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RESEARCH ARTICLE

Prenylated chalcone xanthohumol associates with histones in breast cancer cells–a novel target identified by a monoclonal antibody

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Scope: The intracellular fate of xanthohumol (XN) from hops is an underexplored field in the research for the molecular mechanisms causing its wide range of effects in chemoprevention and gene expression involved in hepatic metabolism.
Methods and results: We aimed to elucidate possible targets for binding of XN in a human mammary carcinoma cell line (MCF-7/6), using a mAB. We investigated the overall solubility and stability of XN in growth medium and the cellular uptake and distribution of XN in MCF-7/6 cells using an optimized immunocytochemistry technique. After incubation of MCF-7/6 cells, with 10 μM XN for 0.5 h up to 6 h, we observed primarily a granular nuclear staining, which intensified with increasing exposure times. Immunoprecipitation of cell lysates (treated with 10 μM XN for 2 h) revealed binding of XN to a fraction of proteins with a molecular weight below 20 kDa. Further analysis of the protein mixture via LC-MS/MS (Q-TOF) resulted in the identification of specific members of the histone family, i.e. histone H2A, H2B, and H4. The identity of histone H2A was confirmed using immunodetection with a specific anti-histone H2A antibody.
Conclusion: In summary, we did successfully apply a mAB against XN in immunocytochemistry and precipitation with highly unexpected results.

Keywords:
Histones / Immunocytochemistry / Immunoprecipitation / MCF-7/6 / Xanthohumol

1 Introduction

Xanthohumol (XN) (3′-[3,3-dimethyl allyl]-2′,4′,4-trihydroxy-6′-methoxychalcone), a yellowish prenylated chalcone, was first isolated in 1913 by Power et al. [1] from the resin of hops (the female inflorescences of the hop plant (*Humulus lupulus* L.)) and later identified as a new natural chalcone by Verzele et al. [2]. It is the most important prenylflavonoid in hop (from 0.1 up to 1% (w/w)) and is, therefore, present in beer and hop-derived dietary supplements targeting the relief of menopausal symptoms [3]. Nevertheless, in beer, the concentration of XN is low (e.g. 28 μg/L in European-style pilsner), due to low solubility and thermal isomerization to the more soluble prenylflavanone isoxanthohumol during the brewing process [4]. Isoxanthohumol is a pro-estrogen as it can be further converted into the potent phytoestrogen 8-prenylnaringenin by demethylation effected by liver enzymes or intestinal gut bacteria as described by Nikolic et al. [5] and Possemiers et al. [6]. Hops are mainly used as a flavoring agent in beer, but over the last decade the health beneficial properties of hop-derived terpenophenolics have gained increased attention, including the possible use of the prenylflavonoid XN as a chemopreventive agent. The news that the drinking

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Abbreviations: **4-HBPH**, 4-hydroxybenzophenone; **CAPS**, *N*-cyclohexyl-3-aminopropanesulfonic acid; **DAPI**, 4′ 6-diamidino-2-phenylindole; **IP**, immunoprecipitation; **TBS**, Tris-buffered saline; **XN**, xanthohumol

of beer could have positive effects on mutagenesis and DNA adduct formation triggered the start of the search for possible constituents in beer with chemopreventive actions [7]. Miranda et al. [8] initiated the research into the anti-cancer bioactivity of XN, by screening it in a number of human cancer cell lines. XN was further described to inhibit metabolic activation of procarcinogens through P450 enzymes [9, 10] and also to induce Phase 2 enzymes that can help in the detoxification of carcinogens [11,12]. Gerhauser et al. [13] confirmed the broad-spectrum chemopreventive activity of XN, acting by multiple mechanisms at the stages of initiation, promotion, and progression of cancer. In particular, besides the Phase I and II modulation of carcinogen metabolism, they established that the anti-inflammatory properties of XN by inhibition of cyclooxygenase enzymes, also contribute to the potential antitumor-promoting mechanisms of the molecule. Van Hoecke et al. [14] described the anti-invasive properties of XN in vitro with breast cancer cell lines, mediated by the upregulation of the E-cadherin/catenin complex. Additionally, the prevention of oxidative damage by scavenging reactive oxygen species [15] and nitrogen oxide production [13, 16], but on the other hand also potential prooxidative properties of XN may trigger the antiproliferative mechanism and the induction of apoptosis [17,18]. Obviously, in the area of cancer prevention, this chalcone has already been extensively studied and proved to be a particularly interesting molecule, at least in vitro. Furthermore, XN can serve as an anti-microbial and -viral agent [19–22], promote the osteogenic differentiation [23], ameliorate metabolic disorders [24–26], and has anti-inflammatory capacity. The latter can be mediated through nuclear response factor 2-antioxidant responsive element signaling [27], or by the inhibition of the activation of transcription factor nuclear factor-kB [28] and inhibition of the expression of nuclear factor-kB-dependent proinflammatory genes [29, 30]. Any possible therapeutic application is highly dependent on the overall bioavailability of XN. Caco-2 cell experiments showed that, although the intestinal absorption of XN is extremely low, it accumulates in the cytosol of intestinal cells due to high protein binding [31]. Its low intestinal absorption is also reflected in the urinary excretion after oral administration, which tends to be very low and seems to be dose dependent [32, 33]. Furthermore, XN is susceptible to microbial degradation and transformation in the intestine, in vitro experiments also showed glucuronidation and sulfatation in the liver as well as in the intestine and P450 enzymes are responsible for its degradation [34–37]. Although several molecular working mechanisms have been suggested and most of the publications have described downstream effectors, especially the characterization of the primary molecular target of XN is an underexplored field of research. The aim of the present study was to investigate the cellular localization of XN and to identify possible intracellular binding partners in a human breast cancer cell line (MCF-7/6). For this purpose, a recently developed specific monoclonal antibody against XN was used in combination with immunocytochemical and precipitation techniques.

2 Materials and methods

2.1 Chemicals

XN was available in our laboratory [6] and the mouse anti-XN IgG_1 antibody was an antibody manufactured in-house as described previously in Wyns et al. [38]. BSA, *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS), 4′ 6-diamidino-2-phenylindole (DAPI), Tween-20, and Nonidet P-40 were purchased from Sigma-Aldrich (St. Louis, MO, USA). PBS, Tris-buffered saline (TBS), trypsin/EDTA, fetal calf serum, DMEM, HAM's F12, NuPage lithium dodecyl sulfate sample buffer, penicillin, streptomycin, goat anti-mouse IgG Alexa 594 and Sypro Ruby Protein gel staining were supplied by Invitrogen (Carlsbad, CA, USA) and amphotericin B by Bristol-Meyers Squibb (Brussels, Belgium). NaCl, Tris, and 4-hydroxybenzophenone (4-HBPH) were purchased from Acros Organics (Morris Plains, NJ, USA) as well as all chemicals and solvents (all of analytical reagent grade) for the analytical work. A 2D Quant Kit for protein concentration quantification was purchased from GE Healthcare (Uppsala, Sweden) and Protein G agarose for immunoprecipitation (IP) was obtained from Pierce (Rockford, IL, USA). Modified sequence grade trypsin (porcine) was purchased from Promega (Madison, WI, USA). Other materials and equipment for gel electrophoresis were obtained from Bio-Rad (Hercules, CA, USA). The solvents for the liquid chromatography were all of LC-MS quality (Biosolve, Valkenswaard, The Netherlands).

2.2 Cell culture

The human mammary adenocarcinoma (MCF-7/6) cell line (from a pleural effusion of mammary adenocarcinoma), was kindly provided by Dr. H. Rochefort (Unité d' Endocrinologie Cellulaire et Moleculaire, Montpellier, France). This subline differs from the parental MCF-7 cell line because it has a non-functional E-cadherin complex and can express non-muscle myosin IIA [39]. These cells were grown and cultured in a 50:50 (v/v) mixture of DMEM/HAM's F12 with 10% heat-inactivated fetal calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. The cells were incubated in a 100% water-saturated atmosphere with 10% CO_2 at 37°C.

2.3 Quantification and stability of XN in cell culture medium via HPLC-UV

The overall solubility of XN after supplementation to the cell culture medium was investigated, as well as the stability of XN in the medium at the culturing temperature of 37°C. Growth medium (at 37°C) was spiked with a solution of XN in 100% ethanol, so that final concentration of 10 μM was reached. Reference samples, spiked with a known amount

of XN in ethanol and also blank samples (medium without XN) were included. The culture media, spiked with XN were kept at 37°C for different time periods (30 min, 1 h, 2 h, 4 h, 6 h, and 24 h). Each time point was tested in triplicate. Afterward, all the samples were spiked with 100 μL of internal standard (4-HBPH; 1 mg 4-HBPH in 10 mL MeOH) and extracted three times with ethyl acetate. The organic solvent layers were evaporated under a gentle nitrogen stream. The resulting residue was dissolved in 500 μL MeOH/H_2O (50/50) + 0.025% formic acid and 20 μL was injected in the HPLC for analysis. For the chromatography, a Waters Alliance 2695 Separation Module (Waters, Milford, MA, USA) equipped with a Waters 996 DAD was reported by Possemiers et al. [6]. Detection was done simultaneously at 370 nm for the quantification of XN and at 295 nm for the internal standard. Data processing was performed with the Waters Millenium 3.2 software.

2.4 Cellular uptake of XN in MCF-7/6 cells

MCF-7/6 cells were seeded in 6-well plates (Nunc, Roskilde, Denmark) at a density of 5×10^4 cells/mL on day one. On day three, half of the medium was aspirated and fresh medium spiked with XN (dissolved in 100% EtOH) was added, so that the final concentration in the well was 10 μM. The cells were incubated for different time periods (30 min, 1 h, 2 h, 4 h, 6 h, and 24 h). A blank control (100% EtOH without XN) was included in each experiment and each time point was set up in six replicates. After the end of the treatment period, the medium was aspirated and transferred to a tube. Then, each well was washed twice with 1 mL of medium and each wash was collected in the respective tube. After three further washing steps with 1 mL PBS, 100 μL lysis buffer (150 mM NaCl–1% Nonidet P-40–50 mM Tris-HCL; pH 7.4) was added to each well and the plates were placed on ice for 30 min. After lysis, the cells were scraped of the plate and washed with 900 μL PBS and the resulting lysates were frozen at −20°C until analysis. Before analysis, the collected medium from the wells and the lysates were all spiked with 100 μL internal standard and subjected to a solvent–solvent extraction with ethyl acetate that was repeated three times. The pooled organic solvent from the triple extraction step was dried under a gentle nitrogen stream and the residue was dissolved in 200 μL methanol. Twenty microliters of each sample was analyzed with HPLC-UV as described in section 2.3.

2.5 Immunocytochemistry

Round glass coverslips were placed in a 24-well plate and MCF-7/6 cells were seeded as a suspension with a density of 3.5×10^4 cells/mL on day one. On day three, half of the medium was replaced by growth medium supplemented with XN (dissolved in 100% EtOH) or an identical volume of 100% EtOH (blank), so that a final concentration in the well of 10 μM XN was reached. The ethanol concentration in the wells was 0.35% (v/v) for every condition. Treated cells were incubated for different time periods at 37°C (30 min, 1 h, 2 h, 4 h, 6 h, and 24 h). During the treatment, the cells were also visualized microscopically to monitor overall cell health. After aspiration of the culture medium, the cells were washed twice with PBS (37°C) and fixed for 20 min in 3% paraformaldehyde in PBS at room temperature. After fixation, the coverslips were washed in PBS and cells were permeabilized with 0.2% Triton-X after 10-min incubation with NH_4Cl. Then, after three washing steps with TBS, the fixed cells were blocked with 5% BSA in TBS for 30 min at room temperature. After the blocking step, blanks and positive controls were incubated with primary antibody, i.e. anti-XN antibody, overnight at 4°C. The negative control (cells treated with 10 μM XN for 6 h) was incubated with an isotype-matched irrelevant mouse IgG_1 antibody. The next day, after three washing steps, the coverslips were incubated with goat anti-mouse Alexa 594 antibody for 90 min at room temperature, and 5 min before the end samples were counterstained with DAPI (400 ng/mL). After washing, the coverslips were mounted in glycergel (Dako, Glostrup, Denmark) on a microscopic slide and kept in the dark at 4°C until evaluation. The immunocytochemical localization was optically verified with a Leitz Dialux 20 microscope (Leica, Wetzlar, Germany) using the NPL Fluotar 23 50/1.00 oil immersion objective. Afterward, confocal images were captured with a Leica Sp5 AOBS confocal microscope (Leica, Mannheim, Germany). Images were taken using a 63× HCX PL Apo 1.4 oil objective. DAPI was excited with an UV diode laser at 405 nm and Alexa 594 with an HeNe Laser at 543 nm. Z-sections were made with step size 0.5.

2.6 Sample preparation for IP

MCF-7/6 cells were seeded in culture flasks (25 cm^2) and when the cells reached approximately 70–80% confluency, they were treated with XN for 2 h at a final concentration of 10 μM. Blanks treated with ethanol (0.35% (v/v)) served as a negative control. After the treatment, the medium was aspirated and the cells were washed twice with ice-cold PBS, then the cells were lysed on ice for 30 min with a non-denaturing lysis buffer with a complete protease inhibitor cocktail (Roche, Penzberg, Germany). The cells were scraped from the flasks and the supernatant of the centrifuged lysates was stored at −20°C. The protein concentration of the lysates was determined using a 2D Quant Kit (Invitrogen, Carlsbad, CA, USA).

2.7 IP with an anti-XN antibody

The isolation and concentration of possible target proteins of XN was performed by IP with Protein G and a specific

in-house monoclonal anti-XN antibody. In particular, 40 μL settled resin (with Protein G cross-linked to agarose) was washed twice with 500 μL lysis buffer (see section 2.4 for composition). Lysates (500 μL) were pre-incubated under slow agitation with 100 μL antibody (1 mg/mL) for 1 h at 37°C, and then added to the beads. The samples with the beads were placed on a rocker overnight at 4°C. The next day, after five washing steps with 500 μL cold lysis buffer and collection of all the supernatants, the pellet of beads with bound proteins was recovered and saved for one-dimensional gel electrophoresis.

2.8 One-dimensional gel electrophoresis (1D-PAGE)

The samples from the IP, the supernatants from the washing steps in the IP and the original cell lysates were denatured with 40 μL NuPage lithium dodecyl sulfate sample buffer supplemented with 4 μL of freshly made 1 M DTT and incubated at 90°C for 5 min. After centrifugation, the denatured and reduced protein samples were loaded on a 15% or 18% Tris-HCl polyacrylamide gel (Biorad, Hercules, CA, USA) and electrophoresis was performed by applying 150 V for 30 min, followed by 200 V for 1 h. The gel was fixed for 30 min in a 10% MeOH, 7% acetic acid solution, and afterward the proteins in the gel were stained overnight using Sypro Ruby Protein Gel staining (Invitrogen). Protein patterns were scanned and digitized using the VersaDoc Imaging System (Biorad).

2.9 Western blotting

The 18% gel and nitrocellulose membranes (Bio-Rad) were incubated for 15 min in CAPS buffer (pH 11) with 20% MeOH and electrophoretic transfer of proteins was performed by tank blotting in a Trans Blot Cell (Biorad) with CAPS buffer at 120 V for 110 min. Successful protein transfer was checked using Ponceau S solution staining (Sigma).

2.10 Protein identification by LC-MS/MS (Q-TOF)

The in-gel porcine trypsin digestion of the proteins of interest from the IP was performed as described earlier [40]. Briefly, gel pieces were excised from the gel and washed twice in 50% ACN in 50 mM ammonium carbonate for 10 min. After reduction with 10 mM DTT at 56°C for 10 min and alkylation with 100 mM iodoacetamide for 45 min at room temperature (both in 50 mM ammonium carbonate), and succeeding washing steps, the pieces were dehydrated with MeCN using a Speed-Vac concentrator (Eppendorf, Le Pecq, France). Then, they were rehydrated with 10 ng/μL sequence grade-modified trypsin for 30 min on ice. After digestion overnight at 37°C, the liquid was collected and the resulting peptides were extracted with 50% ACN followed by an extraction with 100% ACN. The pooled extract was finally completely dried using the Speed-Vac concentrator. Prior to analysis, peptides were resuspended in H_2O + 0.1% formic acid and were then analyzed and identified by LC-MS/MS, using a Q-TOF Ultima Mass spectrometer (Waters). The data were processed using Mascot distiller and searched against the Swissprot human database, using the in-house Mascot daemon searching algorithm. Identification was considered positive with a p-value < 0.05.

2.11 Histone H2A immunodetection

The blot was blocked with 1% BSA in 0.3% Tween-20 in PBS for 1 h and incubated with histone H2A rabbit anti-human polyclonal antibody (LifeSpan Biosciences, Seattle, WA, USA) at a concentration of 1:1000 in blocking buffer. Overnight incubation with the primary antibody was followed by three washing steps (5 min) and incubation with HRP-labeled goat anti-rabbit IgG (1:20 000; Pierce) (1 h). The ECL detection was carried out using Supersignal West Dura Extended Duration Substrate (Pierce) and protein patterns were scanned and digitized using the VersaDoc Imaging System (Biorad).

3 Results

3.1 Solubility and stability of XN in in vitro conditions

First, we investigated the solubility of XN in the culture medium under the conditions of cell culture. XN is a rather apolar molecule, with very poor solubility in aqueous media, but dissolves well in ethanol and DMSO [3]. Since the organic solvent concentration in cell culture should be kept as low as possible, it was necessary to check for possible solubilization problems that could arise for XN during the experiments. Although the concentration of XN is quite low during our experiments (10 μM), we performed this investigation since good dissolution had to be assured. An extraction procedure for XN from cell culture medium was optimized and the final method existed of three solvent–solvent extraction steps of spiked medium (three concentrations) with ethyl acetate by intensive vortex mixing. The recovery of XN from the medium was for the 10 μM concentration 91.8% (± 7.9%). This extraction method was also used in the stability experiment with XN (10 μM incubated for 30 min, 1 h, 2 h, 4 h, 6 h, and 24 h). The recovery of XN from the growth medium for all time periods was good with an average of 83.3% (± 10.0%). No detectable degradation products were observed using HPLC-UV.

3.2 Measurement of the uptake of XN in MCF-7/6 cells

XN was added to the medium of the MCF-7/6 cells at a concentration of 10 μM and the cellular uptake was estimated by measuring the remaining concentration of XN in the medium at the end of each incubation period (Fig. 1). The

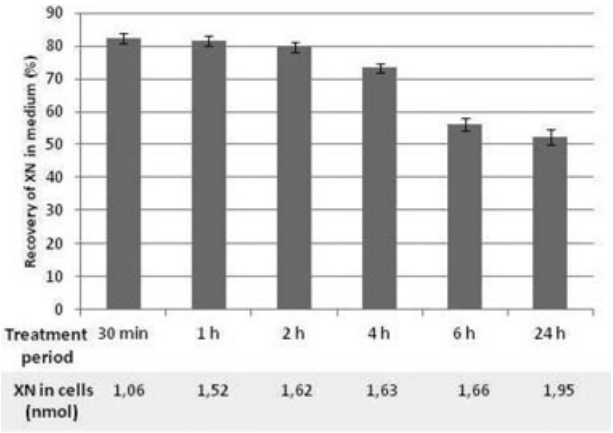

Figure 1. Recovery of XN (10 μM) in medium of MCF-7/6 cells and amount of XN retrieved in cells, after different incubation periods ($n = 6$; mean ± SD).

quantification of XN in the collected media from this experiment revealed that already after 30 min about 17.8% (± 1.49 %) of the original level XN has associated with the cellular fraction. This situation remains more or less stable until after 4 h, when 26.7% (± 1.5%) uptake is reached. After 6 h, about half of the original amount of XN can be retrieved in the medium of the cells and this does not change until the 24-h time point and end of the experiment. Figure 1 also shows the concentration of XN measured in the cell lysates from the uptake experiment. A slight increase in XN concentration over time is noticeable, but even the sum of these and the medium fractions, does not lead to the total dose of the administered XN. Hence, there must be another reason, e.g. loss during sample preparation, association with nonextractable cellular material or rapid biotransformation, for the discrepancy between XN concentrations in the medium and those in the cell fractions.

3.3 Immunocytochemical localization of XN in MCF-7/6 cells

Through indirect immunofluorescence, we investigated the subcellular distribution of XN inside MCF-7/6 cells. First of all, an optimal staining procedure had to be obtained for this experiment and several factors, including fixation method of the cells, concentrations of primary and secondary antibodies, temperatures, and incubation periods, were optimized. Finally, an optimized and reproducible procedure, described in section 2.5, was obtained. Preliminary experiments revealed that after a treatment period of 6 h with 10 μM XN, staining of XN in the cells could be obtained. A particularly granular pattern was observed with a high nuclear density, while a slight staining could be observed in the cytoplasm, with no specific organelle or cellular structure highlighted. The blank (vehicle control, stained with anti-XN antibody and secondary antibody) and the negative control (treatment with XN 10 μM 6 h, stained with unlabeled irrelevant mouse IgG1 and secondary antibody) gave no staining at all (Fig. 2). Furthermore, the same optimized staining procedure was repeated at least five times on cells that were treated for 30 min, 1 h, 2 h, 4 h, 6 h, and 24 h with 10 μM XN. As can be observed in Fig. 2, already after 30 min the same pattern as with the 6-h treatment can be observed, although with a weaker staining. Treatment with a concentration of 50 μM XN for the same time periods gave similar results (pictures not shown here). Confocal imaging showed that with increasing treatment period, the nuclear staining pattern only intensified and also emphasized that there was no staining of the nucleoli. These findings provided us with the information that XN was widely distributed in the nucleus. Nevertheless, several localizations like cytoplasmic and nuclear membranes, cytoskeletal structures, and certain organelles (e.g. ER, mitochondria . . .) could be excluded based on the typical granular and mainly nuclear pattern.

3.4 Identification of target-binding proteins of XN in MCF-7/6 cells

IP with an anti-XN antibody was used to isolate possible target proteins of XN in MCF-7/6 cells. The proteins from the lysates after treatment (10 μM XN for 2 h) that were retained by IP were subjected to 1D-PAGE. As depicted in Fig. 3, this resulted, for the treatment group, for the 10 μM treatment 2-h time point in clear positive staining of a series of proteins in the low-molecular weight area below 20 kDa. IP of untreated cells (6 h, vehicle control) did not produce the same pattern of protein bands in that area. Subsequently, the relevant proteins were further identified after in-gel digestion. Mass spectrometric analysis revealed that the bands of interest from the treatment group were belonging to the histone protein families. More specifically, histones H2A, H2B, and H4 with molecular weights of 14 kDa (H2A and H2B) and 11 kDa, respectively (that corresponds to their location on the gels) were identified (all with p-value < 0.05). In order to confirm the identity of one member of this protein family, immunoblotting with anti-histone H2A antibody was performed.

3.5 Immunodetection of histones in MCF-7/6 cell lysates

One of the cell lysates (10 μM XN, 2-h treatment) in which, after IP, histones were identified as the binding proteins of XN in vitro (section 3.4), was subjected to immunodetection with an anti-histone H2A antibody. The results of this control experiment are summarized in Fig. 4. In the lane of the untreated cell lysate that also underwent IP, no histone H2A could be detected. On the other hand, in the treated cell lysate, one of the bands below 20 kDa was identified as histone H2A. Histone H2A was also identified in cell lysate before IP, although with a lower intensity. Thus, IP with the anti-X

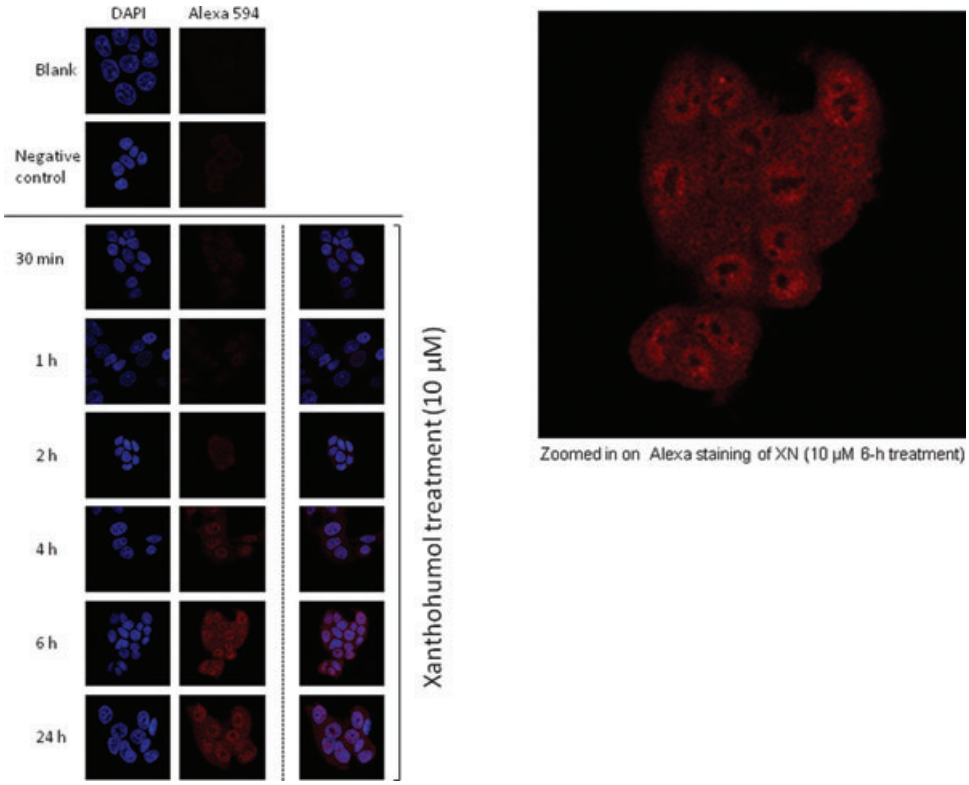

Figure 2. Immunostaining of XN with anti-XN antibody in MCF-7/6 cells. Nucleus stained with 4′ 6-diamidino-2-phenylindole (DAPI) and anti-XN antibody with Alexa 594 anti-mouse IgG (blank: no XN ; negative control : XN and mouse IgG1 as primary antibody).

antibody resulted in an enrichment of histone H2A in our sample.

4 Discussion

This study aimed to gain insight into the in vitro uptake and the subcellular fate of XN in a breast cancer cell line (MCF-7/6) and to identify possible intracellular-binding partners to provide more information about the underlying mode-of-action of bioactivities reported for XN. Initially, we studied the solubility and stability of XN in cell culture medium at experimental conditions, an aspect often forgotten in molecular studies that can greatly influence the results. It was shown previously that XN does not readily dissolve in aqueous solutions and presumably is neither so stable in it [4, 41]. Our experiments showed that at 37°C, XN dissolves as good as completely in the culture medium and the stability is also satisfactory. Only after 6 h of incubation about 25% of the original amount of XN could not be retrieved, but after 24 h, this was still the case. We concluded that the solubility and stability of XN in our cell culture medium were appropriate for further experiments. The differences in solubility and stability of XN with previous reports may be due to the nature of the growth medium, which differs from water and may cause cosolvation or contain certain lipophilic components that enhance dissolution and stability. Motyl et al. [42] recently investigated the solubility of XN in cell culture work and concluded that a minimum of 10% serum in the medium increases the solubility and prevents the absorption to various plastic materials. They also assumed possible interactions with serum proteins in in vitro experiments, which may impact upon the uptake of XN in the cells. Additionally, we investigated the cellular uptake of XN in MCF-7/6 cells in vitro. Already after 30 min, a significant portion of XN was associated with the cellular fraction and after 6 h, about 45% of the total amount of XN added could not be retrieved from the medium fraction. We also observed that, although the added amount of XN tends to decrease rapidly, this trend slows down afterwards (1 h and 2 h). This observation may be due to association with the cell wall, rather than an effective uptake, but could not be clarified from this type of experiment. After 24 h, approximately the same amount is recovered from the medium as with 6 h, which may indicate that after a rapid and strong accumulation in the cells, a saturation plateau is reached. Pang et al. [31], who studied the uptake of XN in intestinal cells, observed a strong intracellular accumulation of XN. Through ultrafiltration and LC-MS techniques, they provided evidence that XN bound to cytosolic proteins in Caco-2 cells treated for 1 h with 10 µM XN. Furthermore, covalent binding of XN with cystein residues of proteins like Keap 1 by a Michael-type addition has been reported as a possible first step in the mechanism of inducing chemopreventive activity [43]. Our immunocytochemistry experiments showed that XN can rapidly locate intracellularly, both cyto- and nucleoplasmic, but tends to accumulate more in the nucleus over time in

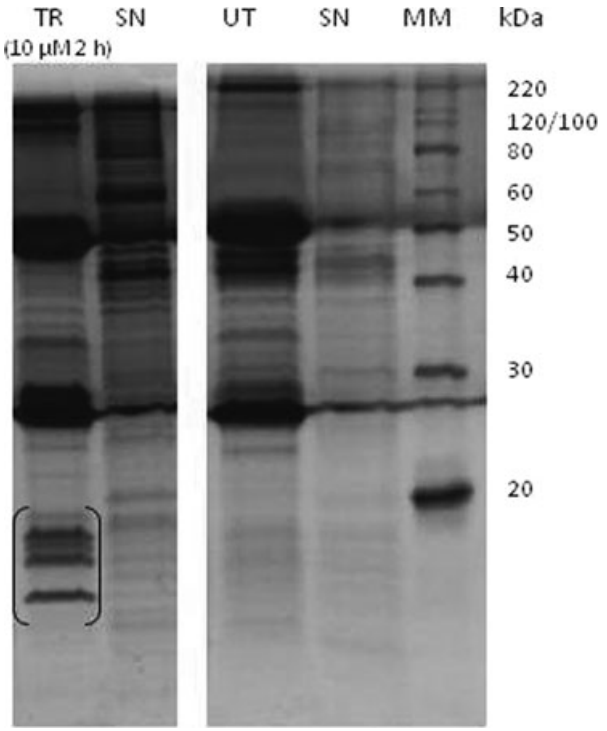

Figure 3. 1D-PAGE of lysates from untreated (UT) MCF-7/6 cells after isolation with IP; detection of histones H2A, H2B, and H4 in cell lysates after incubation with XN (10 μM 2 h) after isolation with IP. The proteins (below 20 kDa and between brackets) were identified by MS. Next to each lane with the isolated proteins from IP, the supernatants (SN) from that IP was spotted, a molecular marker (MM) was used for indication of the molecular weights.

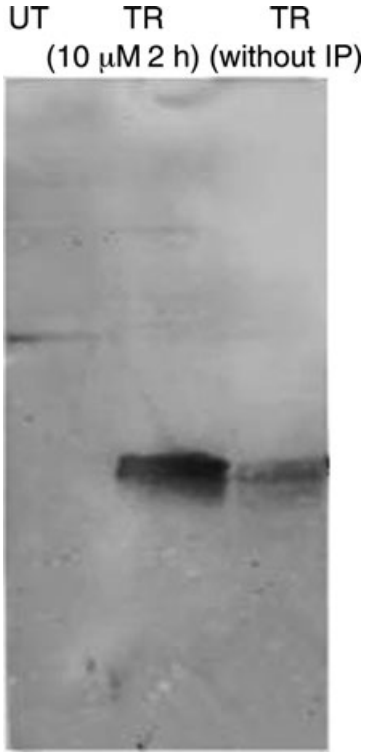

Figure 4. Western blot with immunodetection with anti-histone H2A antibody. Untreated cell lysate after IP (UT); treated cell lysate after IP (TR 10 μM 2 h) and treated cell lysate without IP (TR without IP), both with visualization of histone H2A at 14 kDa.

comparison to the cytoplasm. Six hours exposure to a 10 μM concentration of XN, resulted in a clear nuclear staining, with a granular pattern. On the other hand, the faint, typically aspecific, staining in the cytoplasm that already appeared after 30 min did not further increase in intensity to the same extent as the nuclear staining. These observations, which were highly reproducible, instructed us to isolate/purify the binding partner of XN in conditioned cell lysates of MCF-7/6 cells by means of IP. Using Q-TOF MS, we were able to identify histones (i.e. histone H2A, H2B, and H4) as the proteins that were differentially detected on the 1D-PAGE of immunoprecipitated XN-positive cell lysates. The identity of one of the bands of interest in the low-molecular region (below 20 kDa) was confirmed by means of Western Blotting and immunodetection. The application of our specific monoclonal antibody against the prenylated flavonoid XN, opens up a whole field of research opportunities related to the unraveling of the exact mode-of-action of XN. To the best of our knowledge, histones have not been reported previously as possible protein targets of XN in breast cancer cells. Histones are very basic proteins that form an octameric core, consisting of an H3-H4 tetramer and two H2A-H2B dimers, around which the DNA strand is coiled. The modification of histone proteins is an epigenetic mechanism that effects the chromatin structure and gene expression. Acetylation for instance, one of the posttranslational modifications, which is regulated by opposing activities of histone acetyltransferases and histone deacetylases (HDACs), affects the function of transcription factors and the subsequent oncogenic transformation. Epigenetic regulators, influencing chromatin modifications at the histone core, have been under investigation in recent times, as promising therapeutics in cancer therapy. Dietary polyphenols, which have been suggested as potential chemopreventive agents, are also thought to modulate epigenetic alterations in cancer cells, but the precise mechanism how they regulate and effect modifications is hardly unraveled. Link et al. recently [44] reviewed the role of polyphenols like curcumin (curry), (-)-epigallocatechin-3-gallate, green tea, and several others that can indirectly induce histone modifications acting as histone acetyltransferase and histone deacetylase inhibitors. It would be interesting to investigate if the introduction of those inhibitors into the type of experiment we performed, would impact upon XN binding on histones. Methylation and demethylation of proteins, on the other hand, plays also an important role in nuclear hormone receptor-regulated transcription. XN, to the best of our knowledge, is not known for its direct action on steroid receptors. In estrogen receptor-dependent cell lines like MCF-7/6, XN may influence nuclear hormone signaling indirectly by interference in histone modification processes. Additionally, it would also be recommended to

repeat experiments in steroid-free conditions to clarify the possible role of XN in NR-mediated transcription. Clearly, these are all assumptions based on preliminary findings and basic knowledge of nuclear signaling pathways. Hypothetically, in our case, we could assume that the association of XN with histones, may interfere with the posttranslational modifications of histones and therefore explain its versatile spectrum of effects on cancer cells. Nevertheless, this interaction needs to be worked out in detail, e.g. what amino acids or specific sites are targeted, what kind of interaction is there between XN and histones, is it directly, and furthermore, is there a modulation of the gene expression accomplished. To check the universal character of this finding, other cell lines would have to be applied in this type of experiment. Additionally, medicinal chemistry approaches could be applied for the optimization of derived molecules targeting the same application. Obviously, future studies are necessary to reveal the biological relevance of the binding of XN to histones and its consequences and the availability of a specific monoclonal antibody and the established, optimized techniques from this study has already opened new perspectives and may spur further research in this field.

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