Amoxicillin haptens intracellular proteins that can be transported in exosomes to target cells

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Short title: Identification and fate of AX cellular targets

Abstract

Background

Allergic reactions to β -lactams are amongst the most frequent causes of drug allergy and constitute an important clinical problem. Drug covalent binding to endogenous proteins (haptentation) is thought to be required for activation of the immune system. Nevertheless, neither the nature nor the role of the drug protein targets involved in this process is fully understood. Here we aim to identify novel intracellular targets for haptentation by amoxicillin (AX) and their cellular fate.

Methods

We have treated B-lymphocytes with either AX or a biotinylated analogue (AX-B). The identification of protein targets for haptentation by AX has been approached by mass spectrometry and immunoaffinity techniques. In addition, intercellular communication mediated by the delivery of vesicles loaded with AX-B-protein adducts has been explored by microscopy techniques.

Results

We have observed a complex pattern of AX-haptentated proteins. Several novel targets for haptentation by AX in B-lymphocytes have been identified. AX-haptentated proteins were detected in cell lysates and extracellularly, either as soluble proteins or in lymphocyte-derived extracellular vesicles. Interestingly, exosomes from AX-B-treated cells showed a positive biotin signal in electron microscopy. Moreover, they were internalized by endothelial cells, thus supporting their involvement in intercellular transfer of haptentated proteins.

Conclusions

These results constitute the first identification of AX-mediated haptentation of intracellular proteins. Moreover, they show that exosomes can constitute a novel

vehicle for haptenated proteins, and raise the hypothesis that they could provide antigens for activation of the immune system during the allergic response.

Key words

Amoxicillin, cellular protein targets, drug adducts, exosomes, hapteneration

Introduction

β -lactams are the antibiotics most commonly prescribed and most frequently involved in hypersensitivity reactions induced by specific immunological mechanisms, which represent a growing impedance for their clinical use (1-3). Due to the current prescription tendencies, amoxicillin (AX) provokes most allergic reactions (AX) (4), whereas reactions to clavulanic acid are on the rise (5, 6). Frequently, β -lactams induce immediate hypersensitivity reactions mediated by specific IgE antibodies (7), that must be preceded by a sensitization phase where antigen presenting cells (APC) uptake and process the antigen and finally produce the class switching of lymphocytes B to IgE production (8). There are three main hypotheses on the mechanisms of antigen presentation. In the "hapten hypothesis" (9), the drug, or hapten, binds covalently to endogenous proteins that are processed and presented by cells (10). The "danger signal hypothesis" proposes that both, drug-induced cytotoxic damage and its immune recognition, contribute to the allergic reaction (11). Finally, the "P-I (pharmacological interaction) concept" (12, 13) proposes high-affinity non-covalent interactions between the drug and immune receptors such as MHC or TCR. Interestingly, in certain cases, IgE from allergic patients does not recognize AX alone or conjugated to exogenous structures. This supports the importance of endogenous structures in the formation of the antigenic determinants involved in allergic responses to AX, and thus in AX recognition (14, 15). Therefore, efforts are been devoted to the identification of AX-carrier proteins and their potential role in the allergic process. Most studies to date have been directed to the identification of AX-target proteins in plasma or serum (16). Early studies reported penicillin addition to specific lysine residues on HSA (17), and later transferrin was identified as an ampicillin target. Subsequently, circulating albumin-hapten complexes formed by piperacillin, benzylpenicillin and other β -lactams have been detected (18-20). In recent studies we identified HSA, transferrin and light and heavy chains of immunoglobulins, as targets for haptentation by AX in

serum in vitro (7), with HSA Lys190, being the only site for haptenation identified in ex vivo samples (21). In theory, drugs in general and AX in particular could also form adducts with cellular elements, either in the circulation or along the administration route. Nevertheless, the identity and fate of the cellular targets of AX are poorly understood. To this aim, we have developed and validated immunological methods using specific anti-AX antibodies (22), as well as a biotinylated AX derivative which facilitates the detection of adducts with superior sensitivity, using analog concentrations closer to the expected pharmacological AX concentrations (23). Here we have addressed the identification and subcellular distribution of AX-haptenated proteins in B-cells, a cell type that can be involved in antigen presentation (24, 25). Our results show for the first time that several intracellular proteins are selectively haptenated by AX and secreted both in soluble as well as in extracellular vesicular (EV) fractions, which can participate in the intercellular transfer of AX-adducts. Taken together, these results provide a whole set of novel structures potentially involved in sensitization to AX, as well as new working hypothesis for the development of drug allergy through the vehiculization and/or processing of drug adduct-bearing exosomes.

Materials and methods.

Reagents

AX-B was prepared as previously described (23), Amoxicillin (Clamoxyl) was from GlaxoSmithKline. Enhanced chemical luminescent reagent (ECL) and Horseradish Peroxidase (HRP)-Streptavidin were from GE Healthcare. Sypro-Ruby and Ampholytes were from BioRad. Acetone was from Merck. Neutravidin beads were from Thermo Fisher. Iodoacetamide, protein G-agarose, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), N-hexanoyl-D-sphingosine (C6 ceramide), N-hexanoyldihydrosphingosine (dihydroceramide C6), GW4869, penicillin G, streptavidin-Gold particles of 10 nm Ø, anti-HSP70 and anti-β-Actin were from Sigma. Fluorescein-5-isothiocyanate (FITC), Alexa-488 streptavidin, Texas Red-streptavidin (TXR-Streptavidin) and anti-rabbit-Alexa 647 were from Molecular Probes. Antibodies against α-enolase (sc-100812), RhoGDI (sc-360) and ICAM-1 (sc-7891) were from Santa Cruz Biotechnology and anti-penicillin from AbDSerotec.

Cell culture and treatments

The lymphocytic B cell line RPMI 8866 was from Sigma-Aldrich. Bovine aortic endothelial cells (BAEC) were from Lonza. Cells were cultured in RPMI 1640 (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) or 10% calf serum, respectively, without antibiotics in a humidified atmosphere. For B-lymphocyte treatments, 8×10^7 cells per experimental condition were washed 4 times with 10 ml of serum-free medium to exhaustively remove serum proteins, resuspended in 3 ml of serum-free medium and treated overnight (o/n) with AX or AX-B at the indicated concentrations, before collection of cells and conditioned medium. In experiments to assess the secretion of AX-B-haptenated proteins, excess AX-B was removed by washing cells with serum-

free medium. Cells were then resuspended in 3 ml of serum-free medium and cultured for additional 24 h before cells and conditioned medium collection. Cell viability was assessed by trypan blue dye exclusion.

Sample preparation

Cells and conditioned medium were separated by centrifugation at 1200 x g 5 min at 4 °C. Cells were lysed in homogenization buffer as previously described (26). The conditioned medium was fractionated by sequential centrifugation. The extracellular membrane fraction was obtained by centrifugation at 15000 x g, 5 min at 4°C. The resulting supernatant was centrifuged at 200000 x g, 30 min at 4 °C to obtain the EV fraction (pellet) plus extracellular soluble proteins (supernatant). EV pellets were resuspended in 60 µl of PBS. Extracellular soluble proteins were subjected to ultrafiltration with Amicon Ultra-4 10K filters to remove unreacted AX or AX-B, and concentrated down to a final volume of 2 ml (conditioned medium Soluble Proteins).

Western blot and detection of adducts between AX or AX-B and cellular proteins

Cell lysates (30 µg of protein estimated by the BCA method), 30 µl of membrane fraction, 30 µl of exosomes and 60 µl of extracellular Soluble Proteins were analyzed by SDS-PAGE and Western blot as described (26). For 2D electrophoresis, cell lysates (200 µg of protein), 60 µl of EVs or 1.8 ml of Soluble Proteins fraction were precipitated with ice-cold acetone (27), dried, resuspended with 280 µl of 2D sample buffer (23, 28), and analyzed in duplicate. One gel was transferred to Immobilon-P membrane and the other stained with Sypro Ruby. AX-B-protein adducts were detected by blotting membranes with HRP-Streptavidin. AX-modified proteins were detected by incubation with anti-AX monoclonal antibody AO3.2 (7), and proteins of interest with the corresponding antibodies using ECL detection.

Proteomic analysis

After 2D electrophoresis, spots positive for AX or AX-B were located in the Sypro-Ruby stained gels, processed by trypsin digestion and analyzed by MS (7). MS data were used to interrogate the NCBI non-redundant protein database SwissProt 2014_03 (542782 sequences; 193019802 residues) using MASCOT software 2.3 (Matrix Science).

Purification of AX-B modified proteins by avidin-based chromatography.

Biotinylated proteins from 500 μ l of extracellular Soluble Proteins fraction from control and AX-B treated cells were purified by adsorption onto Neutravidin beads under stringent conditions (28). Blots were developed with HRP-Streptavidin or antibodies against the proteins of interest.

Protein immunoprecipitation

The direct modification of the proteins of interest was assessed by immunoprecipitating samples from control or AX-B-treated cells with specific antibodies and detection of the associated biotin signal as described (29).

Electron microscopy

EVs were analyzed by electron microscopy (EM) (30). Briefly, 5 μ l of exosomes fixed with 2% PFA were adsorbed onto Formvar-carbon coated EM grids, washed, fixed and contrasted. Then, samples were blotted with methyl cellulose. Grids were inspected for gold particles associated to EV under a JEOL 1230 electron microscope operated at 100 kV. Micrographs were recorded at 40000x magnification. Associated biotin was detected by EVs incubation with Streptavidin-Gold prior to fixation.

Fluorescent detection of AX-B incorporation into B-cells

RPML 8866 cells (5×10^5) in glass-bottom 35 mm cell culture dishes (MAT-TEK Corporation) were treated with 0.5 mg/ml AX-B o/n in serum-free RPML1640, washed with PBS and fixed with 4% PFA. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS, blocked with 3% BSA in PBS and incubated with Alexa-488 streptavidin 1:500 for 1 h at r.t. Nuclei were stained with DAPI. AX-B incorporation into B-lymphocytes was visualized by confocal fluorescence microscopy in a Leica TCS SP2 confocal laser spectral microscope, as described (31).

Internalization of AX-B-modified EVs into BAEC

EVs isolated from control or AX-B-treated cells were resuspended in PBS and consecutively labeled with 100 μ M FITC in PBS for 1 h at r.t. as a tracer, and Streptavidin-Texas Red (1:200) for 30 minutes at r.t. to specifically detect biotin moieties. Labeled EVs were added to the incubation medium of BAEC cultured in glass-bottom dishes for 3 h. Cells fixed as above were subjected to immunofluorescence detection of RhoGDI, as a cytoplasm marker, as described (28). Incorporation of EVs into BAEC was assessed by confocal fluorescence microscopy and analyzed for each section.

Statistics

Results are represented as mean $\pm$ SEM of at least 3 independent experiments. Differences between experimental conditions were analyzed by non-parametric test when normality tests were not supported by the data. For normal data population, ANOVA statistical analysis was applied. Experimental conditions were considered different when $p < 0.05$.

Results

β -lactams bind selectively to intracellular proteins.

Current knowledge on intracellular AX targets is limited. We recently showed that the pattern of AX-haptenated proteins is cell-type-dependent (23). To identify AX cellular targets we chose a lymphocytic B cell line, RPMI 8866, because B lymphocytes can act as APC in the allergic response (32) and this cell line possesses the molecular machinery for antigen presentation (33). Incubation of B-lymphocytes with Penicillin G, AX or AX-B generated protein adducts, although with non-superimposable patterns (figure 1A). AX-B allowed the detection of protein adducts with higher sensitivity. Moreover, we confirmed AX-B entry into cells by confocal microscopy (figure 1B), which showed a particulate localization of the biotin signal compatible with vesicular traffic and processing.

Detection of AX-B haptenated proteins in B-lymphocytes.

To obtain a global perspective of AX adduct formation in the cellular context we analyzed several fractions from control and AX-B-treated B-cells and their conditioned medium. We detected a wide number of haptenated proteins in 1D gels of the various fractions, with common or selective biotin-positive bands, marked by arrow heads and asterisks, respectively (figure 2A). Several bands were present in extracellular fractions (EVs and soluble proteins), suggesting that some modified proteins could be secreted from cells (see below). To confirm fractionation adequacy, blots were probed with antibodies against proteins characteristic of each compartment: anti-ICAM-1 (immune cell membranes and EVs (34)), anti-actin (cytoskeleton), anti- α -enolase (cytoplasm) and anti-Rho-GDI (cytosol).

AX-B-haptenated proteins were further analyzed by IEF 2D SDS-PAGE of the Soluble Extracellular Proteins (figure 2C). Staining with HRP-streptavidin showed multiple

specific biotin-positive spots in AX-B samples, whereas only endogenous biotinylated proteins were present in control samples, consistent with the results of 1D gels.

AX and AX-B share a closely related haptenation pattern.

To exclude potential interferences of the biotin moiety with the haptenation process, we explored whether AX and AX-B induced similar haptenation patterns before proceeding to the identification of AX-B-haptenated proteins. Figure 2B shows the patterns of AX-haptenated proteins obtained by immunological detection, which as shown above, requires treating cells with higher concentrations of antibiotic. This approach confirmed the presence of AX-haptenated proteins in all fractions obtained, with a distribution similar to that obtained for AX-B. Validation of the fractions obtained was carried out as in figure 2A.

Coincidence of AX and AX-B-haptenated proteins was confirmed by 2D-electrophoresis and immunoblot of the soluble extracellular fraction. Equivalent spots positive for AX-B or AX are depicted by circles in figure 2C. The patterns of total protein staining with Sypro-Ruby of soluble fractions from AX and AX-B samples were also superimposable.

These results support the use of AX-B as a “bona fide” tool to study protein haptenation by AX.

Identification of proteins haptenated by AX/AX-B.

To identify the protein targets for haptenation by AX and AX-B, the spots marked in figure 2 were analyzed by peptide fingerprinting by MALDI-TOF MS. The main targets identified (detailed in Table 1) include HSP70, EF-2, actin and α -enolase. Although all of them are fairly abundant proteins, the biotin signal observed for each protein did not directly correlate with their abundance, indicating a certain degree of selectivity. Neither

BSA, nor other components of bovine serum were identified in this screen, thus showing the efficacy of the washing steps.

Avidin-based affinity chromatography followed by Western blot (figure 3A) confirmed the presence of EF-2, HSP-70, β -Actin and α -enolase in the fraction eluted from avidin, but only from AX-B-treated cells, indicating that these proteins are modified by AX-B. Moreover, immunoprecipitation showed positive biotin signals associated with EF-2, α -enolase and HSP70 (figure 3B). Haptenation of actin could not be evaluated by this approach due to poor performance of the antibody. Taken together, our results unequivocally show the haptenation of intact cellular proteins by AX-B, as well as their presence in the conditioned medium of AX-B-treated B-lymphocytes.

AX-haptenated proteins are actively and selectively secreted by B-lymphocytes.

Because haptenated proteins found in the extracellular medium of B-lymphocytes could behave as “allergenic messengers”, we explored whether they were haptenated intracellularly, and subsequently secreted, or extracellularly. For this, AX-B-treated B-lymphocytes were extensively washed to remove the extracellular proteins and any unreacted AX-B, and incubated with fresh medium o/n, after which, conditioned medium was collected and analyzed to detect released intracellularly-haptenated proteins. This showed that haptenated proteins were released from cells with time (figure 4A). Finally, cells were lysed and conditioned medium was fractionated as above. This confirmed the presence of haptenated proteins both in EVs and in extracellular soluble proteins (figure 4B, upper panels). A role for cytotoxicity in protein and/or vesicle release seems unlikely since cell viability was not affected by treatments or cell culture conditions (Ct: 2.92 % $\pm$ 0.57 %; AX-B: 2.9 % $\pm$ 1.43%, mean $\pm$ SEM of three determinations). Moreover, the patterns of markers for the different fractions do not support the non-specific release of cytosolic proteins, like RhoGDI, which is not detectable in the extracellular soluble fraction, whereas α -enolase appears selectively

enriched in this fraction, consistent with a potential active secretion process (figure 4B, lower panels).

This supports that proteins can be haptenated by AX-B intracellularly and exported either as EVs cargo or in soluble form. The nature of EVs bearing AX-B-haptenated proteins was studied by EM (figure 4C), which showed vesicles with size and morphology typical of exosomes (35). Exosome secretion can be regulated by multiple mechanisms, including those dependent on the complex endosomal sorting machinery, tetraspanins and the lipid composition of the endosomal membranes. Treatment with C6 ceramide or the neutral sphingomyelinase inhibitor GW4869 induces or represses exosome secretion, respectively (36). Interestingly, we observed that EVs fractions from cells treated with C6 ceramide displayed a higher content of AX-B-haptenated proteins, as well as of the exosomal markers ICAM-1 and HSP70 (figure 4D).

Dihydroceramide C6, which is structurally similar to C6 ceramide but does not stimulate exosomal release, was without effect. Conversely, EVs from B-lymphocytes treated with GW4869 showed reduced levels of AX-B-haptenated proteins and ICAM-1 (figure 4D). Therefore, these results point to a direct relationship between the abundance of AX-B-haptenated proteins in EVs and exosome release.

Identification of AX-haptenated proteins in exosomal fractions

Analysis of EVs by 2D-electrophoresis showed that although the pattern of biotin-positive spots was not identical to that of soluble proteins, the main spots were coincident (figure 4E). Identification of these AX-B targets by proteomic techniques (Table 1) and Western blot of the membranes from 2D gels (data not shown), confirmed that α -enolase, EF-2 and HSP70 were also secreted in exosomes as AX-B-haptenated proteins. Incorporation of AX-B into exosomes was directly evidenced by incubation of secreted EVs with streptavidin-gold beads and EM (figure 5A). These

results unveil the relevance of exosomes as carriers of proteins haptenated intracellularly.

Nevertheless, exosomes from various cell types circulate in plasma in health and disease (37, 38). Therefore, besides the serum proteins previously identified as AX-carriers, components of exosomes already present in the circulation could also be haptenated by AX or other drugs. Thus, we explored the formation of adducts between AX-B and proteins in exosomes from control cells *in vitro*, and compared the pattern of haptenated proteins with that of exosomes secreted by AX-B-treated cells (Fig. 5B). AX-B bound to proteins in isolated exosomes, although the profile of biotin-positive polypeptides showed differences in the intensity of some bands compared to that of secreted exosomes. These observations suggest that exosomes may be relevant carriers of haptenated proteins by various mechanisms.

Haptenated proteins packed in exosomes can be imported by target cells.

Finally, we evaluated the potential role of exosomes as intercellular carriers of AX-B-haptenated proteins. For this, EVs obtained from control or AX-B-treated B-lymphocytes as in figure 4C were labeled with FITC, as tracer, and incubated with TXR-streptavidin, to specifically visualize the biotin moiety. These EVs were added to the culture medium of BAEC as a relevant cellular model, since aortic endothelial cells have been shown to act as “non-professional” APC (39), as other endothelial cell types (40-42). Remarkably, confocal microscopy revealed fluorescent vesicles at different stages of internalization, appearing either at the cell periphery, or in perinuclear localization, compatible with endosomal compartmentalization (figure 5C). Importantly, EVs from control RPMI8866 cells showed only green fluorescence (FITC labeling), whereas EVs from AX-B treated RPMI8866 cells showed also a distinctive red fluorescence, reporting the presence of AX-B-haptenated proteins. These results show that EVs from B-lymphocytes, carrying AX-B-haptenated proteins had been

incorporated into endothelial cells. Altogether these results represent the first evidence of AX-haptenated proteins packed in exosomes and delivered into target cells, raising the possibility that exosome-mediated hapten delivery may also contribute to the modulation of the allergic response to AX.

Discussion

It is commonly accepted that due to its small size, AX antigenicity should be conditioned to haptening of the patient's proteins (9). However, the responsible antigenic determinants and the mechanisms involved remain unclear (6). Importantly, clinical data suggest that structural determinants from the carrier protein(s) may also play a role in the recognition of AX-derived structures by the patient IgE (14). Therefore, to achieve better diagnostic and potentially therapeutic strategies, it is important to characterize AX carriers in detail. Besides the serum proteins identified as AX-targets (7, 23), cellular components could also be haptened by AX.

Here we report the first identification of intracellular targets haptened by AX, their active secretion either as soluble proteins or packed in exosomes, and the incorporation of the carrier vesicles into target cells. These proteins represent novel candidates for diagnostic clinical markers. Remarkably, AX-allergic patients can show a selective response to AX or a broader response towards other β -lactams (43).

Identification and characterization of the common and specific targets for various β -lactams could afford insight into the mechanisms underlying the selectivity of the allergic response.

Notably, the pattern of targets modified by AX and AX-B were quasi identical. This, together with their similar reactivity (23), has allowed us to use AX-B as a "bona fide" tool to identify targets for haptening by AX, taking advantage of its higher sensitivity. Targets reported in this work are fairly abundant proteins. Further work will be needed to identify less abundant proteins potentially relevant for the allergic process or of diagnostic interest.

Importantly, we have detected intact haptened proteins in cells conditioned medium and determined that haptening can take place inside cells and modified targets can

subsequently be secreted either as soluble proteins or in EVs. To the best of our knowledge this is the first study showing secretion of drug-protein adducts in exosomes, which could have important consequences for drug adverse effects, particularly, β -lactam-induced allergic reactions. Most interestingly, a common set of AX-targets including EF-2, HSP70 and α -enolase are secreted from cells both in soluble and exosomal fractions. The selective modification of these targets may imply that they possess structural features favorable for AX binding. In future works we will explore these factors and confirm whether these proteins are also haptenated by AX in other cell types or in the circulation. Interestingly, HSP70 and α -enolase from several species are well-known allergenic proteins for humans (44-46). It would be tempting to speculate that drug modification of the endogenous proteins may render them allergenic as well.

Importantly, modulation of exosome shedding impacts the presence of AX-B-haptenated proteins in EVs, indicating that exosomes play a key role in transport of AX-B-modified proteins. Here we have confirmed that exosomes carrying AX-B-modified structures can be incorporated into target cells, thus transferring their “information”. Although the fate of internalized exosomes is beyond the scope of this study, theoretically they could supply preformed MHC-peptide complexes or undergo degradation yielding haptenated peptides for antigen presentation (47).

Exosomes act as “messenger” vesicles involved in intercellular transfer of biological molecules (48, 49), modulating physiological and pathophysiological processes (50, 51). Therefore, haptenated proteins could putatively be transported by these vesicular carriers to induce the allergic response in distant cells. Our results show that haptenation can also occur in isolated exosomes in vitro. Consequently, the possibility exists that AX could haptenate proteins present in circulating exosomes derived from

diverse cell types (50-52). This widely opens the array of potential candidates contributing to the formation of AX antigenic determinants.

Therefore, our results highlight a vesicular pathway for delivery of exosome-encapsulated, newly identified AX-haptenated proteins that could potentially contribute to the allergic response.

Acknowledgements

We are indebted to María Jesús Carrasco for her invaluable technical support. We thank the staff of Centro de Investigaciones Biológicas Cellular Culture, Electron Microscopy, Confocal Microscopy and Proteomic facilities, as well as the Proteomic Unit from Universidad Complutense de Madrid.

Funding

This work was supported by grants SAF2015-68590R, SAF2012-36519 from MINECO, Spain and RIRAAF/RD12/0013/0008 from ISCIII to D.P-S.; by grants CTQ2013-41339-P from MINECO, Spain and RD12/0013/0003 from ISCIII to E. P-I.; and by grants PI12/02529 and PI15/01206 from MINECO- grant cofunded by ERDF, Spain, RIRAAF/RD12/0013/0001 from ISCIII, CTS-06603 from Junta de Andalucía , grant cofunded by the ERDF, PI-0545-2010, PI-0551 and PI-0699-2011 from Junta de Andalucía to M.J.T and M.B.

Author contributions

FSG and JMGM performed experiments and analyzed data

YV and EPI prepared and characterized biotinylated amoxicillin and critically read the manuscript.

MB and MJT critically read the manuscript and provided insightful information and discussion valuable for design and interpretation of data.

FSG and DPS designed the study, analyzed data and wrote the manuscript.

Conflict of interest

The authors of the manuscript state that there are no conflicts of interest

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Figure legends.

Figure 1. Haptenation of B-lymphocyte proteins by β -lactams. A. RPMI 8866 B-lymphocytes were treated with 10 mg/ml of Penicillin G, 2.5 mg/ml of Amoxicillin or 0.5 mg/ml of AX-B for 16 h in RPMI1640 medium without serum. The concentrations of the antibiotic used were chosen depending on the sensitivity of the corresponding detection tool. Thirty micrograms of protein from cell lysates were analyzed by Western blot to detect the formation of the corresponding adducts or the biotin signal. Representative band patterns of at least 3 different experiments for every condition are depicted. B. B-lymphocytes were incubated with 0.5 mg/ml AX-B for 16 h in RPMI1640 medium without serum. Cell preparations were incubated with Alexa-488-Streptavidin as described in Material and Methods to detect the biotin signal. Nuclear DNA was stained with DAPI. Scale bar, 10 μ m.

Figure 2. Detection of AX-B- and AX-haptenated proteins in cells and conditioned medium. Cells were treated with 0.5 mg/ml AX-B (A) or 2.5 mg/ml AX (B) for 16 h, as above in RPMI1640 medium without serum. Cells were harvested and conditioned medium collected and fractionated to analyze extracellular Membrane fraction, Extracellular vesicles and Soluble Proteins by SDS-PAGE and Western blot. Arrowheads mark biotin-positive bands that appear in all fractions. A polypeptide band that appears only in cell lysates is indicated with an asterisk. Blots were incubated with HRP-Streptavidin to detect the biotin signal, and with antibodies against the indicated proteins to confirm the accuracy of the fractionation procedure. Representative blots of at least 3 experiments are depicted. C. Soluble proteins present in the conditioned medium of cells treated with 0.5 mg/ml AX-B or 2.5 mg/ml AX were analyzed for the formation of adducts with AX-B or AX by 2D SDS-PAGE as described in material and methods. Blots incubated with HRP-Streptavidin or anti-AX antibody A03.2 for biotin or AX adduct detection, respectively, and gels stained with Sypro Ruby for protein detection are depicted. Representative images for at least 3 experiments are shown.

Numbers indicate spots that are positive both for AX and AX-B haptenation and were selected for MS analysis.

Figure 3. Validation of AX-B targets identified by MS by avidin affinity

chromatography and immunoprecipitation. A. Soluble proteins isolated from conditioned medium of control and AX-B-treated cells were purified by avidin-based affinity chromatography and analyzed by SDS-PAGE. The presence of biotin and proteins of interest in the avidin-bound fraction (eluate) were assessed by incubation of the blots with HRP-Streptavidin or the corresponding antibodies. B. Proteins of interest were immunoprecipitated with the indicated antibodies. The immunoprecipitates were analyzed for the presence of a biotin signal associated with the corresponding proteins and of the proteins of interest with the indicated antibodies. In the case of Hsp70, TrueBlot® was used for detection. Representative blots from 3 different experiments are shown.

Figure 4. Exosomes from AX-B-treated B-lymphocytes bearing haptenated

proteins are secreted by cells. A. Control and AX-B-treated cells were extensively washed to eliminate unreacted AX-B from the medium. Conditioned medium collected at 0 (T_0) and 16 h (T_{16}) of an o/n incubation period was analyzed for biotin positive polypeptides by SDS-PAGE and Western blot. A representative blot from three different experiments is shown. B. Exhaustively washed AX-B-treated cells were then cultured in serum free medium for an additional 24 h period. Both, cells and conditioned medium were collected and fractionated to analyze the indicated fractions. Blots were incubated with HRP-Streptavidin to detect the biotin signal, and with the indicated antibodies to assess the accuracy of the fractionation. Representative blots from three experiments are depicted. C. EVs isolated from conditioned medium of B-lymphocytes were observed by EM as described in Materials and Methods. Typical vesicle shapes are depicted in the representative micrographs shown. Scale bar, 200

nm. D. B-lymphocytes were treated with or without AX-B for 16 h, the unreacted excess of AX-B washed out and the cells treated with 10 μ M C6 ceramide, 10 μ M dihydroceramide C6 or 5 μ M GW4869 for additional 24 h. The EVs fraction from conditioned medium was analyzed by Western blot to detect the biotin signal, as well as ICAM-1 and HSP70 as exosome markers. Representative blots from three different experiments are shown. E. B-lymphocytes were treated in absence or presence of 0.5 mg/ml AX-B, the unreacted antibiotic was exhaustively washed and the newly secreted EVs, isolated as described in Material and Methods. The complete EVs fraction was precipitated and processed to analyze biotin positive proteins by 2D SDS-PAGE. A replicate sample was used to detect total protein by Sypro Ruby staining. The AX-B targets previously identified in the extracellular soluble protein fraction were located in the blots and gels of EVs fraction and identified by MS. Spots excised are numbered in the figure. Spots 2, 3 and 4 correspond to α -enolase, EF-2 and Hsp70, respectively.

Figure 5. Secreted EVs bear and deliver AX-B-modified proteins to target cells. A.

Secreted EVs isolated from conditioned medium of B-lymphocytes treated with or without 0.5 mg/ml AX-B were incubated with 10 nm Streptavidin-gold particles and visualized by EM as described in Materials and Methods. Particles bound to vesicles are indicated by arrowheads. Representative images from three different experiments are shown. Scale bar, 200 nm. B. B-lymphocytes were treated with or without 0.5 mg/ml AX-B and the secreted EVs isolated as described in Material and Methods. Control EVs were then treated with 0.5 mg/ml AX-B *in vitro* for 16 h and compared with EVs secreted by AX-B-treated cells by SDS-PAGE. Anti-ICAM-1 was used as an exosome marker. The biotin signal was densitometered and represented to compare the band patterns of biotin positive polypeptides. Dotted line, *in vitro* AX-B labeled EVs; solid line, EVs obtained from AX-B-treated cells. Representative blots from three different experiments are shown. C. Secreted EVs from control or AX-B-treated B-lymphocytes were isolated and labeled with FITC and TXR-Streptavidin. Endothelial

cells were then incubated with either EVs preparation for 3 h, fixed and stained with anti-RhoGDI. Cells were observed by fluorescence confocal microscopy to analyze the presence of EVs internalized by endothelial cells. Representative images of three experiments are depicted. Scale bars, 20 μm .

Table 1. MS identification of selected AX-B-modified proteins from extracellular Soluble Proteins and EVs fractions.

Fraction	SPOT	Acc. Number	Gene name	Score	Limit score	MW	Isoelectric point
Soluble protein	1	P60709	β -actin (ACTB)	351	59	40536	5.55
Soluble protein	2	P07437	β -tubulin (TUBB)	246	59	50096	4.75
Soluble protein	3	Q71U36	α -1A Tubulin (TBA1A)	317	59	46797	4.96
Soluble protein	4	Q53GZ6	HSP70	157	59	71083	5.28
Soluble protein	5	P13639	EF-2	100	59	96246	6.41
Soluble protein	6	P06733	α -enolase (ENOA)	162	59	47453	7.01
EVs	1	P08133-2	Isoform 2 of Annexin A6	73	59	72720	5.42
EVs	2	P06733	α -enolase (ENOA)	83	59	47453	7.01
EVs	3	P13639	EF-2	195	59	96246	6.41
EVs	4	Q53GZ6	HSP70	69	59	71083	5.28
EVs	5	P50990	T-complex protein 10	139	59	60153	5.42

Results obtained for the identification of AX-B modified proteins from extracellular Soluble Proteins and EVs fractions picked from 2D Sypro Ruby-stained gels. The fraction, number of the spot, accession number, gene name, experimental score, limit score, molecular weight and isoelectric point are included. Limit score refers to the minimum score for an identification to be considered positive by MASCOT software 2.3 analysis.

Figure 1.

A

B

Figure 2.

Figure 3.

A

B

Figure 4.

Figure 5.