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Species-specific Toxicity of Aristolochic Acid (AA) *in vitro*

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(Title Page, Page 1) Abbreviations: AA, Aristolochic Acid; PKCm p1, Porcine kidney cortex cells of passage 1 (male); HKCm p1, Human kidney cortex cells of passage 1 (male); RKCm p0, Rat kidney cortex cells of passage 0 (male)

Abstract

Differences in toxicity and carcinogenicity of the nephrotoxic compound aristolochic acid between rodents and humans suggest a species-dependent mechanism of action. The goal of this study was to investigate constitutive differences in the susceptibility of renal cortex cells originating from human, rat and porcine origin *in vitro*. Effects of 24 and 48 h AA exposure on cell number and MTT reduction were studied. Furthermore, using the effective concentrations causing 20 and 50 % reduction (cell number), cell cycle, ^{3}H-thymidine incorporation and DNA damage analyses were conducted. AA cytotoxicity was observed in all cell types in a time- and concentration dependent manner with species-specific differences, with porcine cells being the most sensitive. AA had a comparable effect on the cell cycle in primary human and porcine cells and the rat NRK-52E cell line following 48 h exposure, also corroborated by the reduced ^{3}H-thymidine incorporation in NRK-52E cells. In addition, DNA unwinding, suggestive of enhanced DNA damage, was observed in primary porcine cells. These results provide an initial insight into the sensitivity and suitability of different *in vitro*-systems and suggest that primary porcine renal cortex cells could be a valuable *in vitro*-system to study AA toxicity.

Introduction

Aristolochic acid (AA) is a nephrotoxic and carcinogenic compound (Mengs et al. 1982; Mengs 1987; Mengs 1988), which has been demonstrated to be genotoxic and mutagenic both *in vitro* and *in vivo* (Gotzl and Schimmer 1993; Arlt et al. 2001; Lebeau et al. 2001; Lord et al. 2004; Zhang et al. 2004). Metabolic activation of AA has been shown to be necessary for the generation of AA-DNA adducts (Schmeiser et al. 1997; Stiborova et al. 2001; Stiborova et al. 2003), which may at least in part explain its carcinogenicity. While the kidney cortex seems to be the primary target for toxicity (Mengs 1987; Depierreux et al. 1994; Lebeau et al. 2001; Lebeau et al. 2005), tumour induction in rodents was shown to occur in kidney cortex, forestomach, renal pelvis and urinary bladder as well as the lung, uterus and lymphoid tissue (Mengs et al. 1982; Mengs 1988). In contrast, AA exposure in humans leads to a rapidly progressive renal fibrosis, the so-called Chinese Herb Nephropathy (CHN), followed by a high prevalence of urothelial cancer (Vanherweghem et al. 1995; Cosyns et al. 1999; Nortier 2000; Nortier and Vanherweghem 2007). Although the carcinogenic potential of AA in humans appears to be limited to urothelial tissue, AA-DNA adducts have also been found in renal cortical and corticomedullary tissue of CHN patients (Schmeiser et al. 1996; Bieler et al. 1997). These differences in toxicity and carcinogenicity between rodents and humans suggest a species-dependent mechanism of action.

Cytotoxicity determinations represent an initial step toward the characterisation of species-dependent differences in toxicity *in vitro*. Although cytotoxicity assays can not allow discrimination of distinctive mechanistic responses, description of constitutive differences in the susceptibility of cells originating from different species can be achieved using *in vitro* systems. Furthermore, cytotoxicity assays are valuable for the pre-selection of cells *(primary or continuous)* sufficiently susceptible to the toxin in question, in this case aristolochic acid. Finally, knowledge of the concentration range that induces cytotoxicity enables better definition of concentrations to be employed in more in-depth descriptive and mechanistic studies, e.g. species and/or cell type specific gene expression and proteomic analyses.

The use of human primary cells is a major advantage of *in vitro* systems. In this case, it would enable characterisation and evaluation of possible differences in the mechanistic action of AA in humans and rodents. However, the availability and quality of biopsy material from direct (primary care) facilities for the establishment of primary human cell culture is highly variable and commercially available cells are expensive. Variability in quality can result in limited reproducibility and data quality. Given the renal anatomical and physiological proximity of pigs and humans (Terris 1986; Soucek et al. 2001; Bagetti Filho et al. 2008), and the constant availability of fresh material as a by-product of pork production, the use of primary porcine cell models could be considered as a valuable potential alternative.

In order to address the question of species dependent toxicity of AA and to investigate if porcine renal cells could serve as a sufficiently sensitive *in vitro* model, the effect of AA on cell number and MTT reduction was investigated in renal cortical cells of rat, pig and human. Apart from species influence, *in vitro*-specific effects as the cell type (*primary or continuous*), passaging as well as gender-specific effects were considered. Using the effective concentrations causing 20 and 50 % reduction of cell number (48 h exposure) the effect of AA on cell cycle, ^{3}H-thymidine incorporation and DNA damage were determined.

Materials and Methods

Cell culture media and materials

Dulbecco's modified Eagle's ***medium*** (DMEM), DMEM Ham's F12, DMEM High Glucose (D-Val) as well as the antibiotics, penicillin/ streptomycin, foetal bovine serum (FBS Gold) and trypsin/ EDTA were purchased from PAA (Pasching, Austria). MEM-DVal was obtained from Cell Culture Technologies GmbH (Gravesano, Switzerland). Medium was supplemented with FBS as well as 100 U/ ml penicillin and 0.1 mg/ ml streptomycin (Tab. 1). Media for rat primary cells were supplemented with REGM SingleQuots® supplements and growth factors from Cambrex (Verviers, Belgium), containing transferrin (0.5 µl/ ml), insulin (1 µl/ ml), hydrocortsol (1µl/ ml), EGF (1 µl/ ml) as well as with 50 ng/ ml prostaglandin E1 from Acros (Geel, Belgium) and 5 ng/ ml selenic acid from Sigma (Taufkirchen, Germany). Standard ***cell culture material*** was obtained from Greiner Bio-one (Frickenhausen, Germany) and Sarstedt (Nuembrecht, Germany). Primaria™ plasticware were from Becton Dickinson (Heidelberg, Germany). Collagen type 1 (rat tail) was purchased from Sigma (Taufkirchen, Germany). Collagen coating (6 µg/ cm^2) was carried out immediately prior to use.

Tissue Samples

Human renal biopsy material was obtained from the urology departments of two local hospitals. This was in collaboration with Prof. Dr. Norbert Pfitzenmaier, subsequent to patient information and receipt of the signed patient's agreement forms. Tissue samples as well as patient data were handled in compliance with the stipulations put forth by the ethics committee of the University of Konstanz and in fulfilment of German law and the Declaration of Helsinki pertaining to personal data protection and handling of human biopsy material. Biopsy material originated from patients with an age ranging between 64 to 85 years.

Kidneys from German hybrid pigs were obtained from a local abattoir. ***Rat kidney samples*** were obtained from Long Evans rats, which had an average weight of 352 ± 8.8 g (SD) and an age of 20 to 27 weeks. Rats were obtained from an in-house breeding program conducted at the animal research facility at the University of Konstanz.

Cell Preparation and Culturing

An overview of cell culture media and material used for the different *in vitro*-systems is given in table 1. Isolation and culture of ***primary human*** (HKC) and ***primary porcine*** (PKC) kidney cortex cells (primarily proximal tubular cells) was carried out as previously described (Dietrich et al. 2001), with the following differences: collagenase digestion (collagenase type 1 from *Clostridium histolyticum*, Sigma, Taufkirchen, Germany) was carried out in 50 ml HBSS containing 1 mg/ ml collagenase (type 1), 4 mM $CaCl_2$ and 1 mM $MgCl_2$ at 37°C for 25 min under continuous stirring. After collagenase digestion, the cell suspension was filtered through a 200 µm gauze, directly followed by a second filtration using an 40 µm gauze. The cells were seeded at a density of $1x10^4$ cells/ cm^2, were incubated under standard humidified conditions at 37 °C and 5 % CO_2 and checked daily for contamination (Tab. 1). The use of D-valine containing medium (MEM-DVal) as seeding medium allow a positive selection of epithelial cells expressing D-amino-acid oxidase, thus preventing fibroblast growth.

Primary porcine cells which were exposed at passage 0 were seeded directly at $1x10^4$ cells/ cm^2 into 24-well plates (Tab. 1). An initial medium renewal was carried out 24 h after preparation. Seeding medium was replaced by DMEM Ham's F12 medium on day 2. The cells were exposed to AA in fresh medium three days after preparation. For primary human and porcine cultures which were exposed as passage 1, the first medium change was carried out 48 h after preparation. On day 5 after preparation cultures were passaged using trypsin/ EDTA and seeded at $1x10^4$ cells/ cm^2 into culture plates using DMEM Ham's F12. Cell cultures were allowed to recover for at least 24 h following trypsinisation before exposure.

Isolation and culturing of ***primary rat*** cells was carried out as previously described (Dietrich et al. 2001) with following differences: for each replicate, two rats were anesthetised by CO_2 narcosis and sacrificed by exsanguination. After separating renal cortex from medulla, pooling and washing of renal cortex pieces, pellets of the washed renal cortex pieces were resuspended in 100 ml of HBSS containing 1 mg/ ml collagenase (Type 1 from *Clostridium histolyticum*), 4 mM $CaCl_2$, 1 mM $MgCl_2$, and were incubated on a magnetic stirrer at 37 °C

for 25 min. After collagenase digestion, the cell suspension was filtered through a 200 µm gauze, directly followed by a second filtration using a 40 µm gauze. Enrichment of proximal tubular cells by Percoll density-gradient centrifugation (GE Healthcare, Munich, Germany) was carried out as described previously (Dietrich et al. 2001). The upper 10 ml of all tubes were collected into two tubes filled to 50 ml with HBSS, and washed twice. The resulting pellet was resuspended in medium. Cell vitality was checked by trypan blue exclusion. Renal proximal tubular cells were seeded into collagen coated 24-well plates (TC) at a density of 1 x 10^4 cells/ cm^2 (Tab. 1). An initial medium renewal was carried out 96 hours after seeding. Toxin exposure began concurrent to medium renewal using DMEM Ham's F12.

The ***continuous cell lines*** NRK-52E (rat), IHKE (human) and LLC-PK1 (pig) cell lines were maintained under standard humidified conditions (37 °C and 5% CO_2) and checked daily for contamination. The NRK-52E cell line (DSMZ No. ACC 199) originated from a male Osbourne-Mendel rat (De Larco and Todaro 1978). The IHKE cell line was derived from primary human foetal kidney epithelial cell culture and was transformed by nickel exposure (Tveito et al. 1989). The LLC-PK1 epithelial cell line (ECACC no. 86121112) was derived from renal cortex tissue from a male juvenile Hampshire pig (Hull et al. 1976). Cell lines were passaged by trypsinisation at a confluence level of approximately 80 % and were seeded for exposure experiments at a density of 1x10^4 cells/ cm^2. Cell cultures were allowed to recover for at least 24 h following trypsinisation before exposure.

Aristolochic Acid Exposure

Aristolochic Acid (41% AAI and 56% AAII) was purchased from Sigma (Taufkirchen, Germany). Stock solutions (20 mM) were prepared in sodium bicarbonate (0.1 M) by dissolving in an ultrasound bath for 30 min and sterilised by filtration through 0.2 µM filter units (FP30/ 0,2 CA-S; Schleicher and Schuell, Dassel, Germany) prior to aliquoting and storage at -20 °C. Stock solutions were thawed only once for preparation of serial dilutions (in $NaHCO_3$, 0.1 M). Dilutions were freshly prepared prior to exposure and aliquots were stored at -20 °C until use for toxin renewal.

For ***AA-exposure*** proliferating cell cultures were exposed to nominal concentrations of 0.01 – 1000 µM AA for 24, 48 or 72 h. Negative and vehicle controls (5 % $NaHCO_3$) were also included. Rat primary, NRK-52E and LLC-PK1 cells were exposed for 24 and 48 h only as these cells reached confluency within 48 – 72 h due to their rapid proliferation rate. Medium and toxin were renewed every 24 h, whereby cells were washed in phosphate-buffered saline (PBS) prior to replenishment of fresh medium and toxin.

Cytotoxicity Determinations: Cell number counting and MTT reduction assay

For ***cell number counts***, trypsin/ EDTA was added subsequent to media removal and washing of cells with PBS. Cultures were incubated under standard conditions for approximately 30 min prior to cell number determination using a Coulter® counter.

For the ***MTT reduction assay***, 500 µl of fresh medium and 25 µl of MTT solution (5 mg/ ml; Sigma, Deisendorf, Germany) were added and the cells were incubated for a period of 150 min under standard conditions. For primary rat cells, 25 µl of MTT solution was added directly to the medium prior to incubation. The supernatant media were then discarded and the cells with the intracellular dye were solubilised with 95 % isopropanol/ 5 % formic acid at room temperature for 15 min. MTT reduction was assessed at an absorbance of 550 nm using a microtiter plate reader (SLT, Crailsheim, Germany).

Fluorescence activated cell sorting analysis (FACS) for cell cycle distribution

Primary human (HKCm p1) and primary porcine (PKCm p1) cell cultures, as well as the rat NRK-52E were exposed for 48 h using the following concentrations of AA: 1.0 µM, 1.8 µM, 10.4 µM (HKCm p1); 0.05 µM, 0.5 µM and 3 µM (PKCm p1); 0.1 µM, 1.3 µM, 6.3 µM (NRK-52E). AA concentrations represent the NOEC and the effective concentrations causing 20 and 50 % effect (cell number, 48 h exposure). Colchicin-treated cells (0.05 µM) (Fluka, Buchs, Switzerland) served as positive control. After treatment cells were trypsinised (4 min, 37 °C) and collected by centrifugation (174 x g, 5 min). Cells were washed three times (PBS) prior resuspension in PBS. For fixation ethanol (70%) was added to final dilution of 1:5 and cells were incubated at 4 °C at least over night (maximum 72 h). After centrifugation (2000 x

g, 5 min), cells were resuspended (PBS) and incubated with 100 µg/ ml RNAase A (Sigma, Taufkirchen, Germany) and 40 µg/ ml propidium iodide (Sigma, Taufkirchen, Germany) for 30 min at 37 °C in the dark. Flow cytometry analysis was conducted using FACSCalibur (Becton Dickinson, Heidelberg, Germany) and Pro™-Program (Becton Dickinson, Heidelberg, Germany). For each sample 20.000 events were acquired.

^{3}H-thymidin incorporation in NRK-52 cells: corroborative studies

After 48 h AA exposure of NRK-52E (0.1, 1.3 and 6.3 µM AA) cultures were replenished with medium containing 1 µCi/ ml ^{3}H-Thymidin (Hartmann-Analytic, Braunschweig, Germany). After 3 h of incubation at standard conditions, medium was removed and cells were washed three times in ice-cold PBS. For cell lysis 2% SDS solution (w/v) was added followed by a 15 min incubation period at room temperature. Cell lysis was supported by mechanical disruption and dd$H_2$0 was added to a final 1:2 dilution. After adding trichloroacetic acid (2.5 %) (Roth, Karlsruhe, Germany), suspension was centrifuged at 12.000 g for 2 min. After discarding the supernatant, 2 M NaOH was added to the DNA precipitate. Solubilisation over night (room temperature) was followed by addition of HCl to a final concentration of 1.7 M. ^{3}H-thymidin content was measured via scintillation counting on a LS6500 Scintillation Counter (Beckman Coulter, Krefeld, Germany).

Fluorescence-detected alkaline DNA-unwinding (FADU)

DNA damage (DNA strand breaks) was indirectly analysed using the FADU Assay (Moreno-Villanueva et al. 2007), representing an optimised version of a previously described assay (Birnboim and Jevcak 1981; Pfeiffer 2001). Primary porcine cell cultures (PKCm p1) of 30 - 40 % confluence (72 hours after seeding passage 1) were exposed for 2.5 h and 5 - 150 min. The 48 h exposure of PKCm p1 was conducted as described above. After medium removal and washing with PBS, cells were removed by trypsinisation (5 min, 37 °C), followed by gently knocking-off adherent cells and careful resuspension. After centrifugation (200 x g, 4 min, 4 °C) the cell pellet was resuspended (0.25 M meso-inositol, 10 mM sodium phosphate, 1 mM MgCl, pH 7.4). Cell number was determined using the Coulter® counter

and adjusted to 3 x 10^5 cells/ ml. Four or eight technical replicates of each sample (70 µl each) were transferred to a cooled FADU 96-well plate (Greiner-Bio One, Frickenhausen, Germany; modified). X-ray-treated (4.05 Gy) (C.H.F. Müller GmbH, Hamburg-Fu., Germany) cells served as positive controls. Cell lysis, DNA unwinding and Sybr®Green I- incorporation (Invitrogen, Karlsruhe, Germany) was carried out by a TECAN-roboter (TECAN AG, Hombrechtikon, Switzerland) as described (Kappes et al. 2008; Moreno-Villanueva 2008). Fluorescence was determined at 485/ 530 nm with a fluorescence reader (Bio-Tek, Bad Friedrichshall, Germany) and evaluated with Bio-Tek® KC4 software (Bio-Tek, Bad Friedrichshall, Germany). The fluorescence intensity is a measure of the amount of DNA strand breaks. Fluorescence of each sample was normalised to the respective negative control.

Statistical Analysis

Results of ***cytotoxicity tests*** were normalised to the vehicle control. Calculation of endpoints e.g. EC_{20}, etc. was carried out without blank value subtraction. The effective concentrations causing 20, 50 and 80 % effect (EC_{20}, EC_{50}, EC_{80}) were calculated using GraphPad Prism version 4. EC_{20}, EC_{50} and EC_{80} were compared using the Student's T-test ($p<0.05$) and one-way ANOVA with Bonferroni's post-test ($p<0.05$). Unless otherwise stated, data were expressed as arithmetic means ± corresponding 95 % confidence intervals from at least three independent experiments carried out in duplicates. Data of ***cell cycle analysis*** were expressed as arithmetic means ± standard deviations from three independent experiments. Data of ***^{3}H-Thymidin incorporation assay*** were expressed as arithmetic means ± standard deviations from four independent experiments carried out in duplicate. Data of ***FADU assay*** were expressed as arithmetic means ± standard deviations from at least 3 independent experiments, carried out four- or eightfold each. Significance of effects in theses tests were determined using a one-way ANOVA with Dunnett's post-test ($p<0.05$, $p< 0.01$).

Results

Cytotoxicity determined via cell number and MTT reduction

Comparison of cell number with MTT determinations

Lower AA concentrations were found to induce significant cytotoxicity when determined via cell number analysis than with the MTT assay, thus demonstrating the higher sensitivity of cell counting *versus* MTT determinations. Despite this, the dose response curves obtained with cell number and MTT were comparable, as exemplified in Fig. 1.

Time- and concentration-response

A time- and concentration-dependent response to AA exposure was demonstrated in all cell types (Tab. 2), when comparing 24 h with 48 h exposures (Fig. 2). No increased cytotoxicity could be observed when expanding the exposure time from 48 to 72 hours (data not shown).

Species differences

To obtain a rank order of specifies sensitivity, EC_{20}, EC_{50}, EC_{80}'s determined in the different species, and respective cell types, and time-points were compared (Tab. 2). After 24 h exposure porcine cells were the most sensitive primary *in vitro* model, followed by human and rat cells (Tab. 2; Fig. 2A). After 48 h exposure the response was more similar between the different species (Tab. 2; Fig. 2B). In contrast, comparable sensitivity of LLC-PK1 and NRK52E was observed in continuous cell line exposures, followed by the nickel transformed human IHKE (Tab. 2).

Primary cells versus continuous cell lines

Primary cells and continuous cell lines of porcine and rat origin was generally comparable in their sensitivity to AA (Tab. 2). As already indicated above, IHKE were distinctly less sensitive to AA than human primary cells.

Passage dependency

The passaging-effect was studied using passage 0 and 1 of primary porcine kidney cortex cells (PKCm p0 and p1). For all time points, comparable sensitivity was found for cells of passage 1 and 0 (Tab. 3).

Sex-specific differences in primary porcine cells

No statistical significant differences in sensitivity were apparent using male and female primary porcine kidney cortex cells of passage 1 (Tab. 3).

Cell cycle effects (FACS and ^{3}H-thymidine incorporation)

No statistically significant AA-induced effects were observed on the cell cycle (FACS-analysis) of human and porcine primary cells and the rat NRK-52E (Tab. 4), when compared to the corresponding vehicle control. However, when including the negative control for the comparison, a significant effect on numbers of G2/M phase cells was observed at the highest AA-concentration in porcine primary cells, thus suggesting an AA-induced G2/M-phase arrest. Albeit not statistically significant, due to the enormous variation observed, a similar trend was noted for the other cell types tested at the AA-concentrations resulting in 50 % cytotoxicity of the respective cells. Even though no effect in S-phase was visible (FACS-Analysis), ^{3}H-thymidine incorporation assay demonstrated reduced incorporation in NRK-52E exposed to 6.3 µM AA for 48 h (Fig. 3).

DNA damage in porcine cells

Using 48 and 2.5 hours as well as 0 - 150 minute exposures, the DNA damaging effects of AA was determined indirectly using FADU.

48 h AA Exposure

No significant effect on DNA unwinding were seen in primary porcine cells following 48 h exposure to 0.05, 0.5, and 3 µM AA. However, the 3 µM exposure, corresponding to the concentration resulting in 50 % cytotoxicity, resulted in an appreciably higher, albeit not significant, DNA unwinding compared to the concurrent controls (Fig. 4).

2.5 h AA Exposure

The 2.5 h exposure demonstrated a concentration-dependent DNA damaging effect in primary porcine cells (PKCm p1). The first significant response observed at 125 µM AA (Fig. 5), led to 31 % reduction of Sybr®Green I fluorescence. This reduction in fluorescence is equivalent to DNA damage induced by γ-irradiation treatment (2.7 Gy) (Fig. 5, inset).

0-150 min AA Exposure

Treatment of PKCm p1 with 125 µM AA for up to 150 min resulted in significantly reduced Sybr®Green I fluorescence at 90 and 150 min (Fig. 6).

Discussion

Aristolochic acid led to a cytotoxic response in all *in vitro* models used, suggesting that AA uptake must have occurred in all cells. The comparison of cytotoxicity data obtained via cell number analysis with those obtained with the MTT assay, however, demonstrated reduced sensitivity of the MTT assay, irrespective of the species and cell type used. This is not unexpected as similar observations were made previously (Dietrich et al. 2001). However, the higher sensitivity of cell number analysis is critical when cytotoxicity data are used for a comparison with potential cell cycle inhibiting or DNA damaging effects of AA. Indeed, earliest effects of AA on the cell cycle of primary human and porcine cells and the rat NRK-52E cell line were observed at concentrations ≤ 10 µM AA, resulting in 50 % cytotoxicity (determined via cell number analysis). At this concentration AA appeared to induce lowered cell proliferation as shown by a tendentially higher number of cells in the G2/M phase observed in all three cell types tested (Tab. 4) and the reduced ^{3}H-thymidine incorporation per cell observed in the NRK-52E cell line (Fig. 3). An increased number of G2/M-phase cells is suggestive of a prolonged period of the G2/M phase and therefore for DNA damage repair. Indeed, increased DNA damage (FADU), albeit not significantly different due to the inherently high variability of the data, was demonstrated in primary porcine kidney cells of passage 1 exposed for 48 hours to 3 µM AA. The latter findings are supported by results of Li and co-workers (Li et al. 2006) who observed an increase in G2/M phase and DNA damage in the LLC-PK1 porcine cell line after 24 h exposure and using 0.8 (320 ng/ ml) and 3.5 µM (1.28 µg/ ml) of the most potent AA-derivate AAI (Balachandran 2005). The use of an AA-mixture containing AAI (41%) and AAII (56%) could explain the slightly higher concentrations required to induce DNA damage and G2/M-phase shift in the study presented here. However, the comparison of the primary porcine and human kidney cells and the NRK-52E and LLC-PK1 cell lines does suggest that similar effects of AA are found at comparable concentrations.

As all DNA damage and G2/M-phase shift effective AA concentrations were also cytotoxic, as determined via cell number counting, the question arose whether the perceived “cytotoxicity” is due to limited proliferation or enhanced necrosis and apoptosis. Li and co-workers analysed both necrosis and apoptosis and were able to exclude either at 0.8 (320 ng/ ml) and 3.5 µM (1.28 µg/ ml) AAI (Li et al. 2006). The latter observation is also supported by the findings of reduced ^{3}H-thymidine incorporation observed in NRK-52E cells in this study. Moreover, the blatantly higher AA concentrations required to provide for an increased MTT positive reaction as observed in this study also suggests that overt apoptosis/ necrosis did not occur and the lower cell numbers found are primarily a result of limited cell proliferation.

Despite AA-induced DNA damage (Fig. 2), the exposure and release experiments with PKCm p1 did not indicate that the DNA damage incurred via AA exposure was being repaired within the 90 minutes post AA-exposure. As no positive control for testing the repair of AA-type DNA adducts was available, the general DNA repair capacity of PKCm p1 was tested using X-radiation exposure (Fig. 2, inset), which demonstrated sufficient DNA repair activity. Thus the question remains to be elucidated whether PKCm p1 cells maintained sufficient nucleotide excision repair (NER) capacity to repair AA-induced DNA damage or whether the time-frame allowing for NER was too short.

Of the primary *in vitro*-systems tested, porcine primary cells appeared the most sensitive to AA-induced toxicity, followed by rat and human cells. This difference was more prominent after 24 h of exposure than after 48 h, possibly indicating that distinct effects of AA are more abundant after short exposure durations. The latter observation is also supported by the FADU findings demonstrating significant AA-induced DNA unwinding already after 2.5 h exposure, albeit at extremely high concentrations. Differences between the species could be the result of species-specific differences in AA toxicokinetics and dynamics, as suggested by Stiborova et al. (Stiborova et al. 2001), who showed microsomal activation of AA to be species-dependent, with minipigs having the most effective activation system followed by human and rat. However, the lower sensitivity of the primary human cells can possibly be

attributed to the relatively high age of the patients (Schmucker and Wang 1980; Goukassian et al. 2000; Warrington et al. 2004; Hazane et al. 2006) and to patients medical history.

As primary cells are thought to represent the *in vivo* ancestor cells more closely than continuous cell lines (Wilkening et al. 2003), a lower sensitivity of cell lines was expected, as already seen in the case of ochratoxin A-mediated cytotoxity (Dietrich et al. 2001; O'Brien et al. 2001). Thus the comparable sensitivity of primary cells and their corresponding cell lines of porcine and rat origin may suggest that primary cells as well as the cell lines used are similar in their state of dedifferentiation concerning AA toxicokinetics. In contrast to the findings in rat and porcine cells, the human cell line IHKE had a relative low sensitivity. This cell line was established via Nickel(II)-treatment, and are known to present with chromosomal aberrations, altered gene structure and increased expression of p53 (Tveito et al. 1989; Maehle et al. 1992). Moreover, the fetal origin of this cell line, and thus different metabolic capability, could account for its relatively low AA susceptibility (Tveito et al. 1989). Altogether, due to the low AA-sensitivity further mechanistic studies with this cell line appear inappropriate.

In vitro models could serve as a powerful tool to evaluate species-specific toxicity, such as that described here for AA. The possible use of human cells enables the characterisation and evaluation of possible differences in the mechanistic action of AA in humans and rodents. This represents an obvious advantage of *in vitro* systems in addition to time and cost considerations. However, limitations of human cell availability, quality and/ or costs may influence *in vitro* studies. Furthermore, cells of *in vitro* systems may lack relevant physiological functions (Gstraunthaler et al. 1985; Bruggeman et al. 1989; Cummings et al. 2000) which can potentially alter toxin susceptibility. Consequently, a pre-selection of *in vitro*-models sufficiently susceptible/ sensitive to the toxin in question appears prerequisite for the mechanistic examination of toxicity.

From this study, primary porcine cells of passage 1 appear to represent a viable tool for toxicological studies of AA. The major advantages of passage 1 are: i.) more consistent

quality (viability) and quantity of cells and ii.) consistent number of adherent cells at the experimental outset, both contributing to a higher reproducibility of the experiments. These advantages are also supported by the observation that no overt passage-dependent effect was observed in primary porcine cells, thus suggesting that no passage-dependent process affecting AA toxicity occurred. The highly consistent and sensitive cytotoxic response, the comparable effects on cell cycle in primary human and porcine cells and the rat NRK-52E as well as with DNA damaging effects observed in this study, suggests primary porcine cells to be employed in more in-depth studies of AA toxicity.

Given the genetic proximity of humans and pigs and the anatomical and physiological similarity of the kidneys, future directions should consider the use of primary porcine cell models as a potential alternative system. Cells derived from pigs of different ages could serve as an improved testing system for potential compound-mediated effects e.g. for testing of pediatric drugs.

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Table and Figures:

Table 1: Cell culture media and material for the different cell types

	Medium		Cell Culture Material	
Cell Type	**Seeding**	**Culturing**	**Cytotoxity and Cell Cycle-Analysis**	**FADU-Assay**
Primary Human, Porcine Passage 0	MEM-DVal (add 10 % FBS, add P/S)	DMEM Ham's F12 (10 % FBS, P/S)	Primaria™	Standard (Sarstedt)
Primary Human, Porcine Passage 1	DMEM Ham's F12 (10 % FBS, P/S)	DMEM Ham's F12 (10 % FBS, P/S)	Standard (Greiner Bio-one)	Standard (Sarstedt)
Primary Rat Passage 0	MEM-DVAL (5 % FBS, P/S), supplemented	DMEM Ham's F12 (10 % FBS, P/S), supplemented	Standard (Greiner Bio-one) Collagen coated	-
IHKE, LLC-PK1, NRK-52E	DMEM	DMEM	Standard (Greiner Bio-one)	-

Table 2: Cytotoxicity of AA in different cell types

AA Exposure		Primary Cells			Continuous Cell Lines		
Time [h]	**Effective Conc.[µM]**	**Pig**	**Human**	**Rat**	**LLC-PK1 (Pig)**	**IHKE (Human)**	**NRK-52E (Rat)**
24	**EC_{20}**	**2.0** (1.3 – 2.8)	**13.2** (6.6 - 24)	**7.1** (3.5 – 11.6)	**3.5** (2.2 – 5.8)	**72** (56 - 88)	**8.0** (5.4 – 10.2)
	EC_{50}	**12.5** (9.4 – 16.0)	**197** (107 - 334)	**62** (39 - 113)	**34** (23.7 - 49)	**408** (339 - 500)	**21** (14.9 - 28)
	EC_{80}	**70.3** (52 - 107)	**> 1000**	>**1000** (> 1000)	**588** (294->1000)	**> 1000**	**> 500**
48	**EC_{20}**	**0.47** (0.34 – 0.68)	**1.3** (1.1 - 1.8)	**2.4** (1.3 – 3.8)	**0.76** (0.54 – 1.0)	**40** (31 - 51)	**1.3** (1.4 – 2.2)
	EC_{50}	**2.9** (2.4 – 3.7)	**8.3** (6.3 - 11)	**9.7** (7.4 – 12.5)	**5.3** (4.2 – 6.1)	**90** (79 - 101)	**6.4** (4.7 – 8.4)
	EC_{80}	**17.7** (12.5 – 24)	**78** (48 - 123)	**36** (24 - 58)	**26** (19.5 - 34)	**201** (160 - 256)	**26** (17.9 - 39)

Primary cells were of passage 1 (pig, human) and passage 0 (rat). Cytotoxic effects were determined as reduction of cell numbers counted after 24 and 48 hours exposure. Shown are the concentrations of AA causing 20, 50 and 80% effect (EC_{20}, EC_{50}, EC_{80}). Data represent arithmetic means ± CI95 % (n≥3 replicates carried out in duplicate).

Table 3: Cytotoxicity of AA in porcine primary cells of male (passage 0 and 1) and female origin (passage 1)

Cell Type	Effective Conc. [µM]	24h	48h	72h
Passage 0 male	EC_{20}	**3.7** (2.4 – 4.9)	**0.79** (0.6 – 1.0)	**0.5** (0.4 – 0.6)
	EC_{50}	**19.7** (16.9 - 23)	**4.7** (3.8 – 5.5)	**2.7** (2.4 – 3.0)
	EC_{80}	**96** (77 - 120)	**22** (17.6 - 27)	**12.2** (10.3 – 14.0)
Passage 1 male	EC_{20}	**2.0** (1.3 – 2.8)	**0.47** (0.34 – 0.68)	**0.56** (0.42 – 0.75)
	EC_{50}	**12.5** (9.4 – 16.0)	**2.9** (2.4 – 3.7)	**2.6** (2.1 – 3.0)
	EC_{80}	**70.3** (52 - 107)	**17.7** (12.5 – 24)	**9.6** (7.5 – 11.3)
Passage 1 female	EC_{20}	**2.5** (1.7 – 3.4)	**0.55** (2.6 – 1.4)	**0.39** (0.3 – 0.53)
	EC_{50}	**11.5** (9.2 – 14.2)	**2.6** (2.3 – 3.1)	**1.8** (1.5 – 2.1)
	EC_{80}	**61** (46 - 88)	**11.8** (9.7 – 13.9)	**6.8** (5.6 – 7.9)

Cytotoxic effects were determined as reduction of cell numbers counted after 24, 48 and 72 hours exposure. Shown are the concentrations of AA causing 20, 50 and 80% effect (EC_{20}, EC_{50}, EC_{80}). Data represent arithmetic means ± CI95 % (n≥3 replicates carried out in duplicate).

Table 4: Effect of 48 h AA exposure on the cell cycle of PKCm p1, HKCm p1 and NRK-52E

Cell Cycle Phase	Controls		AA [µM]			
Primary Porcine	**Negative**	**Positive**	**0.0**	**0.05**	**0.48**	**3.0**
G0/G1	79 (± 2)	46 (± 3)	77 (± 3)	76 (± 6)	77 (± 5)	71 (± 3)
S	10 (± 2)	14 (± 3)	11 (± 2)	11 (± 4)	9 (± 3)	13 (± 3)
G2/M	11 (± 1)	39 (± 2)	13 (± 1)	12 (± 2)	14 (± 2)	16 (± 2) *
Primary Human	**Negative**	**Positive**	**0.0**	**0.1**	**1.8**	**10.4**
G0/G1	54 (± 11)	27 (± 8)	58 (± 11)	50 (± 10)	50 (± 13)	47 (± 10)
S	16 (± 4)	7 (± 4)	13 (± 6)	19 (± 5)	17 (± 6)	15 (± 3)
G2/M	30 (± 8)	66 (± 8)	29 (± 6)	31 (± 6)	34 (± 7)	38 (± 7)
NRK-52E, Rat	**Negative**	**Positive**	**0.0**	**0.1**	**1.3**	**6.3**
G0/G1	79(± 8)	n.d.	76 (± 7)	78 (± 7)	73 (± 10)	72 (± 7)
S	8 (± 6)	n.d.	10 (± 6)	8 (± 6)	10 (± 4)	12 (± 4)
G2/M	13 (± 3)	n.d.	14 (± 2)	14 (± 4)	17 (± 6)	16 (± 3)

Data of FACS-Analysis. AA concentrations represent the NOEC, EC_{20} and EC_{50} of cell number determinations (48 h). Colchicin (0.05 µM) was used as positive control. Data represent percentages of cells in G0/G1-, S- and G2/M-phase and are expressed as arithmetic means ± SD (n=3 (HKCm p1, PKCm p1), n=4 (NRK-52E)). Statistics: One-way ANOVA with Dunnett's post-test (*$p<0.05$); n.d.: not determined.

Figure 1:

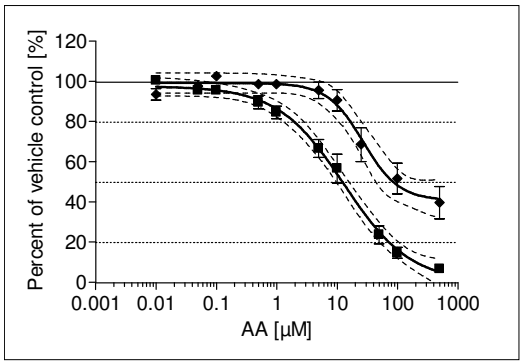

Percent of vehicle control [%]
120
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
1000
AA [µM]

Figure 2:

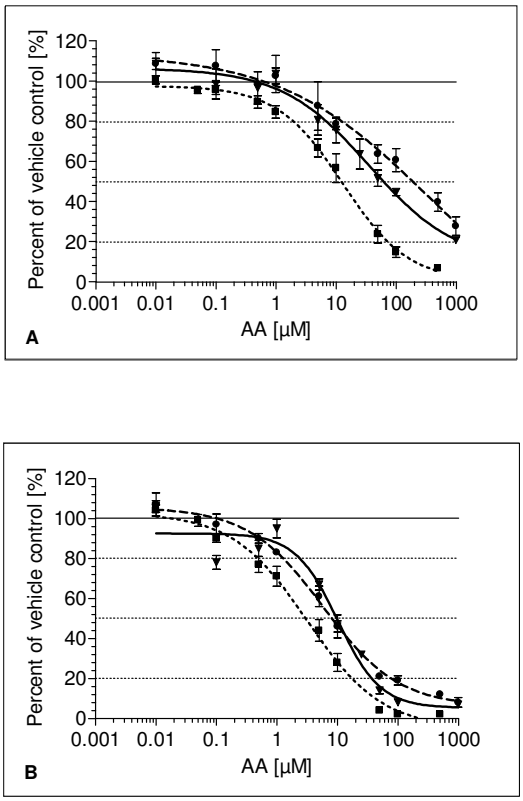

Percent of vehicle control [%]
120
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
1000
AA [µM]
A
Percent of vehicle control [%]
120
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
1000
AA [µM]
B

Figure 3:

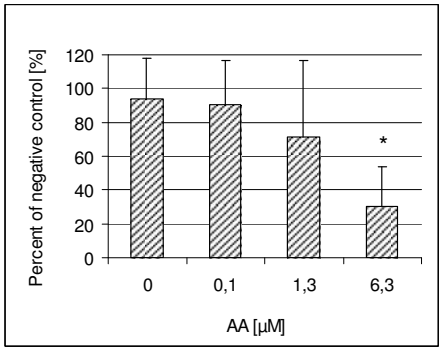

Percent of negative control [%]
120
100
80
60
40
20
0
*
0
0,1
1,3
6,3
AA [µM]

Figure 4:

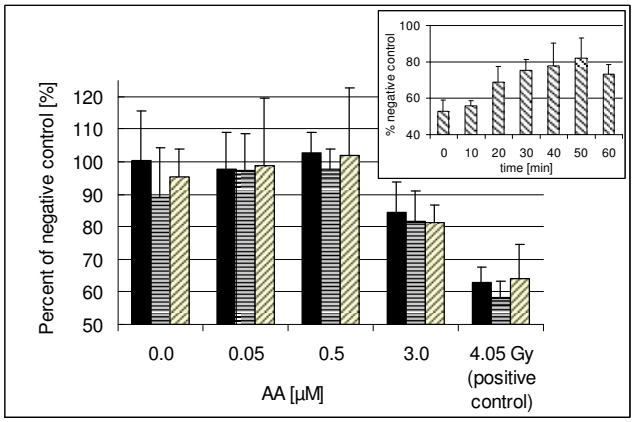

Percent of negative control [%]
120
110
100
90
80
70
60
50
0.0
0.05
0.5
3.0
4.05 Gy (positive control)
AA [µM]
% negative control
100
80
60
40
0
10
20
30
40
50
60
time [min]

Figure 5:

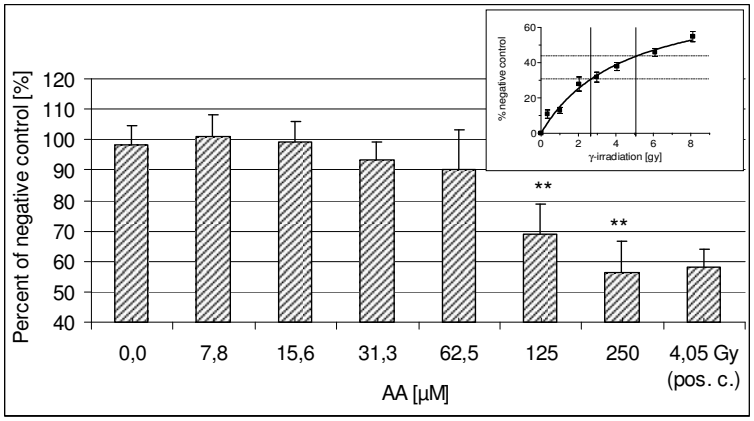
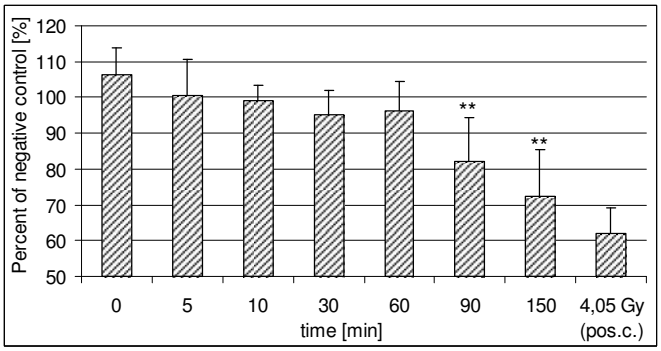

Percent of negative control [%]
120
110
100
90
80
70
60
50
40
**
**
0,0
7,8
15,6
31,3
62,5
125
250
4,05 Gy
(pos. c.)
AA [µM]
%-negative control
60
40
20
0
0
2
4
6
8
γ-irradiation [gy]

Figure 6:


Percent of negative control [%]
120
110
100
90
80
70
60
50
**
**
0
5
10
30
60
90
150
4,05 Gy
(pos.c.)
time [min]

Legends

Fig. 1. Cytotoxicity of AA in primary renal cells of porcine male origin determined via MTT reduction assay (♦) and cell number determination (■) for the 24 hour exposure period. Data points represent arithmetic means ± SEM (n=5 replicates carried out in duplicate). Curves are given ±95 % CI (dashed lines), extrapolated using GraphPad Prism Software (Version 4).

Fig. 2. Cytotoxicity of AA in primary renal cells of rat (▼, solid line), human (●, dashed line) and porcine (■, dotted line) male origin. Cytotoxic effects were determined via cell number counting for the 24 hour (A) and 48 hour (B) exposure period. Data points represent arithmetic means ± SEM (n≥3 replicates carried out in duplicate). Curves are extrapolated using GraphPad Prism Software (Version 4).

Fig. 3. ^{3}H-thymidin incorporation in NRK-52E after 48 h AA exposure. AA concentrations represent the NOEC, EC_{20} and EC_{50} determined via cell number counting (48 h). Data were calculated as Bq per cell and expressed as percent negative control. Shown are arithmetic means ± SD (n=4 replicates carried out in duplicate). Statistics: One-way ANOVA with Dunnett's post-test (* $p<0.05$).

Fig. 4. Decrease in DNA integrity (FADU-Assay) in porcine primary cells (p1) following 48 h AA exposure without (black) and with a subsequent 0.5 h (horizontal lines) and 1.5 h (sloped lines) incubation period allowing for DNA-repair. AA concentrations represent the NOEC, EC_{20} and EC_{50} of cell number determinations after 48 h exposure. Positive control was treated without allowing for repair. Data points represent arithmetic means ± SD (n=3 replicates carried out in quadruplicate). Statistics: One-way ANOVA with Dunnett's post-test ($p<0.05$). Inset: DNA integrity (PKCm p1) after γ-irradiation treatment (4.05 Gy) and subsequent incubation allowing for DNA repair (n=3 ± SD carried out in quadruplicate).

Fig. 5. Decrease in DNA integrity (FADU-Assay) in porcine primary cells (p1) following 2.5 h AA exposure. Data points represent arithmetic means ± SD (n=5 replicates carried out eight-fold). Statistics: One-way ANOVA with Dunnett's post-test (** $p<0.01$). Inset: DNA unwinding

in PKCm p1 after γ-irradiation treatment. Data points represent arithmetic means ± SD (n=3 ± SD carried out nine-fold).

Fig. 6. Decrease in DNA integrity (FADU-Assay) in porcine primary cells (p1) using 125 µM AA and exposure periods from 5 - 150 min. Data points represent arithmetic means ± SD (n=5 replicates carried out eight-fold). Statistics: One-way ANOVA with Dunnett's post-test (**$p<0.01$).

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