Toxicology in Vitro 28 (2014) 156–163

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Atrazine represses *S100A4* gene expression and TPA-induced motility in HepG2 cells

Ludovic Peyre *, Nathalie Zucchini-Pascal, Roger Rahmani

Laboratoire de Toxicologie Cellulaire, Moléculaire des Xénobiotiques, INRA, UMR 1331 TOXALIM Sophia Antipolis, 06903 Sophia Antipolis, France

ARTICLE INFO

Article history:
Received 31 May 2012
Accepted 28 October 2013
Available online 6 November 2013

Keywords:
Atrazine
S100A4
Epithelial-to-mesenchymal transition
Cell migration
HepG2

ABSTRACT

Atrazine (ATZ) is probably the most widely used herbicide in the world. However there are still many controversies regarding its impacts on human health. Our investigations on the role of pesticides in liver dysfunctions have led us to detect an inhibition of FSP1 expression of 70% at 50 μm and around 95% at 500 μM of ATZ ($p < 0.01$). This gene encodes the protein S100a4 and is a clinical biomarker of epithelial–mesenchymal transition (EMT), a key step in the metastatic process. Here we investigated the possible effect of ATZ on cell migration and noticed that it prevents the EMT and motility of the HepG2 cells induced by the phorbol ester TPA. ATZ decreases Fak pathway activation but has no effect on the Erk1/2 pathway known to be involved in metastasis in this cell line. These results suggest that ATZ could be involved in cell homeostasis perturbation, potentially through a S100a4-dependant mechanism.

1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine, ATZ) is a selective triazine herbicide that is slightly soluble in water. In the USA, this xenobiotic is used mainly in corn and sorghum farms to protect crops from broadleaf and grassy weeds. ATZ is the most heavily used pesticide in this nation and can be found at high concentrations in surface and ground water sources as well as in the soil of many agricultural lands (Powell et al., 2011). ATZ is stable at pH 7 in aqueous media and volatilization from water is minimal. In addition, it is poorly absorbed by suspended solids and sediment. Such physical and chemical properties make ATZ a serious contaminant of surface and ground water. ATZ can be absorbed into the blood stream through oral, dermal and inhalation exposure. It is considered as a possible human carcinogen (group C) based on evidence of induction of mammary gland tumor growth in laboratory animals, however data on its carcinogenic potential in humans is lacking (Tchounwou et al., 2001). *In vitro* studies have shown an increase in DNA damage induced by atrazine-based herbicide in lymphocytes (Zeljezic et al., 2006), endocrine disruption resulting in an increased aromatase expression (Laville et al., 2006; Sanderson et al., 2001), arsenic-dependant potentiation of cytotoxicity and transcriptional activation of stress genes (Tchounwou et al., 2001), and growth inhibition of the HepG2 cells at low doses (Powell et al., 2011). ATZ also induces reproductive effects such as decreased sperm motility and birth defects (Betancourt et al., 2006; Winchester et al., 2009), as well as developmental (Gammon et al., 2005) and immune perturbations (Whalen et al., 2003). Some epidemiological studies have linked ATZ to ovarian, prostate, brain, testicular and breast cancer as well as to leukemia and non-Hodgkin's lymphoma (Alavanja et al., 2003; Donna et al., 1989; Engel et al., 2005; De Roos et al., 2003; Mills, 1998; Schroeder et al., 2001) however other authors believe that more consistent and scientifically convincing evidence is required to support such a causal relationship between exposure to ATZ and human cancers (Freeman et al., 2011; Hessel et al., 2004; Rusiecki et al., 2004; Sathiakumar et al., 2011; Simpkins et al., 2011).

Cell migration is a multistep process that requires stimuli such as cytokines or growth factors to enhance cell movement. Transduction of such a message from the cell receptors inwards increases the activity of the cellular machinery leading to a succession of changes including the cyclic formation of cytoplasmic protrusions and focal adhesions disruptions, and induction of contractions mainly orchestrated by the RhoGTPases Cdc42, Rac and Rho (Entschladen et al., 2011). The Src/Fak pathway is known to be involved in the transduction of the signal from the extracellular matrix to the cytoskeleton. Indeed, it allows the phosphorylation of the PKC proteins and the activation of the RhoGTPases downstream (Schaller, 2010). Focal adhesion kinase (FAK) also plays a role in adhesion, invasion, proliferation, apoptosis and epithelial-to-mesenchymal transition (EMT) through the activation of

* Corresponding author. Address: Laboratoire de Toxicologie Cellulaire, Moléculaire et Génomique, INRA, UMR 1331, 400 route des Chappes, BP167, 06903 Sophia Antipolis, France. Tel.: +33 4 92 38 65 48; fax: +33 4 92 38 64 01.
E-mail address: ludovic.peyre@sophia.inra.fr (L. Peyre).

0887-2333/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tiv.2013.10.019

ERK/MAPK and the AKT pathway (Yam et al., 2009). To allow EMT and facilitate cell movement, an increase in the recycling of integrins occurs (Pinzani, 2011) as well as the repression of E-cadherin and the loss of cell–cell adhesions. In such cases, migration is individual and is called amoeboid or mesenchymal (Yilmaz and Christofori, 2009).

S100a4 is a calcium-dependent protein that is used as a clinical marker of fibrosis and metastasis. Increases in expression of this protein are strongly correlated with an aggressive malignant phenotype but also with inflammatory disease. S100a4 has a large number of partners including cytoskeletal proteins (NMMHC, actin, tropomyosin) or other proteins (S100a1, P53, septins, CCN3, liprin β1, MetAP2, P37) with which it interacts by heterodimerization. It affects motility through its direct regulation of myosin IIA assembly. Indeed, S100a4 contributes to the dismantling of myosin filaments at the ends of cytoplasmic protrusions which results in the local enrichment of myosin monomers required for the formation of neo protrusions during migration (Sack and Stein, 2009). S100a4 is also known to affect the function of the LAR family of transmembrane protein-tyrosine phosphatases by binding to liprin β1 and thereby modulating its cellular adhesion leading to the establishment of a migratory phenotype. It also plays a role in invasion by regulating metalloproteases (MMPs) positively and TIMPs (MMPs inhibitors) negatively both of which involve extracellular matrix (ECM) remodeling. Finally, S100A4 also regulates apoptosis through its effects on p53, a protein involved in modulating the expression of genes such as *Bax* and $P21^{Waf1}$ (Helfman et al., 2005; Garrett et al., 2006). S100a4 can inhibit the activation by phosphorylation of p53 through PKC as well as physically interact with p53 thereby modulating its regulatory activity (Grigorian et al., 2001). On cancer cells, suppression of *S100A4* can however lead to the arrest of cell growth (Grum-Schwensen et al., 2005) or to anoikis initiation (Shen et al., 2011). S100a4 can also play the role of extracellular paracrine factor thus participating in invasion and angiogenesis. It has an affinity for various unidentified receptors, including the receptor RAGE. Once linked to this receptor, S100a4 induces the activation of NFκB and the MAPK pathway, leading to the regulation of target genes involved in angiogenesis and tumor progression (Boye and Maelandsmo, 2010). S100a4 expression can be controlled by activation of the TGFβ pathway during EMT and by FGF-2 and its transcription level is modulated by β-catenin/Tcf complex activation (Schneider et al., 2008).

In this study, high concentrations of atrazine (250 and 500 μM) have been used to highlight the major molecular events whereas 25 and 50 μM have been used to demonstrate effects of atrazine on the cell phenotype even at lower doses. However, for clear mechanistic aspects, those concentrations are not relevant of those found in environment and in human body. Indeed, atrazine does not bioaccumulate in fatty tissue or in liver (ATSDR, 2003). For these reasons, such high concentrations can only be found after poisoning and accidental ingestion of the compound (Pommery et al., 1993). The aim of this study was to improve our understanding of the molecular events involved and provide evidence to support the link between atrazine and tumorigenesis. Among all EMT biomarkers tested, S100A4 was the only one that was strongly modulated by ATZ and that presented an interest in terms of its role in migration, invasion, angiogenesis and metastasis. To work in metastable conditions, we used the HepG2 cell line and the phorbol ester TPA which has been described as a potent EMT inducer able to initiate migration of HCC cells (Hu et al., 2008; Murata et al., 2009; Wu et al., 2006). In this way, we hypothesized that ATZ could affect the TPA-induced motility of the HepG2 cells by inhibiting S100A4 gene expression. We found that it could prevent TPA-induced migration without affecting MAPK/ERK signaling. ATZ seems to modulate only the FAK pathway as well as the expression of fibronectin and its receptor Itga5.

2. Materials and methods

2.1. Materials

The human hepatocellular carcinoma cells HepG2 were obtained from ATCC (American Type Culture Collection, Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin solution, sodium pyruvate and Eagle's non-essential amino acids were from BioWhittaker (Cambrex Company, Walkersville, USA). DMSO (dimethylsulfoxide) and chemicals were from Sigma–Aldrich (L'Isle d'Abeau Chesne, Saint Quentin Fallavier, France). Protein assay materials were from Bio-Rad. All fluorescence reagents were from Molecular Probes (Eugene, OR). The herbicide atrazine was from Sigma Aldrich. The antibodies FAK, p-FAK, Erk2 and p-Erk1/2 used for western blotting experiments were from Cell Signaling. Cells were visualized with a Nikon Eclipse TE2000 phase contrast microscope.

2.2. Cell culture and drug treatments

HepG2 cells were maintained in DMEM with 1% penicillin/streptomycin, 1% non essential amino acids and sodium pyruvate, and 10% FBS, in humidified atmosphere at 37 °C containing 95% O_2 and 5% CO_2. After washing with sterile phosphate buffer saline (PBS), cells were detached by trypsinization (trypsin/EDTA) and plated at a concentration of $0.5–2 \times 10^6$ cells in 6-well plates depending on the experiment. For all experimental conditions, FBS was reduced to 5% in DMEM medium.

2.3. Western blot

HepG2 cells were scraped into a hypotonic buffer (20 mM HEPES ph7.5, 10 mM KCl, 15 mM $MgCL_2$, 0.25 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and a phosphatase inhibitory cocktail – PhosphoSTOP, Roche-). The protein concentration in each cell lysate was measured with a BCA Protein Assay Kit (Pierce), with bovine serum albumin (BSA) as a standard. Equal protein amounts were separated by SDS–polyacrylamide gel electrophoresis on 10% gels before being transferred to PVDF membranes. The membranes were then immunoblotted with antibodies for 1 h at room temperature or overnight at 4 °C. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse or anti-rabbit immunoglobulin G; Promega, Madison, WI, USA) for 2 h at room temperature. After washing, the blot was reacted using an ECL detection kit. A signal was acquired using a CDD camera (ChemiGenius2, Syngene) and semi-quantitative analysis was then performed using GeneTools software.

2.4. Reverse transcription-quantitative polymerase chain reaction

Total RNA was isolated by acid phenol extraction. One microgram of total RNA was reverse transcribed using a kit (SuperScript II; Invitrogen Corp, Carlsbad, California) following the manufacturer's instructions. The resultant complementary DNA was diluted 100-fold (conditions set to obtain 98% efficacy), and for each gene (target genes *S100A4, ITGA5, FN1,* $P21^{waf1}$ and *ALBUMIN,* or reference gene *GAPDH*) and each condition (DMSO, atrazine, TPA, TPA + atrazine), a mixture of Taq polymerase, 6.4 mmol/L of magnesium chloride, deoxynucleotide triphosphate, primer, and the probe (https://www.roche-applied-science.com) was added. The cDNA was then amplified in a thermocycler (LightCycler 480; Roche Applied Science, Penzburg, Upper Bavaria, Germany) for 45 cycles using conditions of 95 °C and 60 °C for 10 s each.

Commercially available software (LightCycler 480; Roche Applied Science) was used for relative quantitative analysis.

2.5. Cell migration assay

At 90% confluence HepG2 cells were trypsined and $3x10^6$ of cells per well were added to a 6 well plate. After 24 h of incubation, the confluent tissue formed was scratched 4 times per well with a sterile pipette tip. Cells were washed twice with PBS medium then treated with 0.25% DMSO, 100 nM TPA, 25 or 250 μM atrazine and cotreatment TPA + atrazine at the same concentrations in a DMEM medium. FBS was reduced to 5%. Images were taken immediately with an inverted fluorescence microscope (Nikon) equipped with a CDD camera (ORCA-ER Hamamatsu Photonics) via NIS-Elements AR 2.30 software at 4× magnification. Twenty-four hours later, the cell migration progress was photographed in the same conditions and all data were treated with the TScratch software tool developed for automated analysis of monolayer wound healing assays as described by Gebäck et al. (2009).

2.6. Statistical analysis

Each experiment was repeated at least three times. Data shown are an average ± standard deviation (SD). Statistical analysis of *in vitro* studies was performed using a Student's *t* test. Levels of probability are indicated as $^{*}P < 0.05$ or $^{**}P < 0.01$.

3. Results

3.1. Atrazine represses TPA-induced phenotype changes and modulates S100A4 gene expression

We firstly observed by microscopic analysis morphological changes in HepG2 cells after 24 h of 100 nM TPA, with the apparition of characteristic fibroblast-shaped cells not detectable in the control condition with DMSO (Fig. 1A). We also tested two non toxic concentrations of the herbicide atrazine (50 and 250 μM) and detected no major changes in cell shape. However, when cells were co-treated with 100 nM TPA and 50 or 250 μM atrazine, they displayed a less well-defined mesenchymal shape in contrast with those treated with TPA alone, leading us to suspect that atrazine may repress its action. Interestingly, atrazine also prevented the TPA-induced decrease in gene expression of *Albumin*, a hepatocyte marker for differentiation (Fig. 1B). A preliminary transcriptomic analysis focusing on EMT biomarkers, revealed a dose–response inhibition in the gene expression of the clinical and metastasis marker *S100A4* (Fig. 1C). This down regulation was significant at 48 h with 50 μM atrazine and repression was almost total at high concentration (500 μM).

3.2. Atrazine represses S100A4 overexpression and migration induced by TPA

S100a4 has been implicated in the migration of cancer cells where it is found overexpressed. We therefore wondered whether atrazine was able to prevent the TPA-induced modification of *S100A4* expression. While 24 h of TPA 100 nM led to a strong 9-fold increase in levels of *S100A4*, co-treatment of TPA and the herbicide limited this increase to 2 and 2.5-fold depending on the concentration of atrazine: 250 and 50 μM respectively (Fig. 2A). Based on these findings we hypothesized that atrazine may be able to prevent TPA-induced cell migration. To investigate this, we used the wound healing assay (Fig. 2B) and observed that a low concentration of the herbicide (50 μM) reduced by 50% the TPA-induced migration of the HepG2 cells. A high concentration (250 μM) was sufficient to significantly abrogate the effect of TPA on HepG2 cell migration.

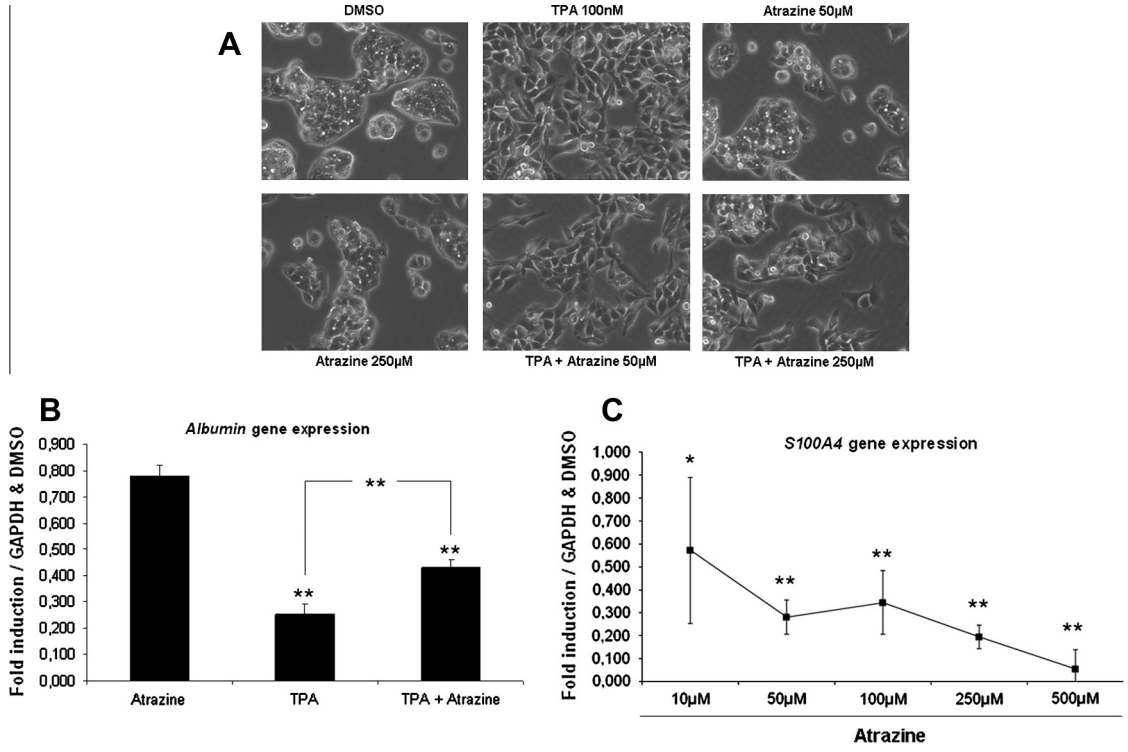

Fig. 1. Atrazine represses the TPA-induced phenotype changes and modulates S100A4 gene expression. (A) HepG2 cells exposed to 100 nM TPA alone, 50 or 250 μM atrazine alone, and co-treatment with 100 nM TPA and 50 or 250 μM atrazine for 24 h. Cell morphology was examined under a light microscope. (B) and (C) Changes in mRNA levels for the EMT-related *S100A4* and the hepatocyte biomarker *Albumin* genes were assessed by real-time RT-PCR. Cells were treated 48 h with 100 nM TPA with or without 500 μM atrazine (B), and 48 h with increasing concentrations of atrazine from 10 to 500 μM (C). Error bars indicate the mean ± SEM of triplicate determinations from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

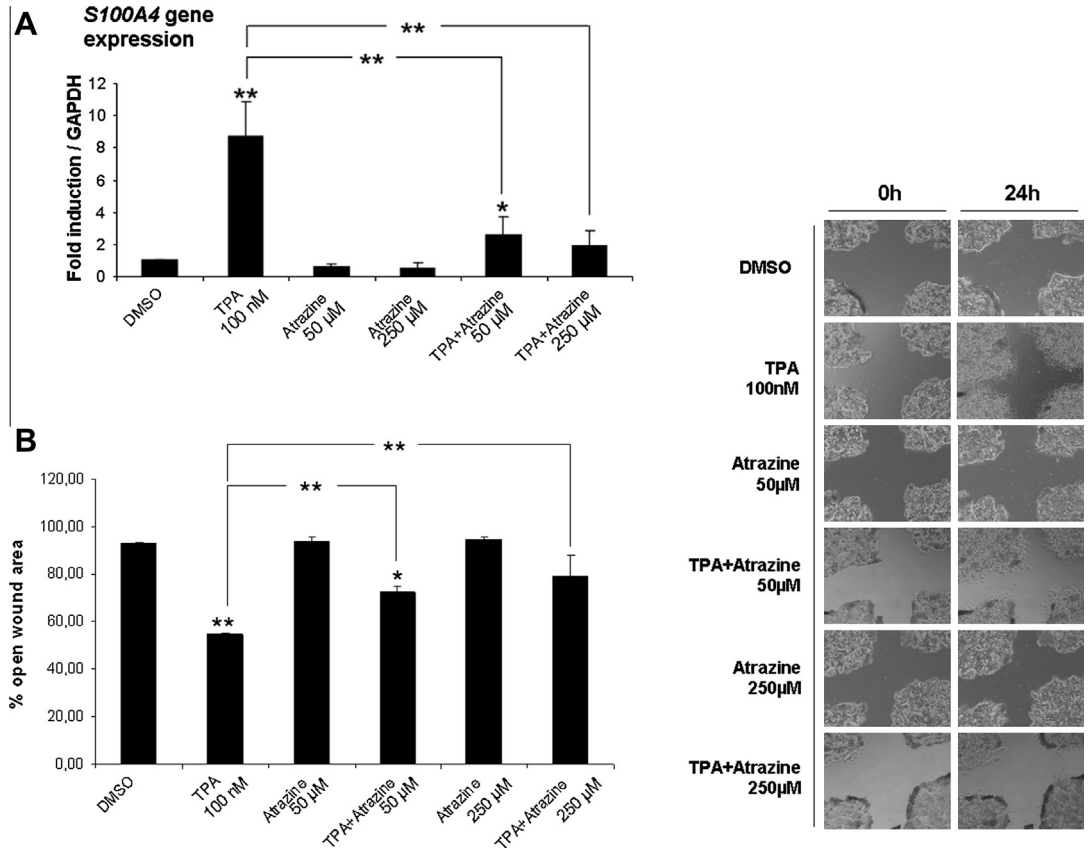

Fig. 2. Atrazine represses S100A4 overexpression and TPA-induced migration in HepG2 cells. (A) *S100A4* (*FSP1*) mRNA levels were assessed by real-time RT-PCR after 24 h of TPA treatment, with or without atrazine (50 and 250 µM). Relative mRNA expression levels (normalized with respect to *gapdh*) were determined and mRNA levels in DMSO-treated condition were set to 1. Error bars indicate the mean ± SEM of triplicate determinations from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Percentages of open wound area at 24 h, in each condition set, as in A, were plotted, with wound width normalized with respect to the initial value 0 h. Data are means ± SEM $^{*}P < 0.05$; $^{**}P < 0.01$.

3.3. Atrazine decreases the phosphorylation of FAK but does not alter the activation of Erk1/2 induced by TPA

During cell migration, activation of the FAK pathway can lead to the downstream activation of the ERK/MAPK pathway. HepG2 cell migration has also been shown to be mainly governed by the early activation of the Erk1/2 pathway (Kodama and Negishi, 2011; Romanelli et al., 2006; Song et al., 2011). With this in mind, we decided to investigate the modulations of Erk1/2 and FAK phosphorylation on tyrosine 925 and thus verify whether the observed effects of atrazine were ERK-dependant. While 100nM TPA alone was sufficient to activate both pathways, 4 h co-treatment with atrazine (25 and 250 µM) only affected FAK phosphorylation (Fig. 3A). Indeed, as shown on densitometry, the atrazine repressed the TPA-induced increase in FAK phosphorylation (Fig. 3C) without affecting the Erk pathway (Fig. 3B) thus indicating an ERK/MAPK pathway independent mechanism of action.

3.4. Atrazine decreases the TPA-dependant increase of ITGA5 and FN1 gene expression and potentiates the overexpression of $P21^{waf1}$

During EMT and cancer cell migration, the overexpression of mesenchymal extracellular compounds, such as fibronectin, is linked to an upregulation of their specific integrin receptors, in this case Itga5. We noticed that TPA was able to induce both *FN1* and *ITGA5* overexpression (Fig. 4B and A respectively). Interestingly, atrazine partially though nevertheless significantly repressed this overexpression at 48 h and at a high concentration (500 µM). Indeed, we noticed a much reduced moderate overexpression (6-fold) of the *ITGA5* gene when HepG2 cells were co-treated with TPA and atrazine by comparison with the expression levels following treatment with TPA alone (10-fold). In the same way, the overexpression of *FN1* induced by TPA alone (2.0-fold) was repressed following co-treatment with atrazine to 1.4-fold. The repressor of Cdk proteins $P21^{waf1}$ acts as a cell cycle inhibitor and induces cell growth arrest. Atrazine has been shown to induce such an event by increasing the $P21^{waf1}$ expression in HepG2 cells (Powell et al., 2011). In concordance with this observation, we found that at a high concentration (500 µM) atrazine increased $P21^{waf1}$ expression by 3.6-fold. TPA alone was found to do the same by increasing $P21^{waf1}$ expression by 6.1-fold. Co-treatment led to a strong increase of 16.5-fold in $P21^{waf1}$ expression, suggesting a possible consequential arrest of cell growth following contact with the two chemicals.

4. Discussion

An increase in *S100A4* expression levels has previously been correlated with cell migration (Jenkinson et al., 2004), invasion of hepatocellular carcinoma (Zhang et al., 2012), metastasis *in vitro* (Cui et al., 2006) and poor prognosis in patients with HCC (Kim et al., 2011). Complementary data have shown that *S100A4* down regulation or repression suppresses cell growth and invasion in different types of cancer (Fujiwara et al., 2011; Grum-Schwensen et al., 2005; Huang et al., 2012; Tabata et al., 2009). In the present study, *S100A4* was shown to be modulated by ATZ in a dose-dependent manner. While the exact mechanisms behind its genic regulation remain obscure, *S100A4* is reported to be involved in several diseases such as rheumatoid arthritis, kidney and liver fibrosis or

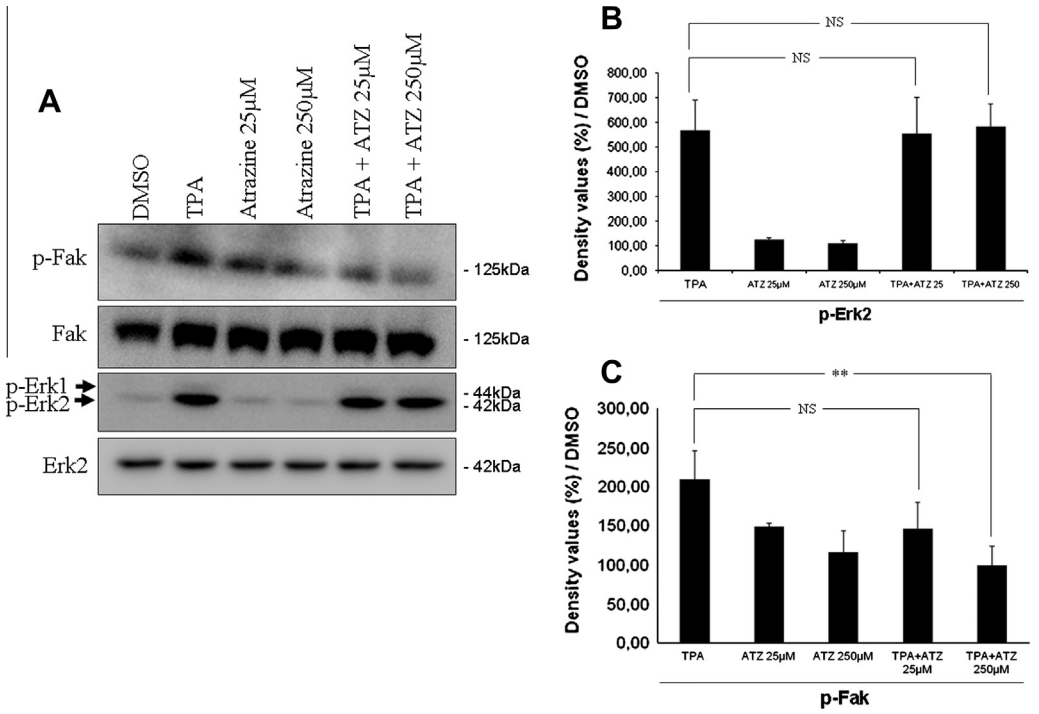

Fig. 3. Atrazine decreases the FAK phosphorylation but has no effect on the Erk1/2 activation induced by TPA. (A) HepG2 cells were exposed to 100 nM TPA alone, 25 or 250 µM atrazine alone and co-treatments of TPA 100 nM and atrazine (25 or 250 µM) for 4 h. Cells were lysed and subjected to western blotting, as described in section 2. The FAK and the Erk1/2 signaling pathways were studied by analyzing the phosphorylation of FAK on tyrosine 925 and the phosphorylation of Erk1/2 on threonine 202/tyrosine 204 respectively, together with total FAK and Erk2 protein levels. (B) and (C) the semi-quantification of chemiluminescence was performed after the acquisition with a CDD camera. P-Erk2 and p-Fak ratio were calculated from band densitometry measured on three independent experiments after normalization by DMSO-control cells value and by respectively Erk2 and Fak total protein. Data are expressed as the mean ± S.D. $^{*}P < 0.05$ and $^{**}P < 0.01$ obtained by a Student's *t* test.

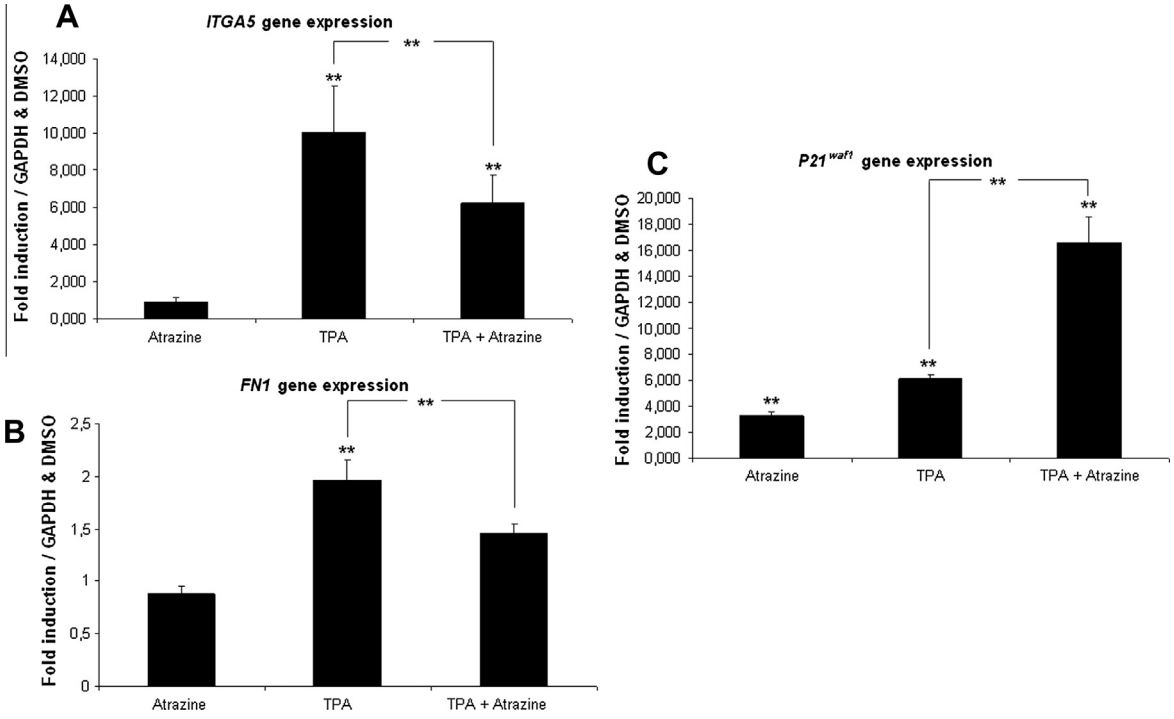

Fig. 4. Atrazine decreases the TPA-dependant increase of *ITGA5* and *FN1* gene expression and potentiates the *P21^{waf1}* overexpression. *Itga5* (A), *fibronectin* (B) and *P21^{waf1}* (C) mRNA levels were assessed by real-time RT-PCR after 48 h of TPA 100 nM alone, atrazine 500 µM alone and co-treatment with TPA and atrazine at the indicated concentrations. Relative mRNA expression levels (normalized with respect to *gapdh*) were determined and mRNA levels in DMSO-treated condition were set to 1. Error bars indicate the mean ± SEM of triplicate determinations from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

neural disease. Since it is also involved in metastasis where it is highly expressed (Mishra et al., 2011) we investigated the effect of ATZ on *S100A4* gene expression and on HepG2 cell phenotype and their ability to migrate. For the first time, we have revealed an original *in vitro* relationship between the herbicide atrazine suspected as being a risk to human health, and inhibition of *S100A4* expression positively correlated with human cancer progression.

In this work, we used atrazine at higher doses than those found in the environment in order to decipher the mechanistic impact of this herbicide on liver cells. Indeed, the problem with most *in vitro* models is their poor relevance to physiological conditions, since we are currently unable to determine the actual concentrations of a given compound available at its cellular and/or molecular target level (plastic adsorption, protein and membrane binding, metabolism...). The most pertinent model to date involves pharmacokinetics in relation to toxicological studies. Indeed, for any given compound, we can only correlate systemic exposure and more specifically the plasma unbound fraction, with one potentially toxic effect. Consequently, potentially toxic effects at the target level are only reliable with detectable plasma concentrations, even if the relation between the plasma unbound fraction and pharmacological effects is unsatisfactory. Unfortunately, human pharmacokinetics data are very scarce for this compound. Moreover, the general population can also be punctually exposed to much higher atrazine doses than those present in the environment during pest control operations, and workers can be regularly exposed to this herbicide during its manufacture, formulation, packing, transportation and storage, as well as in and after its application. For instance, according to Lin et al. (2013), the exposure levels could reach up 151 mg for atrazine manufacturing workers, indicating a much higher occupational risk than that estimated from acute and chronic dietary exposures for the general U.S. population (respectively 0.234–0.857 and 0.046–0.286 μg/kg/day).

TPA dedifferentiated HepG2 cells as demonstrated by their fibroblastic-like shape revealed by microscopy and through the downregulation of *Albumin* gene expression. This marker is only expressed in the liver by hepatocyte cells and closely correlates its differentiation (Hengstler et al., 2005). HepG2 cancer cells maintain many of their original epithelial characteristics and function similarly to well-differentiated hepatocytes (Kelly and Darlington, 1989). Modulation of Albumin gene expression seems to be a good indicator of HepG2 dedifferentiation. Interestingly, ATZ was able to prevent the morphological changes induced by TPA. It also had an inhibitory effect on HepG2 cell migration, FAK activation and overexpression of the mesenchymal markers *S100A4*, *FN1* and its specific receptor *ITGA5*. This would suggest that ATZ could act as a potential repressor of the EMT process by an as yet unknown mechanism. ATZ has been shown to interact with biomembranes and to induce lipid peroxidation as well as an increase of the expression of three connexins Cx26, Cx32 and Cx43 (Campos-Pereira et al., 2012). We postulate that the observed increase in expression of connexins in this study in response to ATZ-dependent liver damage could protect cells from loss of intercellular junctions and EMT.

Moreover, the increase of connexins could result in an increase in calcium transport across the membrane (Decrock et al., 2011) ultimately leading to the arrest of cell growth (Liu et al., 2009). In the present study, we investigated the possible role of ATZ in cell cycle arrest. Indeed, many studies have linked ATZ with inhibition of cell growth (Abarikwu et al., 2011; Manske et al., 2004), also on HepG2 cell line (Powell et al., 2011). We identified a strong overexpression of $P21^{waf1}$ when cells were co-treated with TPA and ATZ compared to lower levels of upregulation found in single conditions (ATZ or TPA). This overexpression could lead to higher levels of cell growth inhibition to those observed in such single conditions (Wen-Sheng, 2003; Wu and Hsu, 2001). In cancer cells, suppression of S100A4 may lead to cell growth arrest (Grum-Schwensen et al., 2005) or to the initiation of anoikis (Shen et al., 2011). In a previous study, we showed that HepG2 cells are resistant to anoikis (Peyre et al., 2012). S100A4 repression and $P21^{waf1}$ upregulation could be two additional events that lead to HepG2 cell growth arrest in response to ATZ.

S100a4 has been shown to interact with different cytoskeleton partners such as F-actin, tropomyosin and the heavy chain of the nonmuscle myosin II (Boye and Maelandsmo, 2010). These interactions are crucial for cytoskeleton rearrangement and cell motility during metastasis (Garrett et al., 2006). Indeed, S100a4 activity has been reported to promote disassembly of myosin-II filament, to increase directional motility and to affect actin–myosin contractility by direct binding to F-actin (Helfman et al., 2005). Repression of this calcium dependent protein by ATZ would be expected to have negative consequences on all of these events and thus on the motility of HepG2 and other cancer cells (Olson and Sahai, 2009). Additional data showed that ATZ can deregulate the expression of ACTB coding for cytoskeleton proteins Actins and involved in various types of cell motility (Lasserre et al., 2009). Actin interacts with S100a4 suggesting that its downregulation could be a supplementary point in favor of the repression of cell migration when hepG2 cells are co-treated with TPA and ATZ.

Integrins are transmembrane heterodimeric receptors that promote interactions between cytoskeleton proteins and the ECM, and allow signal transduction between cells and their microenvironment (Yam et al., 2009). We found that co-treatment with TPA and ATZ prevent the overexpression of *ITGA5* observed following treatment with TPA only. In gastric cancer cells, repression of *S100A4* results in a decrease in integrin α5 expression (Shen et al., 2011). Here we observed the same correlation between S100A4 and ITGA5 thus indicating the possible involvement of the same interaction. This integrin plays a key role in the EMT process (Maschler et al., 2005; Nam et al., 2012). Its increased expression is linked to an increase in levels of its natural ligand fibronectin, and correlates with the initiation of the cellular migration process (Bianchi et al., 2010). Here we also observed when cells are co-treated with TPA and ATZ, a significant repression of the FN1 upregulation induced by TPA alone. Moreover, integrins mediate the transduction signal via focal adhesion protein (FAK). Phosphorylation of Y925 is associated with integrin adhesion and E-cadherin deregulation during Src-induced epithelial mesenchymal transition in colon cancer cells (Brunton et al., 2005) and with Erk activation in human 293 kidney epithelial cells (Schlaepfer and Hunter, 1997). We noted that ATZ decreased the activation of this pathway when co-treated with TPA. ATZ thus seems to perturb the expression of key proteins necessary for cancer cell migration. It prevents the effects of TPA on *FN1*, *ITGA5* and p-FAK overexpression.

Finally, $S100A4^{-/-}$ knockout mice have been shown to develop spontaneous tumors including non-metastatic hepatocellular carcinoma (El Naaman et al., 2004). The proposed explanation is based on the fact that $S100A4^{-/-}$ tissue tumors are P53 positive, but the function of P53 is altered. Under genotoxic stress, such mutated cells are not eliminated by apoptosis thus allowing their selection and leading to the formation of cancer. Here ATZ could have an important role: by repressing *S100A4* gene expression, ATZ could induce such a selection harmful to the organism by promoting the development of spontaneous tumors. Although we have demonstrated the non-metastatic effects of ATZ, repression of S100A4 induced by this herbicide is a concern that warrants further investigation.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgement

The authors received a Public Institutional Funding from INRA. We thank the TOXALIM Research Centre in Food Toxicology for their support.

References

Abarikwu, S.O., Farombi, E.O., Pant, A.B., 2011. Biflavanone-kolaviron protects human dopaminergic SH-SY5Y cells against atrazine induced toxic insult. Toxicol. In Vitro 25, 848–858.

Alavanja, M.C., Samanic, C., Dosemeci, M., Lubin, J., Tarone, R., Lynch, C.F., Knott, C., Thomas, K., Hoppin, J.A., Barker, J., Coble, J., Sandler, D.P., Blair, A., 2003. Use of agricultural pesticides and prostate cancer risk in the Agricultural Health Study cohort. Am. J. Epidemiol. 157, 800–814.

ATSDR, 2003. <http://www.atsdr.cdc.gov/toxguides/toxguide-153.pdf>

Betancourt, M., ResÃ©ndiz, A., Fierro, E.C., 2006. Effect of two insecticides and two herbicides on the porcine sperm motility patterns using computer-assisted semen analysis (CASA) in vitro. Reprod. Toxicol. 22, 508–512.

Bianchi, A., Gervasi, M.E., Bakin, A., 2010. Role of β5-integrin in epithelial–mesenchymal transition in response to TGF-β. Cell Cycle 9, 1647–1659.

Boye, K., Maelandsmo, G.M., 2010. S100A4 and metastasis: a small actor playing many roles. Am. J. Pathol. 176, 528–535.

Brunton, V.G., Avizienyte, E., Fincham, V.J., Serrels, B., Metcalf, C.A., Sawyer, T.K., Frame, M.C., 2005. Identification of Src-specific phosphorylation site on focal adhesion kinase: dissection of the role of Src SH2 and catalytic functions and their consequences for tumor cell behavior. Cancer Res. 65, 1335–1342.

Campos-Pereira, F.D., Oliveira, C.A., Pigoso, A.A., Silva-Zacarin, E.C., Barbieri, R., Spatti, E.F., Marin-Morales, M.A., Severi-Aguiar, G.D., 2012. Early cytotoxic and genotoxic effects of atrazine on Wistar rat liver: a morphological, immunohistochemical, biochemical, and molecular study. Ecotoxicol. Environ. Saf. 78, 170–177.

Cui, J.F., Liu, Y.K., Zhang, L.J., Shen, H.L., Song, H.Y., Dai, Z., Yu, Y.L., Zhang, Y., Sun, R.X., Chen, J., Tang, Z.Y., Yang, P.Y., 2006. Identification of metastasis candidate proteins among HCC cell lines by comparative proteome and biological function analysis of S100A4 in metastasis in vitro. Proteomics 6, 5953–5961.

De Roos, A.J., Zahm, S.H., Cantor, K.P., Weisenburger, D.D., Holmes, F.F., Burmeister, L.F., Blair, A., 2003. Integrative assessment of multiple pesticides as risk factors for non-Hodgkin's lymphoma among men. Occup. Environ. Med. 60, E11.

Decrock, E., Vinken, M., Bol, M., D'Herde, K., Rogiers, V., Vandenabeele, P., Krysko, D.V., Bultynck, G., Leybaert, L., 2011. Calcium and connexin-based intercellular communication, a deadly catch? Cell Calcium 50, 310–321.

Donna, A., Crosignani, P., Robutti, F., Betta, P.G., Bocca, R., Mariani, N., Ferrario, F., Fissi, R., Berrino, F., 1989. Triazine herbicides and ovarian epithelial neoplasms. Scand. J. Work Environ. Health 15, 47–53.

El Naaman, C., Grum-Schwensen, B., Mansouri, A., Grigorian, M., Santoni-Rugiu, E., Hansen, T., Kriajevska, M., Schafer, B.W., Heizmann, C.W., Lukanidin, E., Ambartsumian, N., 2004. Cancer predisposition in mice deficient for the metastasis-associated Mts1(S100A4) gene. Oncogene 23, 3670–3680.

Engel, L.S., Hill, D.A., Hoppin, J.A., Lubin, J.H., Lynch, C.F., Pierce, J., Samanic, C., Sandler, D.P., Blair, A., Alavanja, M.C., 2005. Pesticide use and breast cancer risk among farmers' wives in the agricultural health study. Am. J. Epidemiol. 161, 121–135.

Entschladen, F., ZÃ¤nker, K.S., Powe, D.G., 2011. Heterotrimeric G protein signaling in cancer cells with regard to metastasis formation. Cell Cycle 10, 1086–1091.

Freeman, L.E., Rusiecki, J.A., Hoppin, J.A., Lubin, J.H., Koutros, S., Andreotti, G., Zahm, S.H., Hines, C.J., Coble, J.B., Barone-Adesi, F., Sloan, J., Sandler, D.P., Blair, A., Alavanja, M.C., 2011. Atrazine and cancer incidence among pesticide applicators in the agricultural health study (1994–2007). Environ. Health Perspect. 119, 1253–1259.

Fujiwara, M., Kashima, T.G., Kunita, A., Kii, I., Komura, D., Grigoriadis, A.E., Kudo, A., Aburatani, H., Fukayama, M., 2011. Stable knockdown of S100A4 suppresses cell migration and metastasis of osteosarcoma. Tumour Biol. 32, 611–622.

Gammon, D.W., Aldous, C.N., Carr, W.C., Sanborn, J.R., Pfeifer, K.F., 2005. A risk assessment of atrazine use in California: human health and ecological aspects. Pest Manag. Sci. 61, 331–355.

Garrett, S.C., Varney, K.M., Weber, D.J., Bresnick, A.R., 2006. S100A4, a mediator of metastasis. J. Biol. Chem. 281, 677–680.

Gebäck, T., Schulz, M.M., Koumoutsakos, P., Detmar, M., 2009. TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays. Biotechniques 46, 265–274.

Grigorian, M., Andresen, S., Tulchinsky, E., Kriajevska, M., Carlberg, C., Kruse, C., Cohn, M., Ambartsumian, N., Christensen, A., Selivanova, G., Lukanidin, E., 2001. Tumor suppressor p53 protein is a new target for the metastasis-associated Mts1/S100A4 protein: functional consequences of their interaction. J. Biol. Chem. 276, 22699–22708.

Grum-Schwensen, B., Klingelhofer, J., Berg, C.H., El-Naaman, C., Grigorian, M., Lukanidin, E., Ambartsumian, N., 2005. Suppression of tumor development and metastasis formation in mice lacking the S100A4(mts1) gene. Cancer Res. 65, 3772–3780.

Helfman, D.M., Kim, E.J., Lukanidin, E., Grigorian, M., 2005. The metastasis associated protein S100A4: role in tumour progression and metastasis. Br. J. Cancer 92, 1955–1958.

Hengstler, J.G., Brulport, M., Schormann, W., Bauer, A., Hermes, M., Nussler, A.K., Fandrich, F., Ruhnke, M., Ungefroren, H., Griffin, L., Bockamp, E., Oesch, F., von Mach, M.A., 2005. Generation of human hepatocytes by stem cell technology: definition of the hepatocyte. Expert Opin. Drug Metab. Toxicol. 1, 61–74.

Hessel, P.A., Kalmes, R., Smith, T.J., Lau, E., Mink, P.J., Mandel, J., 2004. A nested case-control study of prostate cancer and atrazine exposure. J. Occup. Environ. Med. 46, 379–385.

Hu, C.T., Wu, J.R., Chang, T.Y., Cheng, C.C., Wu, W.S., 2008. The transcriptional factor Snail simultaneously triggers cell cycle arrest and migration of human hepatoma HepG2. J. Biomed. Sci. 15, 343–355.

Huang, L., Xu, Y., Cai, G., Guan, Z., Cai, S., 2012. Downregulation of S100A4 expression by RNA interference suppresses cell growth and invasion in human colorectal cancer cells. Oncol. Rep. 27, 917–922.

Jenkinson, S.R., Barraclough, R., West, C.R., Rudland, P.S., 2004. S100A4 regulates cell motility and invasion in an in vitro model for breast cancer metastasis. Br. J. Cancer 90, 253–262.

Kelly, J.H., Darlington, G.J., 1989. Modulation of the liver specific phenotype in the human hepatoblastoma line HepG2. In Vitro Cell. Dev. Biol. 25, 217–222.

Kim, H., Choi, G.H., Na, D.C., Ahn, E.Y., Kim, G.I., Lee, J.E., Cho, J.Y., Yoo, J.E., Choi, J.S., Park, Y.N., 2011. Human hepatocellular carcinomas with "Stemness"-related marker expression: keratin 19 expression and a poor prognosis. Hepatology 54, 1707–1717.

Kodama, S., Negishi, M., 2011. Pregnane X receptor PXR activates the GADD45beta gene, eliciting the p38 MAPK signal and cell migration. J. Biol. Chem. 286, 3570–3578.

Lasserre, J.P., Fack, F., Revets, D., Planchon, S., Renaut, J., Hoffmann, L., Gutleb, A.C., Muller, C.P., Bohn, T., 2009. Effects of the endocrine disruptors atrazine and PCB 153 on the protein expression of MCF-7 human cells. J. Proteome Res. 8, 5485–5496.

Laville, N., Balaguer, P., Brion, F., Hinfray, N., Casellas, C., Porcher, J.M., Ait-Aissa, S., 2006. Modulation of aromatase activity and mRNA by various selected pesticides in the human choriocarcinoma JEG-3 cell line. Toxicology 228, 98–108.

Lin, Z., Fisher, J.W., Wang, R., Ross, M.K., Filipov, N.M., 2013. Estimation of placental and lactational transfer and tissue distribution of atrazine and its main metabolites in rodent dams, fetuses, and neonates with physiologically based pharmacokinetic modeling. Toxicol. Appl. Pharmacol. 273, 140–158.

Liu, C.L., Huang, Y.S., Hosokawa, M., Miyashita, K., Hu, M.L., 2009. Inhibition of proliferation of a hepatoma cell line by fucoxanthin in relation to cell cycle arrest and enhanced gap junctional intercellular communication. Chem. Biol. Interact. 182, 165–172.

Manske, M.K., Beltz, L.A., Dhanwada, K.R., 2004. Low-level atrazine exposure decreases cell proliferation in human fibroblasts. Arch. Environ. Contam. Toxicol. 46, 438–444.

Maschler, S., Wirl, G., Spring, H., Bredow, D.V., Sordat, I., Beug, H., Reichmann, E., 2005. Tumor cell invasiveness correlates with changes in integrin expression and localization. Oncogene 24, 2032–2041.

Mills, P.K., 1998. Correlation analysis of pesticide use data and cancer incidence rates in California counties. Arch. Environ. Health 53, 410–413.

Mishra, S.K., Siddique, H.R., Saleem, M., 2011. S100A4 calcium-binding protein is key player in tumor progression and metastasis: preclinical and clinical evidence. Cancer Metastasis Rev.

Murata, K., Suzuki, H., Okano, H., Oyamada, T., Yasuda, Y., Sakamoto, A., 2009. Cytoskeletal changes during epithelial-to-fibroblastoid conversion as a crucial mechanism of des-gamma-carboxy prothrombin production in hepatocellular carcinoma. Int. J. Oncol. 35, 1005–1014.

Nam, E.H., Lee, Y., Park, Y.K., Lee, J.W., Kim, S., 2012. ZEB2 upregulates integrin α5 expression through cooperation with Sp1 to induce invasion during epithelial–mesenchymal transition of human cancer cells. Carcinogenesis 33, 563–571.

Olson, M.F., Sahai, E., 2009. The actin cytoskeleton in cancer cell motility. Clin. Exp. Metastasis 26, 273–287.

Peyre, L., Zucchini-Pascal, N., de Sousa, G., Rahmani, R., 2012. Effects of endosulfan on hepatoma cell adhesion: epithelial–mesenchymal transition and anoïkis resistance. Toxicology 300, 19–30.

Pinzani, M., 2011. Epithelial–mesenchymal transition in chronic liver disease: fibrogenesis or escape from death? J. Hepatol. 55, 459–465.

Pommery, J., Mathieu, M., Mathieu, D., Lhermitte, M., 1993. Atrazine in plasma and tissue following atrazine–aminotriazole–ethylene glycol–formaldehyde poisoning. J. Toxicol. Clin. Toxicol. 31, 323–331.

Powell, E.R., Faldladdin, N., Rand, A.D., Pelzer, D., Schrunk, E.M., Dhanwada, K.R., 2011. Atrazine exposure leads to altered growth of HepG2 cells. Toxicol. In Vitro 25, 644–651.

Romanelli, R.G., Petrai, I., Robino, G., Efsen, E., Novo, E., Bonacchi, A., Pagliai, G., Grossi, A., Parola, M., Navari, N., Delogu, W., Vizzutti, F., Rombouts, K., Gentilini, P., Laffi, G., Marra, F., 2006. Thrombopoietin stimulates migration and activates multiple signaling pathways in hepatoblastoma cells. Am. J. Physiol. Gastrointest. Liver Physiol. 290, G120–G128.

Rusiecki, J.A., De Roos, A., Lee, W.J., Dosemeci, M., Lubin, J.H., Hoppin, J.A., Blair, A., Alavanja, M.C., 2004. Cancer incidence among pesticide applicators exposed to atrazine in the Agricultural Health Study. J. Natl Cancer Inst. 96, 1375–1382.

Sack, U., Stein, U., 2009. Wnt up your mind – intervention strategies for S100A4-induced metastasis in colon cancer. Gen. Physiol. Biophys. 28, F55–F64, Spec No Focus.

Sanderson, J.T., Letcher, R.J., Heneweer, M., Giesy, J.P., van den Berg, M., 2001. Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various

human cell lines and on vitellogenin production in male carp hepatocytes. Environ. Health Perspect. 109, 1027–1031.
Sathiakumar, N., MacLennan, P.A., Mandel, J., Delzell, E., 2011. A review of epidemiologic studies of triazine herbicides and cancer. Crit. Rev. Toxicol. 41 (Suppl 1), 1–34.
Schaller, M.D., 2010. Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. J. Cell Sci. 123, 1007–1013.
Schlaepfer, D.D., Hunter, T., 1997. Focal adhesion kinase overexpression enhances ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. J. Biol. Chem. 272, 13189–13195.
Schneider, M., Hansen, J.L., Sheikh, S.P., 2008. S100A4: a common mediator of epithelial–mesenchymal transition, fibrosis and regeneration in diseases? J. Mol. Med. (Berl.) 86, 507–522.
Schroeder, J.C., Olshan, A.F., Baric, R., Dent, G.A., Weinberg, C.R., Yount, B., Cerhan, J.R., Lynch, C.F., Schuman, L.M., Tolbert, P.E., Rothman, N., Cantor, K.P., Blair, A., 2001. Agricultural risk factors for t(14;18) subtypes of non-Hodgkin's lymphoma. Epidemiology 12, 701–709.
Shen, W., Chen, D., Fu, H., Liu, S., Sun, K., Sun, X., 2011. S100A4 protects gastric cancer cells from anoikis through regulation of αv and α5 integrin. Cancer Sci. 102, 1014–1018.
Simpkins, J.W., Swenberg, J.A., Weiss, N., Brusick, D., Eldridge, J.C., Stevens, J.T., Handa, R.J., Hovey, R.C., Plant, T.M., Pastoor, T.P., Breckenridge, C.B., 2011. Atrazine and breast cancer: a framework assessment of the toxicological and epidemiological evidence. Toxicol. Sci. 123, 441–459.
Song, M.K., Kim, Y.J., Song, M., Choi, H.S., Park, Y.K., Ryu, J.C., 2011. Polycyclic aromatic hydrocarbons induce migration in human hepatocellular carcinoma cells (HepG2) through reactive oxygen species-mediated p38 MAPK signal transduction. Cancer Sci. 102, 1636–1644.
Tabata, T., Tsukamoto, N., Fooladi, A.A., Yamanaka, S., Furukawa, T., Ishida, M., Sato, D., Gu, Z., Nagase, H., Egawa, S., Sunamura, M., Horii, A., 2009. RNA interference targeting against S100A4 suppresses cell growth and motility and induces apoptosis in human pancreatic cancer cells. Biochem. Biophys. Res. Commun. 390, 475–480.
Tchounwou, P.B., Wilson, B.A., Ishaque, A.B., Schneider, J., 2001. Atrazine potentiation of arsenic trioxide-induced cytotoxicity and gene expression in human liver carcinoma cells (HepG2). Mol. Cell. Biochem. 222, 49–59.
Wen-Sheng, W., 2003. ERK signaling pathway is involved in p15INK4b/p16INK4a expression and HepG2 growth inhibition triggered by TPA and Saikosaponin a. Oncogene 22, 955–963.
Whalen, M.M., Loganathan, B.G., Yamashita, N., Saito, T., 2003. Immunomodulation of human natural killer cell cytotoxic function by triazine and carbamate pesticides. Chem. Biol. Interact. 145, 311–319.
Winchester, P.D., Huskins, J., Ying, J., 2009. Agrichemicals in surface water and birth defects in the United States. Acta Paediatr. 98, 664–669.
Wu, W.S., Hsu, H.Y., 2001. Involvement of p-15(INK4b) and p-16(INK4a) gene expression in saikosaponin a and TPA-induced growth inhibition of HepG2 cells. Biochem. Biophys. Res. Commun. 285, 183–187.
Wu, W.S., Tsai, R.K., Chang, C.H., Wang, S., Wu, J.R., Chang, Y.X., 2006. Reactive oxygen species mediated sustained activation of protein kinase C alpha and extracellular signal-regulated kinase for migration of human hepatoma cell Hepg2. Mol. Cancer Res. 4, 747–758.
Yam, J.W., Tse, E.Y., Ng, I.O., 2009. Role and significance of focal adhesion proteins in hepatocellular carcinoma. J. Gastroenterol. Hepatol. 24, 520–530.
Yilmaz, M., Christofori, G., 2009. EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev. 28, 15–33.
Zeljezic, D., Garaj-Vrhovac, V., Perkovic, P., 2006. Evaluation of DNA damage induced by atrazine and atrazine-based herbicide in human lymphocytes in vitro using a comet and DNA diffusion assay. Toxicol. In Vitro 20, 923–935.
Zhang, H.Y., Zheng, X.Z., Wang, X.H., Xuan, X.Y., Wang, F., Li, S.S., 2012. S100A4 mediated cell invasion and metastasis of esophageal squamous cell carcinoma via the regulation of MMP-2 and E-cadherin activity. Mol. Biol. Rep. 39, 199–208.