembrane domains, the MRP1 and Glut1. Western blot of homogenate, membrane fraction and the post-ASB14 treatment samples, ASB14-supernatants and pellets, shows the presence of the probed transporters. Although both molecules, MRP1 and Glut1 were detected in the ASB14-supernatant there is a considerable amount of them remaining in the ASB14-pellet (Fig. 2).

In a first attempt to separate the membrane proteins extracted with ASB14 by 2DE, proteins in the ASB14-supernatants and pellets were prepared using the solubilization Buffer 1 (Protocol I, Materials and Methods). 2DE minigels showed few well focus spots and rather poor resolution with vertical streaking (Fig. 3, A and B). Therefore, to improve the quality and efficiency of the separation, the protocol was modified as follow. We scale up the relative focusing power going from 7 cm 11 cm IPG strips. To this end, major different proteins stops were excised and identified by MALDI-MS indicating a greatly better focusing and superior resolution of protein isoforms. Additionally, we achieved highly reproducible profile separations with independent membrane preparations.

Furthermore, major different proteins spots were excised and identified by MALDI MS indicating that the protocol is compatible with downstream mass spectrometry methods for identification of proteins.