Institut für Pharmazeutische Biologie[1], Universität Frankfurt/M. (Biozentrum), and Institut für Pharmazie[2], Universität Leipzig, Germany

Effects of medicinal compounds on the differentiation of the eukaryotic microorganism *Dictyostelium discoideum*: Can this model be used as a screening test for reproductive toxicity in humans?

K. Dannat[1], J. Tillner[1], T. Winckler[1], M. Weiss[2], K. Eger[2], T. Dingermann[1]

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Prof. Dr. T. Dingermann, Institut für Pharmazeutische Biologie, Universität Frankfurt (Biozentrum), Marie-Curie-Straße 9, D-60439 Frankfurt, Germany
dingermann@em.uni-frankfurt.de

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Dictyostelium discoideum is a single-cell, eukaryotic microorganism that can undergo multicellular development in order to produce dormant spores. We investigated the capacity of *D. discoideum* to be used as a rapid screening system for potential developmental toxicity of compounds under development as pharmaceuticals. We used a set of four transgenic *D. discoideum* strains that expressed a reporter gene under the control of promoters that are active at certain time periods and in distinct cell types during *D. discoideum* development. We found that teratogens such as valproic acid, tretinoin, or thalidomide interfered to various extents with *D. discoideum* development, and had different effects on prestalk and prespore cell-specific reporter gene expression. Phenytoin was inactive in this assay, which may point to limitations in metabolization of the compound in *Dictyostelium* required to exert developmental toxicity. *D. discoideum* cell culture is cheap and easy to handle compared to mammalian cell cultures or animal teratogenicity models. Although the *Dictyostelium*-based assay described in this report may not securely predict the teratogenic potential of these drugs in humans, this organism may be qualified for rapid large-scale screenings of synthetic compounds under development as new pharmaceuticals for their potential to interfere with developmental processes and thus help to reduce the amount of teratogenicity tests in animal models.

1. Introduction

Dictyostelium discoideum is a solitary amoeba that feeds on bacteria in the upper soil layer of decaying leaves. The asexual life style of *D. discoideum* consists of two phases strictly separated in time: a vegetative growth phase, in which the cells ingest bacteria and persist as solitary amoebae, and a developmental phase, in which a multicellular organism is formed to produce dormant spores. The multicellular stage is not obligatory, it depends on the environmental conditions and the availability of food sources in a cell population. The development of *D. discoideum* cells can be devided into four stages [1]: (i) aggregation of amoebae to form a mound; (ii) postaggregation: the appearance of prestalk and prespore cells; (iii) cell type specialization, i.e. the manifestation of the proportions of individual cell types; and (iv) terminal differentiation into stalk and spore cell types and the formation of the fruiting body (Fig. 1).

At the multicellular stage of the *D. discoideum* life cycle two morphogens act together to control morphogenesis and terminal differentiation: cyclic AMP (cAMP) and differentiation inducing factor (DIF). cAMP controls all events during the developmental cycle from aggregation to culmination and acts both as a chemoattractant and a morphogen. Aggregating *D. discoideum* cells orient and move towards gradients of cAMP, which is highest in aggregation centers, such that the cells collect into mounds. Cells perceiving a cAMP signal respond by producing and secreting cAMP, which leads to relay and enhancement of the cAMP signal to run over many neighboring cells in the aggregation field. Intracellular cAMP induces the expression of a variety of genes required for cell type determina-

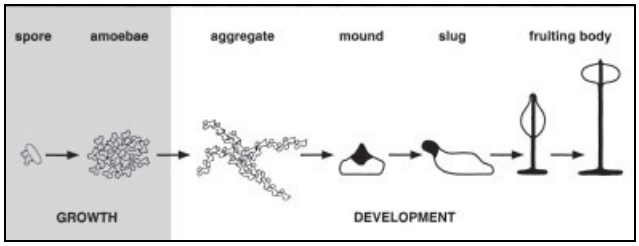


Fig. 1: Development of *D. discoideum*. Spores germinate and live as single-cell amoebae (growth phase). Upon food depletion the cells aggregate and form multicellular organisms through aggregation. Soon after aggregation the cells start to differentiate into two cell types: prestalk and prespore cells. Prestalk cells are located at the tip of the multicellular organism (black). Prespore cells (white) are in the posterior of the organism. Morphogenetic changes and terminal differentiation into stalk cells and spores eventually end with the formation of a fruiting body. An entire developmental program takes 24–30 h at 22 °C

tion. cAMP production in multicellular stages is highest in prestalk regions, which can therefore be considered as an organizing center during morphogenesis of the multicellular organism. Cell fates are determined by cAMP in collaboration with DIF, a chlorinated alkyl phenone that is probably synthesized by prespore cells and broken down by prestalk cells. DIF levels in multicellular stages set the ratio of prestalk and prespore cells and control the cell fate choice between stalk and spore differentiation [2–6].

Recent phylogenetic analysis using a combined protein data set placed *D. discoideum* at the root of the eukaryotic crown organisms. *D. discoideum* seems to be closer related to animals than to plants and fungi. Multicellularity of *Dictyostelium* did apparently not evolve independently of the metazoans, since *D. discoideum* cells use several conserved signal transduction pathways such as STAT proteins, NFκB, MAP kinases, small G proteins, and GSK3 in signal transduction and cell fate determination [2]. *D. discoideum* cells are easy to grow in large amounts in the laboratory. Since *D. discoideum* is a powerful genetic system for the analysis of multicellularity [7], it has recently been added to the list of model organisms for biomedical research by the National Institutes of Health (http://www.nih.gov/science/models/). The 34 Mb chromosomal DNA sequence of the *D. discoideum* genome is currently being sequenced and will be available in early 2003 [7, 8]. As one aspect of the *Dictyostelium* Genome Project a list of more than 30 *Dictyostelium* orthologs of human disease genes has currently been identified and will be functionally analysed using the genetic tools available in *D. discoideum* research (L. Eichinger, University of Köln; personal communication).

Since many of the key regulatory pathways in metazoan development may be conserved at least to a limited extent in *Dictyostelium*, this organism may provide an easy-to-handle model for the inspection of reproductive toxicity of medicinal compounds in humans [9]. The molecular genetic tool box of *Dictyostelium* research offers several cell type-specific promoters that can be linked to reporter genes to analyse gene expression during cell differentiation and morphogenesis [10]. The bacterial *lacZ* gene encodes a β-galactosidase whose activity can be easily detected both *in situ* and *in vitro* when expressed in *D. discoideum* under the control of certain developmentally regulated promoters [9, 11]. For example, a promoter fragment isolated from the gene *cprB* can be used to analyse postaggregative gene expression in cells transfected with a *cprB/lacZ* reporter construct. Similar reporter constructs can be expressed in the laboratory AX2 cells to analyse cell type specific gene expression in prestalk and stalk cells (AX2[*ecmA/lacZ*], AX2[*ecmB/lacZ*]) and prespore and spore cells (AX2[*pspA/lacZ*]) [9, 12].

Using four *D. discoideum* reporter strains that expressed reporter constructs at certain time periods and in a cell type-specific manner, we have shown that strong human teratogens such as valproic acid and methotrexate have profound effects on *D. discoideum* development [9]. Valproic acid strongly delays development by interfering with both stalk cell and spore cell differentiation [9]. Several tested valproic acid analogues displayed effects on *D. discoideum* development also observed in vertebrate teratogenicity models [12]. These results showed that a *Dictyostelium*-based test system may be valuable to predict the reproductive toxicities of newly developed pharmaceuticals. Using several model compounds that are well-known human teratogens, we show in this paper that the *Dictyostelium*-based test system may be considered within its limitations as a relatively inexpensive, less time-consuming, and easy-to-handle microbial alternative to established mammalian teratogenicity assays.

2. Investigations, results and discussion

2.1. Experimental set-up to test human teratogens in Dictyostelium development

The assay described in this report relies on the synchronous development of a *D. discoideum* cell population on the surface of agar. Cells were placed into the cavitites of 24-well culture plates filled with agar under standardized conditions. Critical environmental conditions such as temperature (22 °C), light, and humidity were kept constant. A complete development from aggregation to formation of fruiting bodies (Fig. 1) usually takes 24–30 h. The compounds to be tested for interference with *D. discoideum* development were included into the agar. The compounds were used at concentrations at least twofold below the cytotoxic concentration determined in shaken cell suspensions, expressed as IC_{50} values [9, 13]. Concentrations of compounds tested in the *Dictyostelium* developmental toxicity assay were comparable a previously used sea urchin assay [14, 15] and in the same order of magnitude as therapeutic human plasma concentrations (specified below). However, it seems worth to emphasize that we cannot specify the exact concentrations of drugs actually exposed to developing *D. discoideum* cells at the agar surface. After appropriate time periods multicellular development of *D. discoideum* cells was stopped by the addition of a nonionic detergent. The detergent efficiently solubilizes cells in early and late developmental phases, but not cellulose-coated stalk and spore cells. Hence the detergent serves to permeabilize the cells rather than to lyse them. This method, however, can efficiently stain differentiated cell types for β-galactosidase activity *in situ* [11]. From each "lysate" aliquots were transferred to 96-well plates, CPRG solution was added, and the β-galactosidase activities of lysates were determined with an ELISA reader [9].

The compounds used in this study were selected by their known teratogenicity in humans. The major goal of the experiments was to achieve an overview of whether or not these compounds would be detected in the *Dictyostelium* developmental toxicity assay with a prospective view of how effective this system would be if compounds with unknown teratogenic potentials in humans would be screened in *Dictyostelium*.

2.2. Tretinoin

Tretinoin (all-trans-retinoic acid) is a potent teratogen responsible for a broad spectrum of embryonic malformations [16–20]. Patient plasma concentrations can reach the 1.7–5 μM (0.5–1,5 μg/ml) range depending on the applied dosage [21]. Tretinoin, even at millimolar concentrations, did not affect growth of *D. discoideum* cells in shaken cultures (not shown). However, tretinoin strongly affected *D. discoideum* development in the low micromolar range and displayed specific inhibitory effects on prestalk-specific marker gene expression (Fig. 2). 10 μM tretinoin caused a sharp drop in *ecmA* promoter activity, whereas further increases in compound concentration had little additional effects (Fig. 2). The same was found with the *ecmB/lacZ* reporter construct (not shown). At the time of culmination expression of the prestalk-specific marker

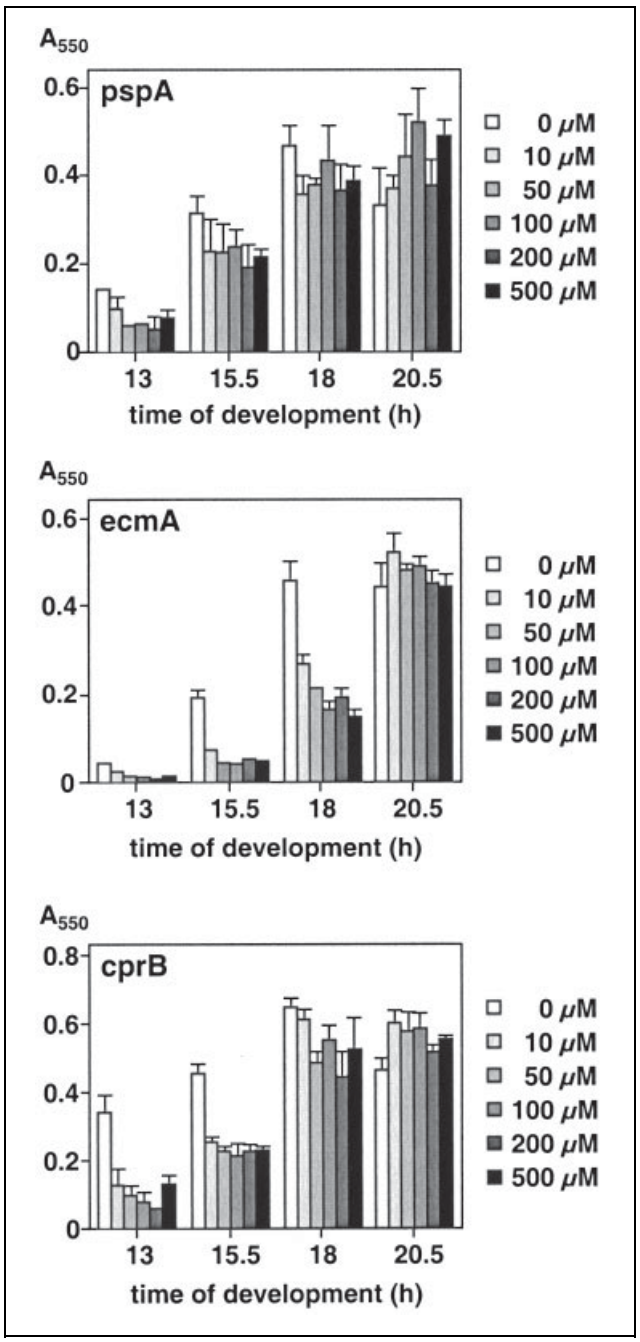


Fig. 2: Effect of tretinoin on reporter gene expression during *D. discoideum* development. Reporter gene activity of AX2[*cprB/lacZ*], AX2[*ecmA/lacZ*], and AX2[*pspA/lacZ*] strains was tested after the indicated time periods of development. The drug was dissolved in DMSO and included into 0.5 g of 1% agar at the indicated concentrations. Controls refer to *D. discoideum* development in the presence of the highest DMSO concentration required to apply tretinoin. Values are shown ± S.D. calculated from four independent experiments

genes almost reached control levels, suggesting that development was delayed but not completely blocked in the presence of the drug, and that cell numbers participating in multicellular development were not significantly reduced by the drug. The *cprB/lacZ* reporter construct, indicative of postaggregative development, was significantly underexpressed in tretinoin-treated cells with the same characteristic drop of β-galactosidase activity in the presence of 10 μM tretinoin compared to untreated cells (Fig. 2). As expected, this effect was most pronounced at time points after the formation of tight aggregates (postaggreagtion). In the postaggregation period key regulatory components are induced or activated which are the prerequisites of proper induction of cell type-specific gene expression and terminal differentiation [2]. Hence effects of tretinoin on postaggregative gene expression may be relevant for the dysregulated prestalk gene expression observed in our assay. The prespore marker *pspA/lacZ* was only slightly affected by the drug (Fig. 2), suggesting that tretinoin interfered predominantly with pathways leading to prestalk gene expression and differentiation.

The intracellular accumulation of tretinoin and possible metabolites was assayed after 48 h incubation of *D. discoideum* cells in the presence of 50 μM tretinoin. The cells accumulated 135 $\mu g/10^9$ cells (0.45 μmol) tretinoin, 48 $\mu g/10^9$ cells 13-cis-retinoic acid, and 11 $\mu g/10^9$ cells 9-cis-retinoic acid, respectively. In addition, the metabolites 4-oxo-retinoic acid (2.6 $\mu g/10^9$ cells) and 5,6-eporetinoic acid (8.9 $\mu g/10^9$ cells) could be detected. Assuming a mean cell volume of 1 pl [13], the accumulated intracellular tretinoin concentration can be calculated as 450 μM. Induction of the prestalk-specific genes *ecmA* und *ecmB* depends on the presence of DIF in the nanomolar range [3]. It is tempting to speculate that due to a structural analogy of tretinoin and DIF the drug may have competed for the binding of DIF to its intracellular receptor(s), thus preventing the induction of stalk cell formation.

The effects of tretinoin on *D. discoideum* development were different from valproic acid effects. At moderate concentrations valproic acid caused a strong delay in the entire developmental process, which terminated with malformed fruiting bodies. This was accompanied by strong inhibition of both prestalk and prespore marker gene expression in our assay [9]. In contrast, tretinoin-treated cells showed normal timing of development and no obvious malformation of fruiting bodies (not shown).

2.3. Diethylstilbestrol

Diethylstilbestrol (DES), a synthetical estrogen, was withdrawn from the market when it was shown to enhance the probability of daughters born to DES-treated mothers to develop vaginal adenocarcinomas [22, 23]. DES was able to completely suppress *D. discoideum* development at concentrations exceeding 50 μM (microscopic observations, data not shown). In the *Dictyostelium* developmental assay DES caused a strong and dose-dependent reduction of *pspA*-mediated reporter gene expression (Fig. 3). The drug also acted inhibitory on the prestalk-specific *ecmA/lacZ* construct. The dose-response curve was biphasic: the β-galactosidase activity expressed from the *ecmA/lacZ* gene was reduced in the presence of low DES concentrations, while it was enhanced at high DES concentrations (Fig. 3). As a result the β-galactosidase activity was at control levels at high DES concentrations (Fig. 3). In support of this observation DES also enhanced the expression of the *ecmB*-regulated expression of β-galactosidase about twofold, but without preceding reduction of reporter gene expression at low DES concentrations (Fig. 3). DES is known as a potent inhibitor of a plasma membrane ATPase purified from *D. discoideum* cells [24]. DES-dependent inhibition of a plasma membrane ATPase would result in an acidification of the cytoplasm, which is known to promote the production of DIF and hence the induction of prestalk-specific genes [25]. This is reflected by the results obtained in our assay.

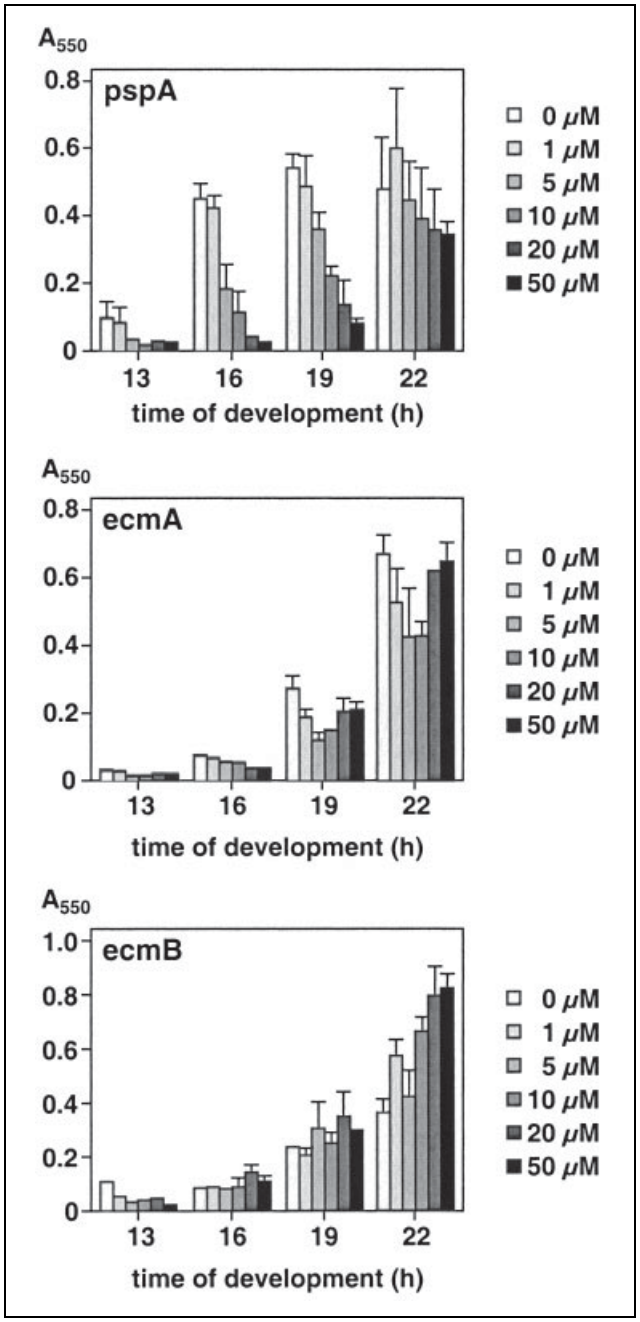


Fig. 3: Effect of DES on reporter gene expression during *D. discoideum* development. Reporter gene activity of AX2[*ecmA/lacZ*], AX2[*ecmB/lacZ*], and AX2[*pspA/lacZ*] strains after the indicated time periods of development. The drug was dissolved in DMSO and included into 0.5 g of 1% agar at the indicated concentrations. Values are shown ± S.D. calculated from three independent experiments

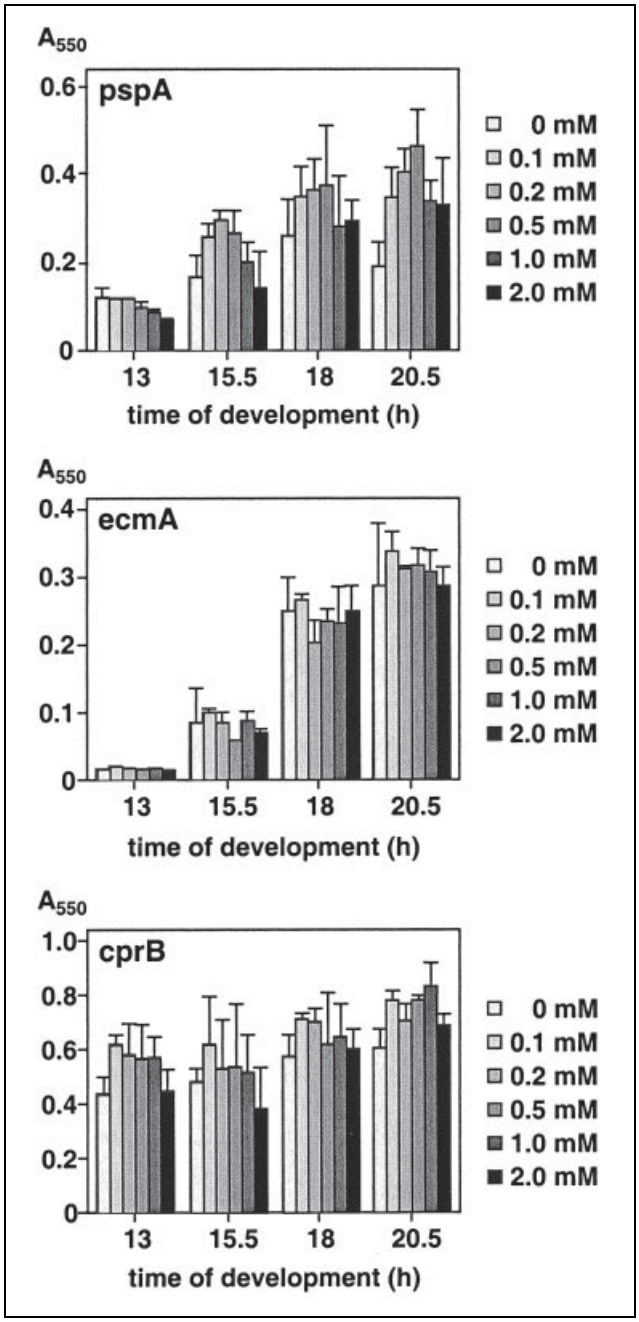


Fig. 4: Effect of phenytoin on reporter gene expression during on *D. discoideum* development. Reporter gene activity of AX2[*cprB/lacZ*], AX2[*ecmA/lacZ*], and AX2[*pspA/lacZ*] strains after the indicated time periods of development. The drug was dissolved in DMSO and included into 0.5 g of 1% agar at the indicated concentrations. Values are shown ± S.D. calculated from three independent experiments

2.4. Phenytoin

Phenytoin (5,5-diphenylhydantoin) is an antiepileptic drug that has a clear teratogenic potential [26–28]. Therapeutic concentrations range from 40–80 μM [29]. The teratogenic effect of phenytoin is attributed to an epoxid formed upon metabolization to 5-(4-hydroxyphenyl)-5-phenyl-hydantoin (5-HPPH) by the liver cytochrome P450 system [30]. We applied phenytoin in our *Dictyostelium* developmental assay as a model substance to test whether a drug that is thought to be hydroxylated by the human liver cytochrome P450 system would be activated by the respective *D. discoideum* enzymes. When *D. discoideum* cells were incubated with 500 μM phenytoin for 48 h in shaking cultures, the cells accumulated 1560 μg/10^9 cells of phenytoin, corresponding to about 6.2 mM intracellular drug. However, only trace amounts of the drug were converted to 5-HPPH (0.36 μg/10^9 cells).

Phenytoin showed no significant cytotoxicity on *D. discoideum* cells growing in shaking cultures (IC_{50} 3.3 mM, data not shown). Interestingly, phenytoin seemed to enhance the expression of the prespore-specific marker gene *pspA/lacZ* by about twofold in a dose-dependent manner (Fig. 4). By contrast, the rates of β-galactosidase accumulation were not significantly different from control when testing the postaggregation (*cprB*) or prestalk-specific

(*ecmA*) marker genes (Fig. 4). The failure of phenytoin to inhibit *Dictyostelium* development even at high concentrations may point to the fact that the amoebae might have been unable to metabolize the drug. Thus, if the *D. discoideum* assay would be applied to identify the potential human teratogenicities of xenobiotics, it should be considered whether or not activation of the compound by the cytochrome P450 system is likely to occur. In this case the *Dictyostelium*-based developmental screening may produce false-negative results.

2.5. Thalidomide

Thalidomide (Contergan™) is a strong teratogen in humans [31]. Typical plasma levels may reach about 4 μM (1 μg/ml) thalidomide [32]. The drug showed a moderate cytotoxicity at relatively high concentrations when incubated with *D. discoideum* cells in shaken cultures (IC_{50} 1 mM, data not shown). Because of the very low solubility of the drug it was impossible to predict the concentration of drug that was in fact exposed to the developing *D. discoideum* cells. Therefore we tested thalidomide concentrations up to the millimolar range that even exceeded the IC_{50} value, but observed no effect of the drug on any of the cell differentiation reporter constructs (Fig. 5).

Thalidomide likely acts teratogenic after metabolization by the cytochrome P450 system. Hence, as discussed above for phenytoin, the absence of effects of thalidomide in our test system may reflect poor metabolization of the compound by *D. discoideum* cells. However, it has recently been discussed that thalidomide may be activated through reactive oxygen species [32, 33]. Alternative to poor metabolization, the failure of thalidomide to exert effects on *D. discoideum* development may have been caused by low exposure of the cells to the drug due to its limited solubility and diffusion in aqueous agar. To test whether limitations in availability of thalidomide dissolved in agar was a reason for the absence of developmental toxicity on *D. discoideum* cells, we preincubated 2 mM thalidomide with starving *D. discoideum* cells in suspension for 3 h and plated the cells afterwards on agar to allow multicellular development. As a result an approx. 50% reduction of both prespore and prestalk-specific expression of marker gene expression in thalidomide-treated cells compared to control cells was observed (Fig. 5).

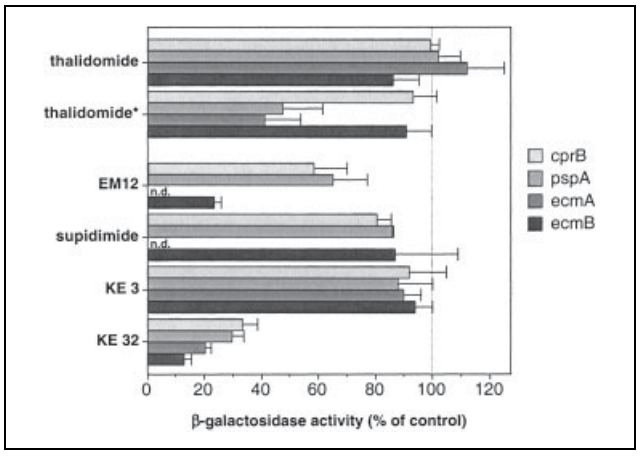
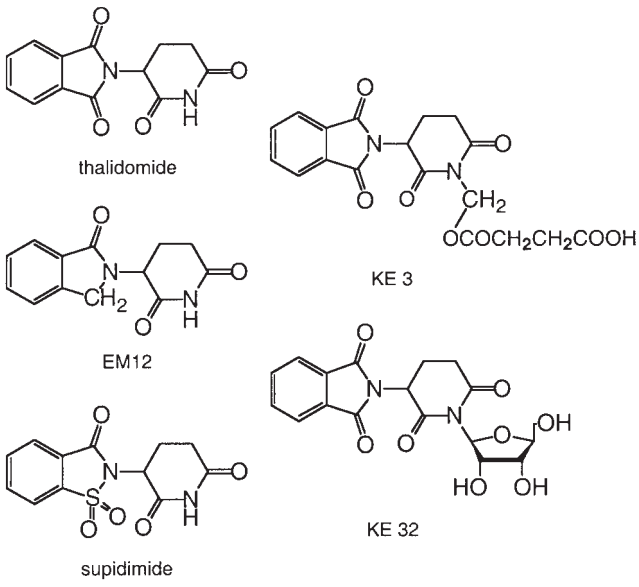


Fig. 5: Effect of thalidomide and thalidomide derivatives on reporter gene expression during *D. discoideum* development. Thalidomide, EM12, and supidimide were dissolved in DMSO. KE 3 and KE 32 were dissolved in buffer used to prepare the agar. The drugs were dissolved in DMSO and included into 0.5 g of 1% agar at 2 mM final concentration. Controls included the solvent concentrations required to set the drug concentrations. Results are shown for experiments with the AX2[*cprB/lacZ*], AX2[*ecmA/lacZ*], AX2[*ecmB/lacZ*], and AX2[*pspA/lacZ*] strains. Due to limitations in compound quantity some measurements were omitted (n.d., not determined). The asterisk (*) denotes that the cells were preincubated with thalidomide for 3 h in shaken culture before plating on agar for multicellular development

The compound EM12 is a thalidomide derivative that was shown in animal models to be a stronger teratogen than thalidomide [34]. Supidimide, a related thalidomide derivative, was completely inactive. We tested both compounds for developmental toxicity in *Dictyostelium*. Both compounds were insoluble in water and required DMSO to prepare stock solutions, from which the drugs were dissolved in the melted agar. When tested under conditions in which thalidomide had no effect on *D. discoideum* development, EM12 proved showed pronounced developmental toxicity, whereas supidimide had no effect (Fig. 5). Hence the teratogenic properties of the compounds determined experimentally in animal models was reproduced in our assay. EM12 acted strongly on e*cmB/lacZ* reporter gene expression, but also the postaggregation marker *cprB/lacZ* and the prespore-specific marker were strongly reduced in expression (Fig. 5).

We chose KE 3 and KE 32 to test whether water-soluble thalidomide analoga would exert stronger effects on *D. discoideum* development than thalidomide itself due to improved accessibility of the cells to the compound when dissolved in the agar. Both KE 3 and KE 32 were completely soluble in water and no addition of organic solvents was required. As shown in Fig. 5, compound KE 3 had no effect in the *Dictyostelium* developmental toxicity assay. This result reflects an example of a test compound that was inconspicuous in the *Dictyostelium*-based assay but may nevertheless require further testing in vertebrate test systems if this compound would be developed to practice. KE 32 showed no cytotoxicity on growing *D. discoideum* cells even at high concentrations (not shown), but showed pronounced developmental toxicity (Fig. 5). This result was surprising since both compound KE 3 and KE32 represent N1′-linked derivatives of thalidomide that were expected to display comparable developmental toxicity. KE 32 showed effects even stronger than those of EM12, and acted on all developmental marker genes tested (Fig. 5). The potency of KE 32 in the *Dictyostelium* developmental



assay must obviously be attributed to the ribose moiety linked to the thalidomide molecule. It is not known whether the strong effect of KE 32 exerted on *D. discoideum* development was due to some kind of active ribose-specific transport of the compound. The results obtained with compound KE 32 in the *Dictyostelium* developmental toxicity assay (Fig. 5) predicts that this compound may be a very strong teratogen in humans that demands further tesing in established vertebrate teratogenicity models. In fact, because compound KE 32 has never been tested in animal teratogenicity models it would be an ideal candidate to test the capability of the *D. discoideum* developmental toxocicty assay to predict potential teratogenicity of newly synthezised drugs in humans.

2.6. Conclusions and perspectives

The assay described in this report takes advantage of the relatively simple developmental program that *Dictyostelium* cells run through when they experience unfavourable environmental conditions such as starvation. The relative simplicity of *Dictyostelium* as a cell differentiation model comes from (i) strict separation in time of growth and development phases; (ii) the possibility to induce synchronous development of a cell population by simply removing the food; (iii) the formation of only two major cell types; and (iv) the availability of molecular genetic tools to analyse developmentally regulated gene expression; (v) easy-to-handle culturing conditions and availability of unlimited cell numbers.

Practical limitations of the *Dictyostelium* developmental toxicity assay to reliably predict the teratogenetic potential of compounds in humans are their solubility in aqueous buffers and metabolization reactions required to generate active derivatives. Since the compounds must be dissolved into the agar that is used to support *D. discoideum* development, there are no exact data about how much of the compound actually reaches the intracellular compartment. This complicates the calculation of doses effective to induce effects.

In order to prevent false-positive results, the cytotoxic potential of each compound to be tested for developmental toxicity should be determined. Cytotoxicity of the test compounds, however, is usually tested in shaken culture. This is thought to result in higher intracellular compound levels compared to exposure of the cells to the drugs on agar surfaces (compare thalidomide data). Hence compound concentrations included in agar may exhibit developmental toxicity rather than cytotoxicity even when applied at concentrations above the calculated IC_{50} values. We have shown that exposure of the cells to the drugs on agar is limited when the drugs are poorly soluble in the aqueous agar environment. Hence preincubation of such compounds with the cells may enhance the sensitivity of the assay.

In the experimental set-up described here the compounds to be tested were present throughout *D. discoideum* development. Temporal delays of early *D. discoideum* development caused by teratogenic compounds may have affected the late terminal differentiation events recorded in our assay by using cell type-specific promoters such as *pspA* and *ecmA*. Hence effects being considered "specific" on these promoters may have been indirectly caused by delays of aggregation/postaggregation gene expression. Nevertheless our assay picked up effects of test substances on *D. discoideum* development and identified them as potential teratogens in humans that would require special attention. The currently used *Dictyostelium* developmental assay can be further improved by using additional genetic markers. Several developmentally promoters are available from the *Dictyostelium* research community to analyse certain aspects of the developmental program in detail. For example, we tested the promoter that regulates the expression of the *csaA* gene, whose product is a cell surface protein critical during both early and late development. The β-galactosidase activity expressed from the *csaA/lacZ* construct was maximal at aggregation (8–12 h post starvation) and then slowly declined. An assay combining with the expression kinetics of the *csaA/lacZ* and the *cprB/lacZ* constructs is thus a powerful measure of the developmental process prior to terminal differentiation.

In order to improve the handling of the assay, especially for the use in high-throughput screenings, one could imagine to adapt monolayer differentiation of *D. discoideum* cells. *D. discoideum* cells attached to a plastic surface can be differentiated into spores and stalk cells without multicellular stages [35–37]. After a certain time period of starvation cells can be coaxed to differentiate into spores by adding 8-Br-cAMP or to form stalk cells by adding 8-Br-cAMP + DIF. If this is performed with cells expressing the appropriate cell type-specific markers, screening of compounds dissolved in the buffer overlaying the starving amoebae would be a significant simplification of the experimental set-up.

We have shown in this and previous studies that the *Dictyostelium* developmental toxicity assay may be an alternative model for the detection of potential reproductive toxicity of drugs under development as therapeutics. *Dictyostelium* is relatively easy to handle and the experimental set-up described here to test for developmental toxicity in *Dictyostelium* has the potential to be adapted for high-throughput screenings. We are aware that a "no interference" result in our *Dictyostelium* test system will nevertheless require testing of interesting compounds in vertebrate systems. On the other hand, compounds that show developmental toxicicty in the *Dictyostelium* system may have a high risk of acting teratogenic in humans and should not developed to practice.

3. Experimental

3.1. Dictyostelium cell culture

The *Dictyostelium* developmental toxicity assay was described in detail elsewhere [9]. Briefly, 3×10^6 *D. discoideum* cells were plated in each well of 24-well cell culture plates (Nunc) filled with 0.5 g of 1% melted agar prepared in 17 mM phosphate buffer (pH 6.8). Dissolved compounds were mixed into the agar before plating. After appropriate periods of multicellular development the cells were permeabilized by the addition of detergent, and β-galactosidase activity was determined by adding chlorophenolred-β-D-galactopyranoside (CPRG) as indicator dye. All compounds described in this study were tested with the four reporter strains AX2[*cprB/lacZ*], AX2[*ecmA/lacZ*], AX2[*ecmB/lacZ*], and AX2[*pspA/lacZ*]. Phenytoin was purchased from Nordmark (Germany) and DES from Bayer Leverkusen (Germany). Tretinoin was from Hoffmann La-Roche and thalidomide from Grünenthal (Stolberg, Germany). Phenytoin, tretinoin, DES, and thalidomide were dissolved in dimethylsulfoxid (DMSO). Controls were performed in the presence of the highest concentration of DMSO required to add the drugs. Final DMSO concentrations used in the assays (0.5–1%) did not interfere with *D. discoideum* development.

3.2. Intracellular detection of compounds and metabolites

Phenytoin and tretinoin were mixed with cells growing in HL5 medium. The cells were harvested by centrifugation, washed twice with 17 mM phosphate buffer (pH 6.8), and stored as cell pellets at −80 °C. Lyophilized cell pellets were used to detect the amount of compounds accumulated by the cells by means of HPLC methods.

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