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Embryotoxic effects of the marine biotoxin okadaic acid on murine embryonic stem cells

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ABSTRACT

Okadaic acid (OA), a marine toxin produced by dinoflagellates, can accumulate in various bivalve molluscs. In humans, consumption of OA induces acute toxic effects like diarrhoea, nausea, vomiting and abdominal pain. OA is a potent inhibitor of protein phosphatase 1 (PP1) and 2A (PP2A), enzymes that are known to be critical regulators of embryonic development. To determine the embryotoxic potential of OA, we performed two independent cellular *in-vitro* assays, both of which are applicable for the detection of teratogenic compounds: (i) the validated embryonic stem cell test (EST) based on the morphological analysis of beating cardiomyocytes in embryoid bodies and (ii) the F9 cell assay quantifying the induction of cell differentiation by measuring the emitted luminescence of a reporter gene. In the presence of OA, beating cardiomyocytes in the EST were inhibited and the reporter gene in transiently transfected F9 cells was activated. Furthermore, OA treatment led to rapid morphological changes including cell rounding, the loss of cell–cell contacts and changed electrical impedance as monitored in real time by the xCELLigence system. The two independent bioassays (EST and F9 cell test) detected OA as a potential embryotoxic compound, since OA influences the differentiation process of cultured murine embryonic cells.

1. Introduction

Okadaic acid (OA) and its analogues, the dinophysis toxins (DTX1, DTX2, and DTX3) are produced by dinoflagellates and can be detected in various species of filter-feeding bivalve molluscs such as oysters, mussels, scallops, and clams. Together, they form the group of OA toxins that can induce so-called Diarrhoeic Shellfish Poisoning (DSP), characterised by acute symptoms such as diarrhoea, nausea, vomiting and abdominal pain (EFSA (European Food Safety Authority), 2008). Literature regarding the acute oral toxicity of OA varies considerably and description of lethal oral dose ranges from 400 to 2000 μg/kg bw (Ito et al., 2002; Le Hegarat et al., 2006; Tubaro et al., 2003).

Experiments conducted on adult Swiss mice given single oral doses by gavage of 50 or 90 μg ^{3}[H]OA/kg body weight (bw) concluded that OA is well-absorbed by the gastrointestinal tract. OA was distributed to all internal organs within 5 min, and remained for up to eight weeks in some tissues (Matias et al., 1999). Additionally, the same group demonstrated that oral doses of 50 μg OA/kg per body weight may pass through the placental barrier of pregnant mice (Matias and Creppy, 1996). However, they administered unphysiological high doses, which do not represent oral consumption. Nevertheless, availability of OA in the placenta is of great relevance, since OA is a potent inhibitor of serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A) (Dawson and Holmes, 1999; Honkanen et al., 1994; Takai et al., 1987) and has, therefore, the potential to disrupt the tightly regulated equilibrium of the phosphorylation and dephosphorylation status during embryonic development. Moreover, dynamic changes in cytoskeleton play a major role in embryo reorganization. It has been

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shown that OA effects the F-actin (Leira et al., 2001) and E-cadherin systems (Malaguti and Rossini, 2002), leading to a changed cell morphology and reduced cell–cell contacts. But so far, only insufficient data about a possible embryotoxicity of OA are available. A study with murine embryonic F9 cells demonstrated that treatment with OA leads to morphological changes, arrest of proliferation and an increase in the expression of differentiation markers (Nishina et al., 1995). And another study using *Xenopus laevis* embryos revealed a dose-dependent embryotoxic impact of OA on mortality, delayed growth and embryo malformation (Casarini et al., 2007).

Regarding this limited investigations, the aim of this study was to analyze the assumed embryotoxic effects of OA in more detail. Thus, two cellular assays especially designed for the identification of embryotoxic compounds and real-time monitoring of treated embryonic cells have been performed.

The EST (Seiler et al., 2006) is based on the morphological analysis of beating cardiomyocytes in embryoid body outgrowths compared to cytotoxic effects on embryonic cells and differentiated adult fibroblasts. The established EST analyzes three different endpoints: (i) cytotoxicity to murine embryonic D3 cells, (ii) cytotoxicity to murine adult 3T3 cells and (iii) inhibition of differentiation of D3 cells into beating cardiomyocytes.

The F9 reporter gene assay is a pre-validated embryotoxicity test system, and is based on the capacity of murine F9 cells to differentiate in the presence of teratogenic compounds (Lampen et al., 1999, 2001, 2005). The Rous Sarcoma Virus (RSV) promoter is repressed in undifferentiated F9 cells, whereas it is activated in differentiated cells (Sleigh, 1987). The F9 assay includes a DNA construct (pRSV-Luc) consisting of the RSV promoter followed by the reporter gene luciferase. Activation of the RSV promoter by embryotoxic substances can be quantified easily by the emitted luminescence.

The xCELLigence System (Roche, Mannheim, Germany) is an innovative method based on the measurement of electrical impedance to analyze cells in real time. The approach allows the monitoring of cell viability, number, morphology, and the degree of cell adhesion – cellular mechanisms playing an important role during embryogenesis.

The performance of these three independent assays allowed the analysis of different individual endpoints more clarifying the assumed embryotoxic potential of okadaic acid on mammalian cells.

2. Material and methods

2.1. Cell culture

All cells were cultivated in a humidified atmosphere of 37 °C and 5% CO_2. The murine embryonic stem cell line D3 was cultivated in DMEM (Gibco, Invitrogen, Karlsruhe, Germany) containing 15% fetal calf serum (FCS; heat-inactivated) (HyClone, ThermoScientific, Bonn, Germany), 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 1% nonessential amino acids (MEM NEAA, Gibco, Invitrogen, Karlsruhe, Germany) and 0.1 mM β-mercaptoethanol. During permanent cultivation, 1000 U/ml leukemia inhibitory factor (mLIF) was added, whereas the assays were performed in the absence of mLIF.

The murine Balb/c 3T3 fibroblast cell line was grown in DMEM containing 10% heat-inactivated FCS (HyClone, ThermoScientific, Bonn, Germany), 4 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin.

The murine embryonic F9 cell line was maintained in DMEM/Ham's F12 (PAA, Pasching, Germany) containing 10% heat-inactivated fetal bovine serum (FBS; PAA, Pasching, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.145 mM β-mercaptoethanol. Transfections were performed without antibiotics.

2.2. Microscopy

Photographic documentation of the cultured embryonic cells was obtained using an inverse microscope (Axio Observer, Zeiss) with an EC Plan-Neo NEOFLUAR 10x/0.3 Ph1 objective and an AxioCam MRc camera.

2.3. Embryonic stem cell test (EST)

The differentiation and cytotoxicity assays were carried out as described in detail by Seiler et al. (Seiler et al., 2006). All assays were reproduced three times.

2.3.1. Differentiation assay

The mouse embryonic stem cell line D3 was kept in permanent culture with the leukemia inhibitory factor (Chemicon, Hofheim, Germany) and spontaneous differentiation into cardiomyocytes was carried out in hanging drops in the absence of mLIF. The culture medium was supplemented with seven different concentrations of okadaic acid (OA) (Sigma–Aldrich, Steinheim) and of 5-Fluorouracil (5-FU) (Sigma–Aldrich, Steinheim, Germany), which was used as a positive control. Approximately 750 cells were forced by gravitation to aggregate thereby forming the so-called embryoid bodies (EBs), which were cultivated for two additional days in suspension. At day 5, the EBs were transferred separately into 24-well plates (Greiner Bio One, Frickenhausen, Germany), in which they were cultured for further 5 days. The possible inhibition of cardiomyocyte differentiation by the test substances was evaluated at day 10 by counting the proportion of EBs containing contracting cardiomyocytes. For each run, a positive control with 0.06 μg/ml 5-Fluorouracil was performed in parallel. The results were represented as concentration-response curves, whereof the inhibition of cell differentiation was determined by the half-maximal concentration, which decreases the development into contracting cardiomyocytes compared to control (ID_{50}).

2.3.2. Cytotoxicity assay

To analyze cytotoxicity, 500 D3 or 3T3 cells were seeded in a 96-well plate (Falcon, BD, New York, USA) in a volume of 50 μl medium per well. After 2 h 150 μl medium supplemented with different concentrations of okadaic acid and 5-Fluorouracil was added. Cells were cultivated for 10 days in the presence of the test substances and test medium was replaced at day 3 and day 5. At day 10, the mitochondrial dehydrogenase activity of the cells was

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quantified after a 2-h incubation with MTT solution (5 mg/ml PBS, Sigma Aldrich, Steinheim, Germany). Absorbance was determined in a spectrometer (GENios Multi-Detections Reader, Tecan, Männedorf, Germany) at 562 nm. For each run, a positive control with 5-Fluorouracil was performed using a concentration of 0.06 μg/ml for D3 and 0.29 μg/ml for 3T3 cells. The cytotoxicity was determined as the half-maximal inhibiting concentration (IC_{50}) from the resulting dose-response curves. IC_{50} and ID_{50} values were applied to a biostatistical prediction model based on linear discriminant functions, allowing the classification of the tested chemicals into three classes: non-embryotoxic, weakly, or strongly embryotoxic.

2.4. F9 reporter gene assay

Twenty four h before transfection 75 000 F9 cells were seeded in a 24-well plate in a volume of 500 μl medium per well. Gene transfer was performed using Metafectene® Pro (Biontex, Martiensried, Germany) according to the manufacturer's instructions. The final DNA content was 0.8 μg RSV-reporter-plasmid (pRSV-Luc) and 0.2 μg SV-β-Galactosidase control vector (pSV-β-Gal) per well. The following day, the medium in each well was replaced with medium containing different concentrations of okadaic acid and 1 mM valproic acid (VPA) as the positive control. Exposure was done in quadruplicate for each concentration. Twenty-four hours later, cells were harvested in 200 μl lysis buffer (0.1 M Tris-acetate, pH 7.8, 2 mM EDTA, 1% Triton X-100). Fifty μl of this cell lysate were pipetted into a white 96-well plate and transferred to a luminometer (Mithras LB 940, Berthold Technologies, Bad Wildbad). The samples were mixed automatically with 150 μl assay buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, 1 mM ATP, pH 7.8) and 50 μl luciferin buffer (1 M luciferin (s039, Synchem, Felsberg, Germany), 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA); luminescence was then measured. To standardize the transfection efficiencies in the different cavities, 50 μl of the cell lysate were pipetted into a transparent 96-well plate and 20 μl of an O-Nitrophenyl-β-D-galacto–pyranoside (ONPG) solution (2 mg/ml) and 100 μl Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol, pH 7,0) were added. Absorbance was determined in a spectrometer (GENios Multi-Detections Reader, Tecan, Männedorf, Germany) at 405 nm and the resulting values were subtracted from luminescence values for normalization of transfection efficiency. The luminescence values in the solvent-treated cells (negative control) were used as reference for the calculation of the luminescence fold change in treated F9 cells. The assay was reproduced three times.

2.5. MTT-assay

F9 cells (6000/well) were seeded in a 96-well plate and incubated for 24 h at 5% CO_2 and 37 °C. Subsequently, cells were treated with different concentrations of okadaic acid for 24 h and the mitochondrial dehydrogenase activity of the cells was quantified after a 2-h incubation with MTT solution (5 mg/ml PBS, Sigma Aldrich, Steinheim, Germany). Absorbance was determined in a spectrometer (GENios Multi-Detections Reader, Tecan, Männedorf, Germany) at 562 nm. In each experiment, 5-fluorouracil (2 μg/ml) was used as positive control.

2.6. Impedance measurement with the xCelligence system

Background of the E-plates was determined in 100 μl medium and subsequently 100 μl of the F9 cell suspension was added (10 000 cells/well). Cells were incubated for 30 min at room temperature and E-plates were placed into the Real-Time Cell Analyzer (RTCA) station. Cells were grown for 48 h, with impedance measured every 30 min. After 2 days cells were incubated with different concentrations of okadaic acid (5–50 nM) and monitored again every 30 min for 24 h. Impedance was represented by the cell index (CI) values (($Z_i - Z_0$) [Ohm]/15[Ohm]; Z_0: background resistance, Z_i: individual time point resistance) and the normalized cell index was calculated as the cell index CI_{ti} at a given time point divided by the cell index CI_{nml_time} at the normalization time point (nml_time). Dose-response curves at different points of time were generated to determine IC_{50} values during the incubation period.

2.7. Statistical analysis

Statistical analyzes were performed with SigmaPlot software. Differences between mean values were determined by one-way ANOVA followed by a Bonferroni *t*-test. Statistically significant differences were set at $p \leq 0.05$.

3. Results

In order to assess whether okadaic acid exhibits teratogenic potential, two independent cellular embryotoxicity assays were performed.

3.1. Embryonic stem cell test (EST)

As previously mentioned, the EST analyzes three different endpoints after a 10-day treatment with the test substances: (i) cytotoxicity to murine embryonic D3 cells, (ii) cytotoxicity to murine adult 3T3 cells, and (iii) the inhibition of differentiation of D3 cells into contracting cardiomyocytes.

The known embryotoxic compound 5-Fluorouracil was used to test the reproducibility and validity of the performed EST. As shown in Fig. 1, 5-FU inhibited the differentiation of D3 cells to cardiomyocytes (Fig. 1A) and was also cytotoxic to D3 and 3T3 cells (Fig. 1B). The following ID_{50} and IC_{50} values were ascertained for 5-FU: ID_{50}D3: 0.057 μg/ml, IC_{50}D3: 0.065 μg/ml and IC_{50}3T3: 0.308 μg/ml.

Subsequently the test substance OA was analyzed and 5-FU was used as positive control in each assay. After a 10-day incubation with okadaic acid, the three toxicological endpoints of the EST were determined and revealed an ID_{50}D3 of 8.55 nM (Fig. 2A), an IC_{50}D3 of 9.87 nM and an IC_{50}3T3 of 7.89 nM (Fig. 2B). Significant inhibition of cardiomyocyte beating ($p < 0.001$) could be demonstrated

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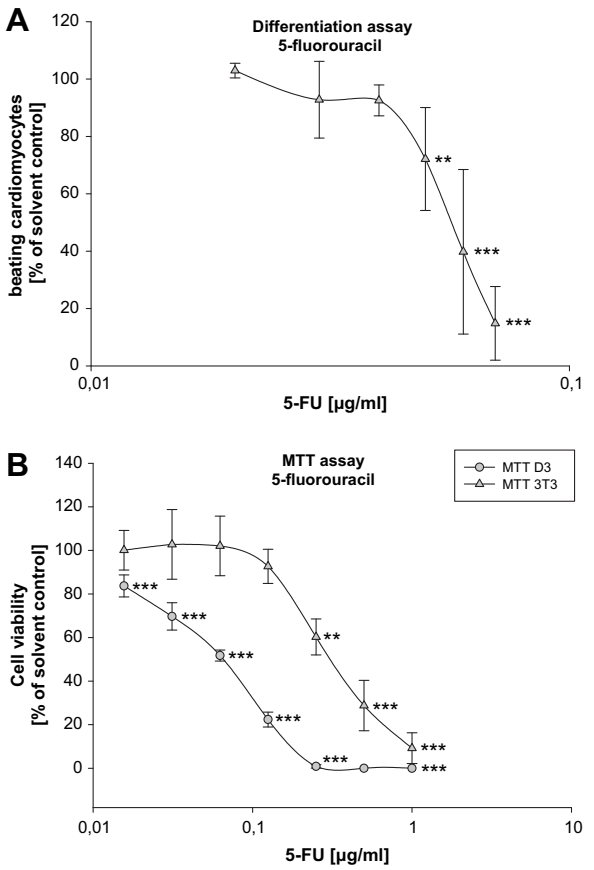

Fig. 1. Embryonic stem cell test (EST) with the known embryotoxic substance 5-Fluoruracil (5-FU). (A) Differentiation assay with D3 cells (ID_{50}D3:0.057 µg/ml). (B) MTT assay with D3 cells (IC_{50}D3: 0.065 µg/ml) and 3T3 cells (IC_{50}3T3: 0.308 µg/ml). Contractility and cytotoxicity are represented as percentage of solvent control (control = 100%). Three independent experiments were performed. Statistical data: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

with the two highest concentrations (12.4 nM and 24.8 nM). Cell viability measured by MTT was significantly (3T3: $p < 0.001$; D3: $p < 0.05$) reduced at the same concentrations, whereas the 3T3 cells showed also significant ($p < 0.01$) cytotoxicity at a concentration of 6.2 nM.

The embryoid bodies showed a change in morphology after treatment with okadaic acid. Incubation of differentiating D3 cells with increasing concentration of OA led to shrunken embryoid bodies which were also less numerous compared with the untreated control (Fig. 3).

3.2. F9 reporter gene assay

To support these data, a further cellular *in-vitro* bioassay, which may also be applied to detect teratogenic compounds, was performed.

The induction of the transiently transfected RSV promoter-driven luciferase reporter gene by different concentrations of okadaic acid is demonstrated in Fig. 4. Highly significant ($p < 0.001$) activation was demonstrated at concentrations of 15.5 nM and above. At a concentration of 21.7 nM, the okadaic acid-induced luminescence of the reporter gene reached similar values as observed for the positive control valproic acid, which was applied at a concentration of 1 mM in each experiment.

3.3. Effects of okadaic acid on embryonic cells

The MTT in Fig. 5 demonstrates that okadaic acid has cytotoxic effects on F9 cells with an IC_{50} of 12.7 nM after incubation for 24 h (Fig. 5).

The xCELLigence system (Roche, Mannheim, Germany) was used to determine the point in time at which different okadaic acid concentrations have their most potent effects. The measured electrical impedance, displayed as cell index (CI) values, can be used to monitor cell viability, number, morphology, and degree of cell adhesion. Untreated and solvent-treated F9 cells demonstrated a similar increase in the cell index values after point of treatment for further 12 h, but then showed a decrease with respect to cell overgrowth. Okadaic acid-treated F9 cells exhibited decreasing cell index values in a dose-dependent manner (Fig. 6A). Six hours after treatment with okadaic acid, an

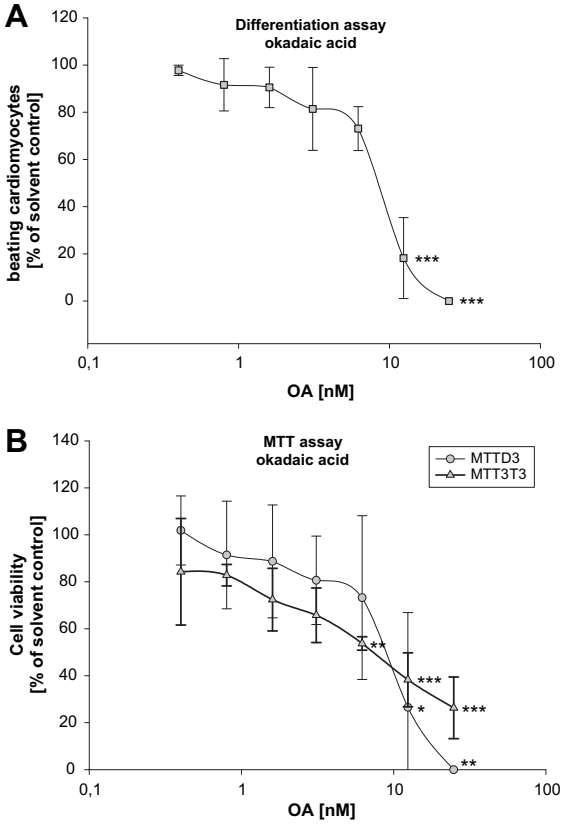

Fig. 2. Embryotoxic effects of okadaic acid (OA) measured using the embryonic stem cell test (EST). (A) Differentiation assay with D3 cells (ID_{50}D3: 8.55 nM). (B) MTT assay with D3 cells (IC_{50}D3: 9.87 nM) and 3T3 cells (IC_{50}3T3: 7.89 nM). Contractility and cytotoxicity are represented as percentage of solvent control (control = 100%). Three independent experiments were performed. Statistical data: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

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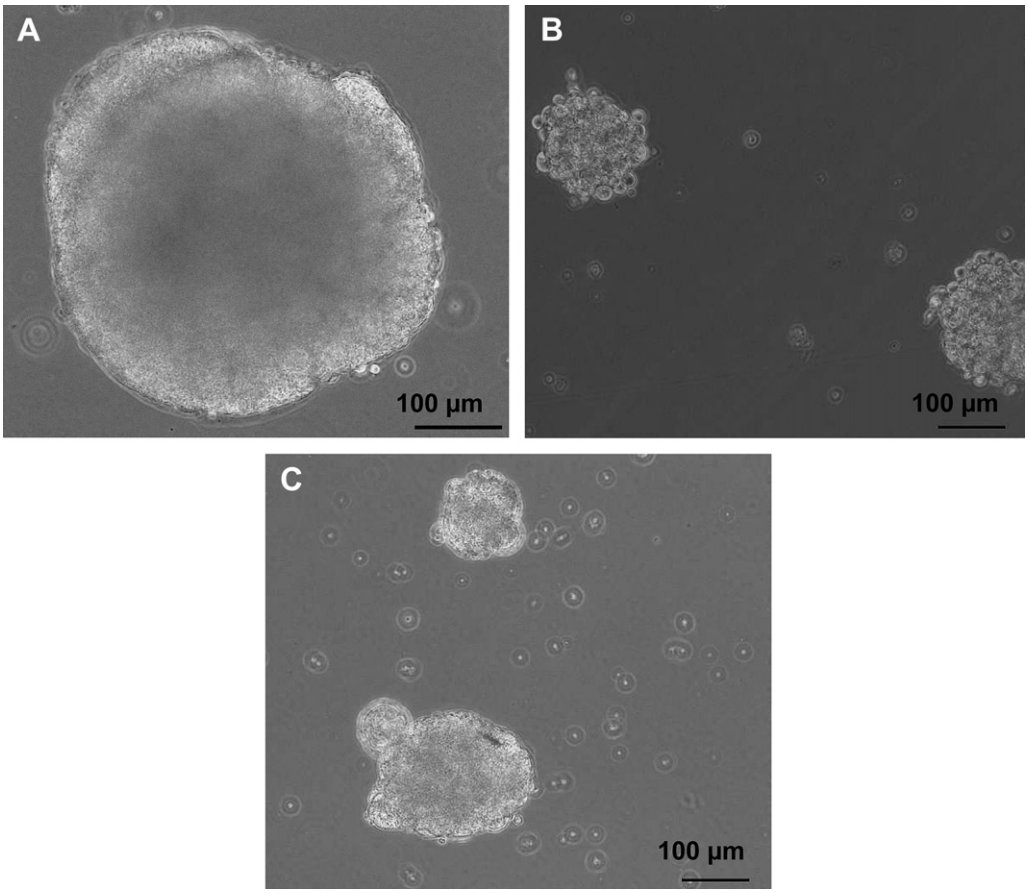

Fig. 3. Change in morphology of embryoid bodies (EBs) of D3 cells after treatment with okadaic acid. (A) untreated EBs; (B) EBs treated for 10 days with 24.8 nM okadaic acid; (C) EBs treated for 10 days with the positive control 5-FU (0.06 µg/ml).

IC_{50} value of 37.2 nM was achieved and reached a value of 7.4 nM after 24 h (Fig. 6B). Thus the IC_{50} value 24 h after treatment as obtained by the impedance measurement is lower than the IC_{50} value measured at the same time by the MTT (IC_{50}: 12.7 nM).

Furthermore, treatment of F9 cells with okadaic acid led to the loss of cell–cell contacts and a more rounded cell shape (Fig. 7). Embryoid bodies of D3 cells incubated with OA exhibited robust changes in their morphologic structure with a decrease in size and number (Fig. 3).

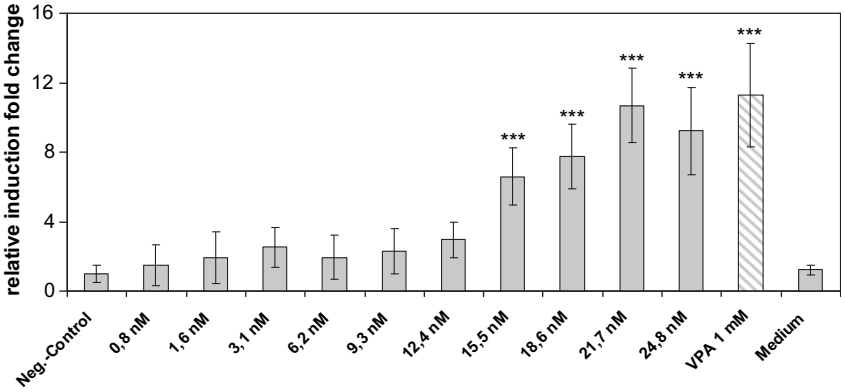

Fig. 4. Teratogenic effects of okadaic acid measured using the F9 reporter gene assay. The induction fold change of the transiently transfected RSV-luciferase reporter gene is shown. The induction values in the solvent-treated cells (negative control) were used as reference for the calculation of the induction fold change in treated F9 cells. F9 cells were treated with different concentrations of okadaic acid and the positive control valproic acid (VPA, streaked column). Three independent experiments were performed. Statistical data: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

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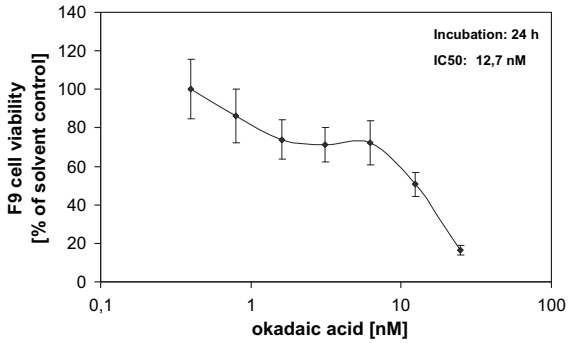

Fig. 5. Cytotoxic effects of okadaic acid on F9 cells measured using the MTT. F9 cells were treated for 24 h with different concentrations of okadaic acid; IC_{50}F9: 12.7 nM. 5-Fluorouracil (2 μg/ml) was used as positive control in each experiment. Independent experiments were performed in quadruplicate.

4. Discussion

This study demonstrates by means of two independent cellular *in-vitro* assays – the EST and the F9 reporter gene assay, that OA exhibits weak embryotoxic effects on cultured murine embryonic cells.

The EST takes two aspects of prenatal toxicity into account: the inhibition of embryonic cell differentiation and the comparison of cytotoxicity effects on embryonic and adult cells. Mitochondrial cytotoxicity, determined by the MTT identifies substance-induced changes in metabolic activity or cell integrity associated with cell death. Thus cytotoxic effects on embryonic cells *in-vitro* may reflect general embryotoxicity *in-vivo*. On the other hand, influences on differentiation events may point for specific malformations or teratogenic events. The term embryotoxicity includes, therefore, both: cytotoxicity on embryonic cells and teratogenicity.

The reproducibility and validity of the performed EST was first tested with 5-Fluorouracil, a well known embryotoxic compound (Fig. 1). The ascertained ID_{50} and IC_{50} values are within the range as indicated by Seiler et al. and the application of the prediction model revealed as expected that 5-FU is highly embryotoxic (Seiler et al., 2006). The successfully validated EST was subsequently performed with the test substance OA (Fig. 2) and the

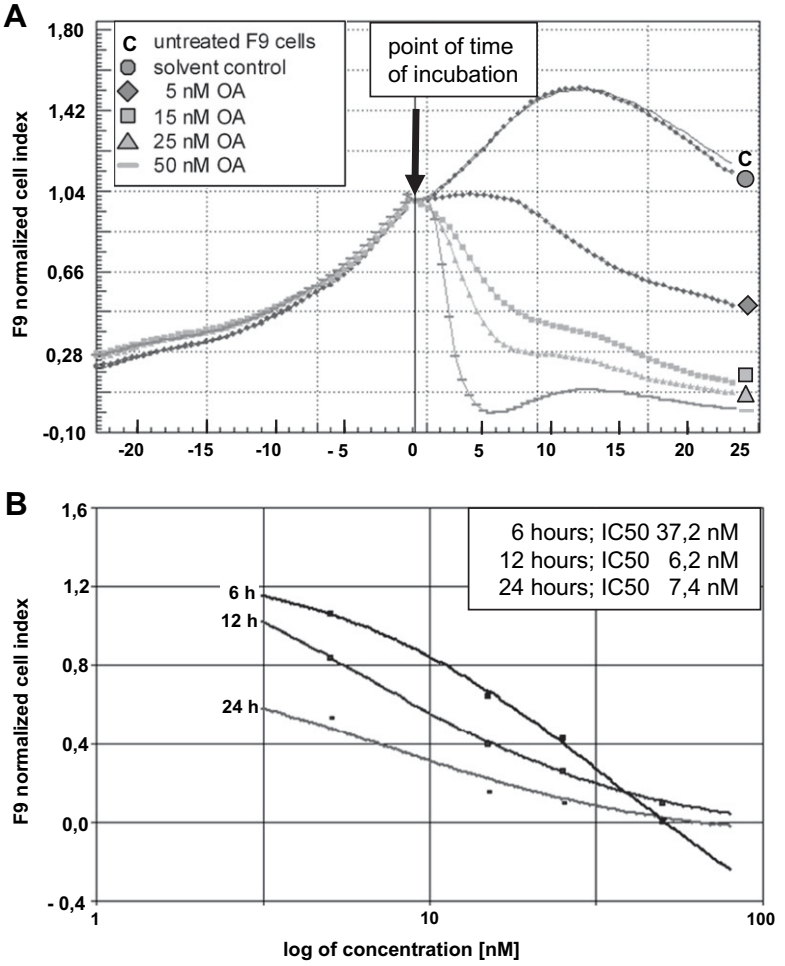

Fig. 6. Dynamic monitoring of F9 cells treated with okadaic acid using impedance technology. (A) Normalized cell index measured over a time of 48 h. (B) Normalized cell index displayed against the logarithm of concentration. Identification of three different IC_{50} values at 6, 12 and 24 h after treatment point. The average of three replicates is displayed.

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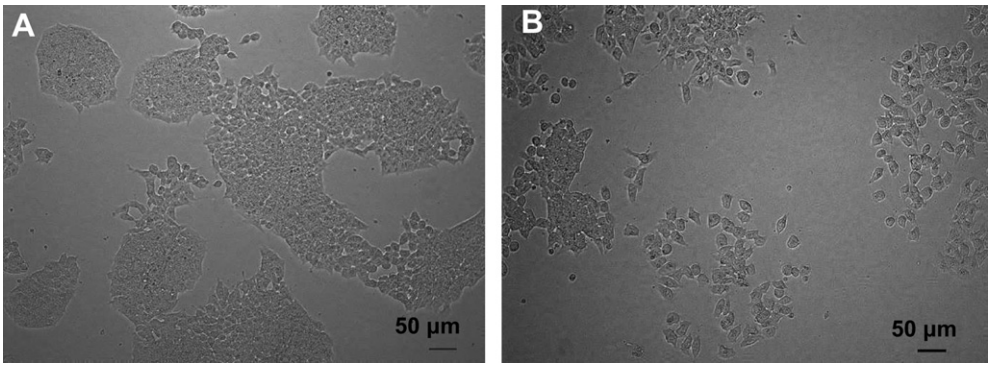

Fig. 7. Change in morphology and loss of cell adhesion of F9 cells. 1-day treatment with okadaic acid (OA). (A) untreated F9 cells (B) OA (5 nM) treated F9 cells.

application of the biostatistical prediction model demonstrated a weak embryotoxic potential for this marine biotoxin. A validation study, which was carried out under the direction of ZEBET and under the coordination of the European Centre for the Validation of Alternative Methods (ECVAM), proved the high predictive potential of the EST with a good concordance to existent *in-vivo* data (Genschow et al., 2002, 2004; Spielmann et al., 2001). OA displayed cytotoxic effects on embryonic D3 cells (IC_{50}D3: 9.87 nM) as well as on adult 3T3 cells (IC_{50}3T3: 7.89 nM), which seem to be even more sensitive. The strong general cytotoxic properties of OA do, therefore, not contribute to its classification as embryotoxic substance. Other known embryotoxic compounds (related to *in-vivo* data) such as diphenylhydantoin or methylmercurychloride exhibit also stronger cytotoxic effects on adult cells than on embryonic cells (Genschow et al., 2004, zur Nieden et al., 2004); but nevertheless they were classified correctly with the biostatistical prediction model of the EST. On the other hand, the impact of OA on the differentiation process of embryonic D3 cells (ID_{50}D3: 8.55 nM) is a little bit more pronounced than to its cytotoxic effects on these cells (IC_{50}D3: 9.87 nM). This leads to the suggestion that OA exhibits weak teratogenic properties independently from its strong cytotoxic potential.

The teratogenic impact of OA was furthermore verified with the F9 reporter gene assay (Fig. 4). This assay focuses on the detection of teratogenicity, since differentiation of F9 cells is investigated. Mouse teratocarcinoma F9 cells undergo only limited spontaneous differentiation under normal cell culture conditions but can be induced to differentiate into endoderm-like cells in response to teratogenic compounds. For example, retinoic acid-induced F9 cell differentiation closely mimics early events of mouse embryogenesis in which cells of the inner cell mass differentiate to either parietal or visceral endoderm (Hogan et al., 1983). Therefore, F9 cells seem to be an excellent *in-vitro* model for the detection of teratogenic compounds. Viral promoters are repressed in undifferentiated F9 cells (Sleigh, 1987), but they are activated in differentiated cells (Sleigh, 1989). The activation of the Rous Sarcoma Virus (RSV) promoter can easily be measured and quantified with the F9 reporter gene assay and results correlate well with the teratogenic impact of compounds (Lampen et al., 1999). This study here demonstrated clearly, that OA has the capacity to activate the RSV promoter in a dose-dependent manner leading to a significant induction at a concentration of 15 nM OA (Fig. 4). An OA-driven induction of differentiation markers in F9 cells could also be demonstrated by Nishina et al. (Nishina et al., 1995). Performing semi-quantitative northern blot analysis they could detect the differentiation markers c-jun and jun B after 24 h by treatment with 20 nM OA. With 10 nM OA, only jun B shows a marginal expression. The F9 reporter gene assay seems, therefore, to be more sensitive and quantitative, since even OA concentrations less than 3 nM result in small, but detectable increases of RSV promoter activation (Fig. 4). However, the activation of differentiation markers by OA treatment in the Nishina study correlates well with our F9 reporter gene data and approve the teratogenic impact of OA.

It may be confusing that the EST and the F9 assay result in the same classification of OA, though this biotoxin affects on the one hand the inhibition and on the other hand the induction of differentiation of embryonic cells. However, this depends on the different endpoints of the respective cellular model: the EST measures in particular mesodermal differentiation into cardiomyocytes while the F9 assay measures the differentiation into endoderm-like cells (Hogan et al., 1983). Both processes are probably induced by separate signal transduction pathways. The dual role of some embryotoxic compounds on distinct differentiation processes is well known. For instance, retinoic acid inhibits myo- and cardiogenesis, while it simultaneously promotes adipogenesis and neurogenesis (Rohwedel et al., 1999). Accordingly, retinoic acid applied in the EST inhibits cardiomyocyte differentiation (Seiler et al., 2006), whereas it induces on the other hand the endodermal differentiation of F9 cells (Hogan et al., 1983). Multiple effects of OA during embryogenesis are also very likely, since OA is a potent inhibitor of protein phosphatase 1 (PP1) and 2A (PP2A) (Dawson and Holmes, 1999; Honkanen et al., 1994; Takai et al., 1987). Protein phosphorylation is known to play an important role in cell differentiation and disturbances in the tightly regulated equilibrium between the phosphorylation and dephosphorylation status can influence many different pathways during embryonic development.

Beside the specific embryotoxic test systems we also analyzed OA effects on F9 cells with the xCelligence System.

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The system monitors cellular events in real time by the measurement of electrical impedance across interdigitated micro-electrodes integrated on the bottom of tissue culture 96-well E-plates. The presence of the cells on the top of the electrodes leads to an increase in electrode impedance and is dependent on the cell number and the quality of the cell interaction with the electrodes. Thus, electrode impedance, displayed as cell index (CI) values, can be used to monitor cell viability, proliferation, morphology, and the degree of cell adhesion.

The impedance measurement showed clearly, that OA-driven effects occur already within the first hour of treatment and even at a concentration of 5 nM OA. The IC_{50} value (24 h) identified by the impedance measurement (7.4 nM) (Fig. 6B) was lower than the IC_{50} value based on the cytotoxicity assay (MTT) (12.7 nM) (Fig. 5). It is likely that the sensitive impedance measurement already detected anterior, early effects like altered morphology or loss of cell adhesion, therefore, resulting in a lower IC_{50} value. Changes in morphology to a more rounded cell shape and loss of cell–cell contacts were also observed by microscopy 24 h after treatment with 5 nM OA (Fig. 7). Nishina et al. demonstrated equal morphological alterations after administration of 10–20 nM OA for 24 h (Nishina et al., 1995). Lower concentrations were not tested in the study. An OA-driven loss of cell adhesion was demonstrated by Leira et al. (Leira et al., 2002). Performing a cell adhesion assay with a neuroblastoma cell line they determined an IC_{50} value of 14 nM OA after a 24-h-treatment. The difference between the IC_{50} values of our impedance measurement and the adhesion assay could be due to the only indirect detection of cell adhesion by the xCelligence system and the usage of different cell lines probably exhibiting different sensitivity to OA.

Frog embryos respond to OA administration with mortality, delayed growth and embryo malformation in a dose-dependent manner (Casarini et al., 2007). Performing the FETAX assay Casarini et al. treated *X. laevis* embryos with 0.1, 1 and 10 nM OA. Mortality was observed after 2-day incubation with 1 and 10 nM OA, whereas delayed growth and embryo malformation was also detected with 0.1 nM OA. The induction of detectable effects by the administered doses correlates approximately with our results, though the endpoints are completely different. Since the investigations of frog embryos on the one hand and mammalian cell culture models on the other hand led to similar results, the transferability to humans got some more reliability.

5. Conclusion

Though the acute toxic effects of OA are well known since many years, investigations regarding the embryotoxic impact of OA are rare. This study clearly demonstrates, that OA exhibits a weak teratogenic potential on cultured murine embryonic cells by using two independent cellular *in-vitro* assays, which are highly capable for the detection of embryotoxic compounds. OA-driven effects include alterations in morphology and loss of cell adhesion detected by microscopy and real-time monitoring of cell-dependent electrical impedance. Minor deviations or interruptions of these cellular processes could damage irreversibly embryonic development.

Therefore, there is an urgent need to assess the risk of orally consumed OA on human unborn life by analysing the passage of OA through the intestinal and placental barrier.

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Conflict of interest

There are no conflicts of interest in the content of this work.

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References

Casarini, L., Franchini, A., Malagoli, D., Ottaviani, E., 2007. Evaluation of the effects of the marine toxin okadaic acid by using FETAX assay. Toxicol. Lett. 169, 145–151.

Dawson, J.F., Holmes, C.F., 1999. Molecular mechanisms underlying inhibition of protein phosphatases by marine toxins. Front. Biosci. 4, 646–658.

EFSA (European Food Safety Authority), 2008. Marine biotoxins in shellfish–okadaic acid and analogues–scientific opinion of the panel on contaminants in the food chain. EFSA J. 589, 1–62.

Genschow, E., Spielmann, H., Scholz, G., Pohl, I., Seiler, A., Clemann, N., Bremer, S., Becker, K., 2004. Validation of the embryonic stem cell test in the international ECVAM validation study on three in vitro embryotoxicity tests. Altern. Lab. Anim. 32, 209–244.

Genschow, E., Spielmann, H., Scholz, G., Seiler, A., Brown, N., Piersma, A., Brady, M., Clemann, N., Huuskonen, H., Paillard, F., Bremer, S., Becker, K., 2002. The ECVAM international validation study on in vitro embryotoxicity tests: results of the definitive phase and evaluation of prediction models. European Centre for the Validation of Alternative Methods. Altern. Lab. Anim. 30, 151–176.

Hogan, B.L.M., Barlow, D., Tilly, R., 1983. F9 teratocarcinoma cells as a model for the differentiation of parietal and visceral endoderm in the mouse. Cancer Surv. 2, 115–140.

Honkanen, R.E., Codispoti, B.A., Tse, K., Boynton, A.L., Honkanan, R.E., 1994. Characterization of natural toxins with inhibitory activity against serine/threonine protein phosphatases. Toxicon 32, 339–350.

Ito, E., Yasumoto, T., Takai, A., Imanishi, S., Harada, K., 2002. Investigation of the distribution and excretion of okadaic acid in mice using immunostaining method. Toxicon 40, 159–165.

Lampen, A., Carlberg, C., Nau, H., 2001. Peroxisome proliferator-activated receptor delta is a specific sensor for teratogenic valproic acid derivatives. Eur. J. Pharmacol. 431, 25–33.

Lampen, A., Grimaldi, P.A., Nau, H., 2005. Modulation of peroxisome proliferator-activated receptor delta activity affects neural cell adhesion molecule and polysialyltransferase ST8SiaIV induction by teratogenic valproic acid analogs in F9 cell differentiation. Mol. Pharmacol. 68, 193–203.

Lampen, A., Siehler, S., Ellerbeck, U., Gottlicher, M., Nau, H., 1999. New molecular bioassays for the estimation of the teratogenic potency of valproic acid derivatives in vitro: activation of the peroxisomal proliferator-activated receptor (PPARdelta). Toxicol. Appl. Pharmacol. 160, 238–249.

Le Hegarat, L., Jacquin, A.G., Bazin, E., Fessard, V., 2006. Genotoxicity of the marine toxin okadaic acid, in human caco-2 cells and in mice gut cells. Environ. Toxicol. 21, 55–64.

Leira, F., Alvarez, C., Vieites, J.M., Vieytes, M.R., Botana, L.M., 2001. Study of cytoskeletal changes induced by okadaic acid in BE(2)-M17 cells by

means of a quantitative fluorimetric microplate assay. Toxicol. Vitro 15, 277–282.

Leira, F., Alvarez, C., Vieites, J.M., Vieytes, M.R., Botana, L.M., 2002. Characterization of distinct apoptotic changes induced by okadaic acid and yessotoxin in the BE(2)-M17 neuroblastoma cell line. Toxicol. Vitro 16, 23–31.

Malaguti, C., Rossini, G.P., 2002. Recovery of cellular E-cadherin precedes replenishment of estrogen receptor and estrogen-dependent proliferation of breast cancer cells rescued from a death stimulus. J. Cell. Physiol. 192, 171–181.

Matias, W.G., Creppy, E.E., 1996. Transplacental passage of [3H]-okadaic acid in pregnant mice measured by radioactivity and high-performance liquid chromatography. Hum. Exp. Toxicol. 15, 226–230.

Matias, W.G., Traore, A., Creppy, E.E., 1999. Variations in the distribution of okadaic acid in organs and biological fluids of mice related to diarrhoeic syndrome. Hum. Exp. Toxicol. 18, 345–350.

Nishina, Y., Sumi, T., Iwai, S.A., Nishimune, Y., 1995. Effects of protein phosphatase inhibition by okadaic acid on the differentiation of F9 embryonal carcinoma cells. Exp. Cell Res. 217, 288–293.

zur Nieden, N.I., Kempka, G., Ahr, H.J., 2004. Molecular multiple endpoint embryonic stem cell test – a possible approach to test for the teratogenic potential of compounds. Toxicol. Appl. Pharmacol. 194, 257–269.

Rohwedel, J., Guan, K., Wobus, A.M., 1999. Induction of cellular differentiation by retinoic acid in vitro. Cells Tissues Organs 165, 190–202.

Seiler, A.E., Buesen, R., Visan, A., Spielmann, H., 2006. Use of murine embryonic stem cells in embryotoxicity assays: the embryonic stem cell test. Methods Mol. Biol. 329, 371–395.

Sleigh, M.J., 1987. Differential regulation of viral and cellular genes in F9 mouse embryonal carcinoma cells. Nucleic Acids Res. 15, 9379–9395.

Sleigh, M.J., 1989. Gene expression and differentiation in F9 mouse embryonal carcinoma cells. Biochem. Soc. Symp. 55, 1–12.

Spielmann, H., Genschow, E., Scholz, G., Brown, N.A., Piersma, A.H., Brady, M., Clemann, N., Huuskonen, H., Paillard, F., Bremer, S., Becker, K., 2001. Preliminary results of the ECVAM validation study on three in vitro embryotoxicity tests. Altern. Lab. Anim. 29, 301–303.

Takai, A., Bialojan, C., Troschka, M., Ruegg, J.C., 1987. Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin. FEBS Lett. 217, 81–84.

Tubaro, A., Sosa, S., Carbonatto, M., Altinier, G., Vita, F., Melato, M., Satake, M., Yasumoto, T., 2003. Oral and intraperitoneal acute toxicity studies of yessotoxin and homoyessotoxins in mice. Toxicon 41, 783–792.

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