Meta-analysis of Supramaximal Effects in *In Vitro* Estrogenicity Assays

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Received December 9, 2009; accepted February 8, 2010

In scientific literature, several estrogenic compounds are reported to induce responses *in vitro* that are significantly higher than that of estradiol (E2). These supramaximal (SPMX) estrogenic effects do not occur consistently and seem to differ depending on the cellular models applied. This study analyzes the possible underlying causes, mechanisms, and drivers for SPMX estrogenic effects in *in vitro* functional assays reported in the peer-reviewed literature. For the 21 natural and industrial chemicals identified as SPMX inducers, the culture and exposure conditions varied greatly among and between the assays. Detailed information on assay characteristics, however, sometimes lacked. Diethylstilbestrol, genistein, and bisphenol A were selected to build a database. The meta-analysis revealed that the occurrence of SPMX effects could be related to a number of specific assay characteristics: (1) the type of serum used to supplement the exposure medium, (2) the end point used to quantify the estrogenic potency (endogenous or transfected), (3) the number of estrogen response elements, and (4) the promoter's nature. An SPMX response was not reported for expression of endogenous genes, assays that used African green monkey kidney (COS-1) cell line or with chloramphenicol transferase as the reporter gene. There were no indications that solvent concentration in culture, exposure period, or cell model influenced the occurrence of an SPMX effect. It is important to understand the mechanism behind this phenomenon because *in vitro* assays for estrogenicity are used extensively to characterize and quantify the estrogenic potency of compounds, mixtures and environmental extracts.

Key Words: estrogenicity; *in vitro*; bioassay; supramaximal; genistein; bisphenol A; diethylstilbestrol; reporter gene assay; risk characterization.

There has been considerable effort over the last two decades to develop *in vitro* assays capable to screen and assess the estrogenic activity of environmental pollutants, industrial chemicals, natural chemicals, and estrogenic pharmaceuticals (reviewed by Charles, 2004; Mueller, 2002, 2004; Sato et al., 2004; Scrimshaw and Lester, 2004; Zacharewski, 1997). These assays include competitive ligand-binding assays (Berthois et al., 1986; Korach et al., 1978; Migliaccio et al., 1992), cell proliferation assays (Maggiolini et al., 2001; Parez et al., 1998; Soto et al., 1995), induction of protein expression (Gehm et al., 1997; Parez et al., 1998), and recombinant receptor/reporter gene assays using various mammalian cell lines (Jausons-Loffreda et al., 1994; Legler et al., 1999; Miller et al., 2000; Pons et al., 1990; Ranhotra and Teng, 2005; Wilson et al., 2004) and yeast strains (Arnold et al., 1996; Routledge and Sumpter, 1996).

One interesting and often ignored feature of many *in vitro* assays is the “supramaximal effect,” occurring when the response for a particular ligand is significantly higher than the maximal response obtained for estradiol, the natural occurring ligand. Figure 1 displays three types of agonistic responses that can be distinguished: agonists that induce a supramaximal (SPMX) effect, compounds that induce “submaximal” responses, significantly lower than that of E2, and those with responses in between supra- and submaximal responses, named “maximal.” The presence of SPMX effects is not consistently reported in scientific literature, and sometimes the reports seem to be contradictory. Figure 1 also shows a “putative supramaximal” curve, where the response is reported as high as E2 maximum but without actually showing its maximum while the shape of the curve suggests that an SPMX response could have been reached at higher concentrations. For interpretation of the assay results, it is important to know whether this effect is related to a real suprastimulation of the gene expression that might also occur *in vivo* or whether it is an artefact from the assay applied.

An SPMX estrogenic response can be traced back at least to 1994 when Makela et al. (1994) discuss “the exceeded 17β-estradiol–induced activation” of both zeranol and zeralenone using an MCF-7 cell proliferation assay. Different names have been coined for the same effect by different authors; for instance, “superagonism” (Gehm et al., 1997, 2004; Legler et al., 1999; Ter Veld et al., 2006), “superagonist” (Basly et al., 2000; Harris et al., 2005; Levenson et al., 2003; Mueller et al., 2004), “superinduction” (Freyberger and Schmuck, 2005), “supramaximal” (Van Lipzig et al., 2005a, 2005b; Wilson et al., 2004), and recently “overactivation” (Soto et al., 2006).
Many authors only mention the presence of a maximal response greater than that induced by estradiol (Buterin et al., 2006; Han et al., 2002; Kitamura et al., 2005; Kuiper et al., 1998; Kuruto-Niwa et al., 2002, 2005, 2007; Makela et al., 1994; Van Der Woude et al., 2005; Wilson et al., 2004), while others do not describe it in their results or mention it in their discussion even when evidently an SPMX response was obtained. Interestingly, reports of the SPMX estrogenic effect are not consistent among publications even for compounds analyzed with the same system. SPMX effects also have been observed in other reporter gene assays particularly for dioxin-like compounds as the Dioxin Receptor mediated Luciferase reporter gene assay (Jonker et al., 2006; Murk et al., 1996; Vrable et al., 2009).

The mechanism for an SPMX effect response remains obscure, only a few authors have discussed possible explanations and no one its implications. Stimulation of the estrogen receptor (ER) and/or coactivators expression or effects on luciferase stability have been suggested as potential underlying mechanisms in T47D cells stably transfected with a luciferase reporter gene construct (Legler et al., 1999). In addition, some authors have used this later statement to support their observations without adding further insights on the mechanistic explanation (Harris et al., 2005; Kuruto-Niwa et al., 2002; Wilson et al., 2004).

It has been questioned whether the ER is actually involved in the SPMX effect. This is based on the low affinity for the ER of compounds like bisphenol A (BPA) or resveratrol, while at the same time, a SPMX effect is observed on functional in vitro assays (Ashby et al., 1999; Freyberger and Schmuck, 2005; Gehm et al., 1997). However, several authors have demonstrated the ability of fluvestrant (ICI 182,780), an ER antagonist, to completely block the response supporting the idea that an SPMX response is ER mediated (Gehm et al., 1997; Mueller et al., 2004; Van Lipzig et al., 2005).

To our knowledge, only two papers have been published that have considered in a systematic manner the SPMX effect problem. Gehm et al. (1997) reported the SPMX effect of resveratrol in MCF-7 cells on the transiently transfected luciferase reporter gene induction and on cell proliferation but not on the induction of progesterone receptor mRNA measured by reverse transcription-PCR. They suggested that the resveratrol SPMX effect might depend on the target gene and the cell type. In addition, using the MDA-MB-231 ER-negative cell line, they demonstrated the requirement of both an ER and an estrogen response element (ERE) in the same reporter gene construct to obtain a resveratrol SPMX effect (Gehm et al., 1997). Further on, they studied resveratrol SPMX activity with a number of MDA-MB-231 subclones stably transfected with a variety of intact, mutated, or none ERα expression vectors and one or two consensus EREs-tk81-Luc reporter gene plasmids (Gehm et al., 2004). Among their most important conclusions were the independence of resveratrol SPMX effect on the transfection procedure and its dependence on the cell type, the target gene, the number of ERs in the clone construct, and the integrity of both activation function domains. The last finding led to the suggestion that the greater activity of ”resveratrol-liganded ER” compared to “E2-liganded-ER” may result from enhanced interaction of these two domains. Increased cofactor binding to genistein (GEN) and/or quercetin-bound ER was recently suggested as a cause for an SPMX response in ER-U2OS-Luc reporter gene assay (Sotoca et al., 2008).

Chrysene metabolites and specifically 2-hydroxy-chrysene (2-OHCHN) SPMX behavior was investigated using the ER-CALUX assay (Van Lipzig et al., 2005b). Besides the blockage effect by the antiestrogenic compound ICI discussed previously, the authors studied the interaction with the aryl hydrocarbon receptor (AhR) and the ER nuclear concentrations under different patterns of stimulation. They found that co-incubation with the AhR antagonist 3’.,4’-dimethoxyflavone (DMF) could not block the 2-OHCHN SPMX effect, which suggest that a “putative role for the AhR in the increased estrogenic response of 2-OHCHN can be excluded.” However, DMF at low concentrations induced an increase in E2 and 2-OHCHN luciferase activity. Besides, higher ER nuclear concentrations were observed with DMF alone or in combination with 2-OHCHN compared to controls. The suppressive effect of the AhR on the ER nuclear concentration and the ER-dependent transcriptional activity is well known (Safe et al., 1991). Therefore, the authors suggest that SPMX estrogenic effect could be partially due to a decreased ability of the AhR to inhibit ER expression, resulting in higher ER concentrations or enhanced ER transcriptional activity. Freyberger and Schmuck (2005) supported this suggestion based on SPMX effects of resveratrol and other flavonoids in MCF-7 cells. Since these compounds have been characterized as AhR antagonists, they claimed the same AhR inhibition mechanism and thus explain the increased flavonoids estrogenic signaling.

![Simulated dose-response shapes as they appear in in vitro literature compared to E2 (filled squares) for which the maximum is set at 100%. Data represent submaximal induction (filled diamonds), maximal induction (filled triangles), SPMX induction (open circles and open squares), and putative SPMX induction (open diamonds). All simulated SEs are ± 10% of the percentage of relative response.](https://academic.oup.com/toxsci/article-abstract/115/2/462/1732312/FIG.1)
Nonetheless, based on the SPMX effect of BPA obtained with a U2-OS cell line supposedly devoid of endogenous steroid receptors, it has been proposed that cross-reactivity of different receptors can be excluded. Instead of this, a possible modulation of the several kinases involved in the ER activation has been suggested as a potential mechanism (Ter Veld et al., 2006).

In summary, there is neither clear explanation given in literature for the mechanism of action causing the SPMX estrogenic effect nor has the discussion over the regularly observed SPMX effects in in vitro assays been consistently addressed. As the quantification of the estrogenic activity of environmental contaminants, both individually as well as in mixtures (Müller et al., 2002), is based on the response relative to that of estradiol, an SPMX effect could lead to overestimation of the estrogenic hazard of the evaluated substance and/or mixture. Besides, reported SPMX effects are not consistent either across studies or among compounds. Therefore, SPMX is an intriguing and relevant phenomenon to toxicology and toxicological risk assessment, and a systematic approach is needed to understand the conditions for the SPMX effect to occur. Especially whether it is an assay-based artifact or a real-life toxicological effect. A better understanding of the SPMX estrogenic effect will help to unravel its possible causes, determine future research, and suggest proper precautions when applying in vitro assays for estrogenicity risk characterization procedures. For this purpose, a literature meta-analysis of publications with functional estrogenic in vitro assays was performed. Compounds with responses above estradiol maximal response were identified. Three of the most frequent SPMX effect inducers—diethylstilbestrol (DES), GEN, and BPA were selected to study further. A database was build with 134 entries compiling the nature, characteristics, and responses of these three compounds on functional in vitro assays. A relative response meta-analysis was performed using a response variable. Correlations of SPMX effect with assays nature and characteristics were established and discussed.

**MATERIALS AND METHODS**

**Compound selection.** Scientific reviews from 1997 to 2006 related to in vitro assays for estrogenic substances were collected and evaluated for compounds with responses above the maximal response observed for estradiol (Charles, 2004; Gray et al., 1997; Mueller, 2002, 2004; Scrimshaw and Lester, 2004; Soto et al., 2006; Zacharewski, 1997). As a result, a total of 42 scientific papers were reviewed to identify compounds that exerted the SPMX effect. Synthetic hormones, plasticizers, flame retardants, pesticides, and natural substances were found to show submaximal (t < 0.05) differences. A descriptive analysis was performed comparing the proportion of experiments reported (degrees of freedom) in the original publication. The response was classified as submaximal for a negative t < 0.05 lower than the critical value, as SPMX for a positive t > 0.05 higher than the critical value, and as maximal when the null hypothesis could not be rejected (p < 0.05). Several publications reported dose-response curves without visible maximum (Fig. 1, last curve). These putative SPMX cases were considered as maximal responses during the analysis.

**Database analysis.** The database was split into three data sets: mammalian cell entries, yeast entries, and rainbow trout entries to account for species differences. A descriptive analysis was performed comparing the proportion of submaximal (t < 0.05), maximal (t > 0.05 different from 0), and SPMX (t > 0.05 significantly different from 0) entries within assay characteristic. The proportion of entries with SPMX effect is given within brackets. Because of the limitation of its application to the data set, the Kruskal-Wallis nonparametric test using t as continuous variable only was applied to substantiate the qualitative results comparing average t values between groups (p values given within brackets). When considered appropriate, subsets of the database were evaluated to underline trends on particular cell models or end points.

**RESULTS**

Twenty-one chemicals including DES, dihydrotestosterone, 4-nonylphenol, 4-tert-octylphenol, BPA, and some of its...
where 

\[ t_c = \frac{[\% C_{\text{max}} - \% E_2\text{max}]}{\sqrt{\frac{S_C^2}{n_C} + \frac{S_{E2}^2}{n_{E2}}}} \]

and the number of independent measurement were the SD was calculated from. 

**Compound response**

Lowest tested concentration, highest tested concentration, maximal response concentration, maximal response compared to E2 maximal response, and compound SD at maximal response.

**Response Variable**

In the mammalian database, 33% of the compound responses fell above the tabulated \( t \) value indicating SPMX, while from the yeast entries 18% were considered SPMX. When discriminating by compound, 13, 32, and 48% of all DES, BPA, and GEN entries, respectively, fell above the critical value and, thus, were considered SPMX. Table 3 shows the percentage of entries considered SPMX, discriminated by cell model and end point.

**Cell Model**

The cell model appeared to be an important factor in the occurrence of the SPMX effect (Fig. 2A). T47D (78%) and U2-OS (75%) cell lines had more frequent occurrences of SPMX compared with, for example, HeLa (42%) or H293 (33%). Indeed, significant differences were found between cell model \( t_c \) averages (\( p = 0.033 \)). When only cell models with luciferase induction as the end point were selected, again cell model \( t_c \) averages showed significant differences between cell models (\( p = 0.018 \), with SPMX proportions of T47D-Luc (86%) and U2-OS-Luc (75%) higher than the other cell models.

Interestingly, estrogenic responses with COS-1 cells never were SPMX; while MCF-7 cells had entries with values well distributed from submaximal to SPMX relative responses (Table 3 and Fig. 2A). Despite the differences, the cell model only accounted for a small fraction of the data variation. There was no apparent effect of the cell model when each compound was evaluated independently. The relative responses for BPA, GEN, and DES in Rainbow trout hepatocytes were always below the estradiol maximum response (Table 3).
Number of database entries and references of maximal induction by the compounds DES, GEN, and BPA compared to E2 per cell model and end point. Cell models: human epithelial breast carcinoma (MF-7), human cervical cancer (HeLa), human ductal breast carcinoma (T47D), human endometrial adenocarcinoma (Ishikawa), human osteosarcoma (U2-OS), human embryonal kidney (H293), African green monkey kidney (COS-1), rainbow trout hepatocytes (Rainbow t hep) and yeast. End points: luciferase induction (Luc), GFP, CAT, β-galactosidase (B-Gal), cell proliferation (cell prol), alkyl phosphatase activity (APhos), vitellogenin production (Vit prod), and progesterone receptor production (PR prod).

<table>
<thead>
<tr>
<th>Cell model</th>
<th>End Point</th>
<th>DES</th>
<th>References</th>
<th>BPA</th>
<th>References</th>
<th>GEN</th>
<th>References</th>
<th>Total no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Luc</td>
<td>3</td>
<td>Sanoh et al. (2003), Demirpence et al. (1993), and Freyberger and Schmuck (2005)</td>
<td>6</td>
<td>Freyberger and Schmuck (2005), Fujimoto and Honda (2003), Kitamura et al. (2005), Matthews et al. (2001), and Yoshihara et al. (2004)</td>
<td>10</td>
<td>Freyberger and Schmuck (2005), Fujimoto and Honda (2003), Harris et al. (2005), Ki et al. (2003), Maggiolini et al. (2001), Le Bail et al. (1998), and Zierau et al. (2005)</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Cell prol</td>
<td>6</td>
<td>Han et al. (2002), Jones et al. (1998), Kanai et al. (2001), Morito et al. (2001), Okubo et al. (2001), and Olsen et al. (2005)</td>
<td>8</td>
<td>Han et al. (2002), Kanai et al. (2001), Olsen et al. (2005), Brotons et al. (1995), Nakagawa and Suzuki (2001), Olea et al. (1996), Parez et al. (1998), and Samuelsen et al. (2001)</td>
<td>14</td>
<td>Maggiolini et al. (2001), Le Bail et al. (1998), Seo et al. (2006), Han et al. (2002), Morito et al. (2001), Kuruto-Niwa et al. (2007), Makela et al. (1994), Yap et al. (2005), Hsieh et al. (1998), Hwang et al. (2006), Kinjo et al. (2004), Matsumura et al. (2005), Schmidt et al. (2005), and Murata et al. (2004)</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>CAT</td>
<td>3</td>
<td>Olea et al. (1996), Parez et al. (1998), and Krishnan et al. (1993)</td>
<td>1</td>
<td>Matsumura et al. (2005)</td>
<td>1</td>
<td>Matsumura et al. (2005)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>PR prod</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAT</td>
<td>1</td>
<td>Shelby et al. (1996)</td>
<td>1</td>
<td>Makela et al. (1994)</td>
<td>1</td>
<td>Makela et al. (1994)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Cell prol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T47D</td>
<td>Luc</td>
<td>1</td>
<td>Wilson et al. (2004)</td>
<td>3</td>
<td>Buterin et al. (2006), Legler et al. (1999), and Meerts et al. (2001)</td>
<td>3</td>
<td>Wilson et al. (2004), Buterin et al. (2006), Legler et al. (1999), and Meerts et al. (2001),</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Cell prol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U2-OS</td>
<td>Luc</td>
<td>2</td>
<td>Ter Veld et al. (2006)</td>
<td>2</td>
<td>Van Der Woude et al. (2005)</td>
<td>2</td>
<td>Van Der Woude et al. (2005)</td>
<td>4</td>
</tr>
</tbody>
</table>
Despite apparent differences in the proportions of sub-maximal, maximal, and SPMX entries within end points in Figure 2B, no significant differences were found between the end points $t_{cc}$ averages. However, transfected chloramphenicol transferase (CAT) reporter gene cells did not produce SPMX responses on either MCF-7 or HeLa cell lines (Table 3). On the other hand, mammalian cells transfected with green fluorescence protein (GFP) reporter gene consistently gave SPMX responses (Fig. 2B), while the same reporter gene transfected into yeast gave SPMX response on three cases out of five (Table 3). There were in addition no differences on the SPMX effect occurrence among cell proliferation measurement methods.

**Cell Culture Characteristics**

Neither solvent nor its concentrations in culture seemed to have a significant effect on the three compounds relative maximum responses in mammalian cell lines. All mammalian entries together did not show any correlation between relative responses and serum concentration. However, within cell proliferation entries, a weak trend toward higher $t_{cc}$ values was observed with increasing serum concentrations in the exposure medium (data not shown). Interestingly, cell proliferation assays consistently never gave SPMX responses when human serum was used.

### TABLE 2—Continued

<table>
<thead>
<tr>
<th>Cell model</th>
<th>End Point</th>
<th>No. $^a$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>H293</td>
<td>Luc</td>
<td>2</td>
<td>Kuiper et al. (1998)</td>
</tr>
<tr>
<td>COS-1</td>
<td>Luc</td>
<td>4</td>
<td>Ashby et al. (1999) and Pennie et al. (1998)</td>
</tr>
<tr>
<td>Rainbow trout hep</td>
<td>Vit prod</td>
<td>2</td>
<td>Olsen et al. (2005) and Pelissero et al. (1993)</td>
</tr>
<tr>
<td>Yeast</td>
<td>B-Gal</td>
<td>6</td>
<td>Morito et al. (2001), Ashby et al. (1999), Arnold et al. (1996), Beck et al. (2005), and Gaido et al. (1997)</td>
</tr>
<tr>
<td>GFP</td>
<td>2</td>
<td>Beck et al. (2005) and Bovee et al. (2004)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>36</td>
<td>64</td>
</tr>
</tbody>
</table>

$^a$ An entry is the report of a specific compound with a given cell model and a given end point. Therefore, a paper which analyzes a variety of compounds or uses a variety of cell models will appear with more than one entry.

### TABLE 3

<table>
<thead>
<tr>
<th>Cell model</th>
<th>End point</th>
<th>$%_c$</th>
<th>$%_n$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Luc</td>
<td>16</td>
<td>19</td>
<td>Ishikawa</td>
</tr>
<tr>
<td>HeLa</td>
<td>Luc</td>
<td>50</td>
<td>4</td>
<td>APhos</td>
</tr>
<tr>
<td>H293</td>
<td>Luc</td>
<td>33</td>
<td>6</td>
<td>CAT</td>
</tr>
<tr>
<td>COS-1</td>
<td>Luc</td>
<td>0</td>
<td>6</td>
<td>Cell prol</td>
</tr>
<tr>
<td>Rainbow trout hep</td>
<td>Vit prod</td>
<td>0</td>
<td>6</td>
<td>B-GAL</td>
</tr>
<tr>
<td>Yeast</td>
<td>B-Gal</td>
<td>50</td>
<td>2</td>
<td>Cell prol</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>17</td>
<td>133</td>
<td></td>
</tr>
</tbody>
</table>

End Points

**Testosterone (T) and Progesterone (PR)**

- **T**: Cell prol, alkanes, and progesterone production (PR prol).
- **PR**: Cell prol, alkanes, and progesterone production (PR).
was used in culture (data not shown but available within Supplementary data 3).

Transfected Cell Lines

Illustrated by Figure 3A, the proportion of SPMX entries for transfected cells was higher (42%) compared with wild-type cells (21%) \( (p = 0.026) \). Transfected T47D and Ishikawa cell lines exhibited higher SPMX proportions (86 and 50%, respectively) compared to the values in proliferation assays with its wild-type cells (50 and 0%, respectively). It is interesting to note that the likeliness of obtaining an SPMX effect apparently was not influenced by the origin of the ER, whether it was endogenous (T47D) or introduced (U2-OS).

Among transfected entries, the stability of transfection had a significant influence on the occurrence of an SPMX effect (Fig. 3B). Considering mammalian cell lines, stably transfected cells had a higher SPMX proportion (59%) compared to transiently transfected cells (26%) \( (p = 0.029) \). Particularly for BPA, the SPMX incidence was higher in stably transfected cells (70%) compared to transiently transfected cells (10%) \( (p = 0.028) \). Also yeast cell lines stably transfected gave a higher SPMX proportion (27%) compared with transiently transfected ones which do not have a single SPMX entry \( (p = 0.022) \).

There were no significant differences between the proportion of SPMX for the three compounds together between cells expressing endogenous receptors (27%) compared to cells with transfected receptors, either ER\( \alpha \) (48%) or ER\( \beta \) (35%), despite a visible trend. Only for luciferase induction as the end point was the number of entries (59) sufficient to compare SPMX in models with endogenous receptors (24%) with those with transfected ER\( \alpha \) (52%) or ER\( \beta \) (35%). In addition, the source of the transfected receptors (i.e., human, rat or mouse) did not significantly influence the relative occurrence of SPMX responses.

A clear increase in the SPMX proportion was observed with increasing number of EREs (Fig. 4A). The \( t_c \) average values of mammalian cell entries grouped by the number of consensus EREs, transfected upstream of the reporter plasmid, were significantly different \( (p = 0.003) \). Furthermore, in yeast transfected with one consensus ERE, the SPMX occurrence was lower than in cells transfected with two EREs \( (p = 0.041) \) (Fig. 4B).

Also, the context of the promoter within the clone construct was related to the incidence of SPMX response of the compounds. When thymidine kinase (tk) was present as a promoter, the proportion of SPMX entries were lower compared to TATA box and C3 promoters (Fig. 5).

DISCUSSION

The existence of SPMX responses and the putative inconsistency of this SPMX occurrence in in vitro assays for estrogenicity presents a challenge for the quantification and interpretation of the estrogenic potency of compounds and environmental extracts. When the SPMX character of a response is ignored, it could lead to overestimation of the relative
potency compared to the current standard E2 dose-response. And when the existence of an SPMX dose-response is recognized, it is difficult to interpret its meaning for estrogenicity assessment. Several ubiquitous compounds have been reported to produce an SPMX effect in in vitro assays for estrogenicity under diverse (seemingly unrelated) circumstances. As results frequently are not consistent across studies, this has lead to scientific debates about the nature of its cause and relevance for the in vivo situation. This study is the first to present a systematic analysis of data published on the estrogenicity of compounds that have been reported to induce SPMX effects. The correlation between the occurrences of SPMX with assay characteristics has been examined, with special attention for cell type, target gene, and clone characteristics.

Upon entering the published information into a database, the lack of detailed information became apparent. For example, unclear compound identification including its purity and lack of information on the identity and concentrations of reagents in the assays made it difficult to interpret the consequences of the absence or occurrence of SPMX effects in published studies. It was impossible to include detailed clone construct characteristics like ERE spacing, promoter spacing, ERE sequence or source, and reporter sequence or source in the analysis; the information provided was not sufficient even when referenced to previous published data.

Another possible source of confusion is the transformation of the response data for graphical presentation. For example, when using induction factors, the maximum value depends greatly on the control value (commonly the value with relatively the greatest variability). In several cases, the absolute data were not available, while in other cases the maximum values were not shown and the curve stopped at 100% with the shape suggesting a putative SPMX response. To avoid inexplicable inconsistencies in future in vitro publications that could perhaps have been related to differences in method details, good reporting practices are crucial.

Three estrogenic compounds were selected—DES, BPA, and GEN—that are frequently tested in addition to E2 to validate new in vitro methods for estrogenicity and to serve as a reference for other potentially estrogenic compounds and samples. Therefore, the total number of publications was sufficient for building the database and for investigating the main trends. Many publications did not aim at comparing the full dose-response relationship between E2 and other agonists and therefore lacked its maximal response. This was the main reason for exclusion of some publications from the database (see Supplementary data 2). Heterogeneity of results between publications was expected, but in addition, ample data dispersion was found for the variables within single categories. For instance, MCF-7 or luciferase entries separately have large variations in the occurrence of SPMX and thus in their \( t \_c \) ranges. This confirmed the inconsistency in the SPMX effect occurrence and its apparent independence of the cell model or end point. None of the variables alone, although significant in some cases, could explain the substantial variation in the occurrence of SPMX. This actually was to be expected given the limitations in the input data mentioned above. Only very dominant explaining variables could have been identified in the current meta-analysis of the available literature data. The best explaining variable as indicated from this analysis should ideally be further studied under experimentally controlled conditions.

There is a general trend within the database toward a higher frequency of SPMX effects with reporter gene assays. The influence of cell culture variables like serum type and concentration, exposure period, and solvent concentrations was not

![FIG. 4. Percentage of submaximal (sub), maximal (max), and supramaximal (supra) entries discriminated by the number of EREs in the reporter plasmid used in (A) mammalian cell lines (n1 = 17, n2 = 13, n3 = 36) and (B) yeast cell lines (n1 = 12, n2 = 10).](https://academic.oup.com/toxsci/article-abstract/115/2/462/1732312)

![FIG. 5. Percentage of submaximal (sub), maximal (max), and supramaximal (supra) entries of mammalian reporter cell lines discriminated by the promoter type: tk promoter (n = 12), TATA box (n = 36), and C3 promoter (n = 8), included with the ERE into the reporter plasmid construct.](https://academic.oup.com/toxsci/article-abstract/115/2/462/1732312)
apparent. The effects of ethanol on the occurrence of SPMX in cell proliferation assays and ERα transcriptional activity in human breast cancer cells has previously been reported (Fan et al., 2000; Singletary et al., 2001). Bhattacharya et al. (2001) suggested that the SPMX effect induced by resveratrol (Gehm et al., 1997) could actually be due to the use of ethanol as solvent. The data in our study, however, do not support this suggested effect of ethanol. Also the concentration of serum has been suggested to influence the occurrence of SPMX (Soto and Sonnenschein, 1984; Stroucken et al., 1994). Again, this indication is not in accordance with the results from our study. Our results do nevertheless suggest that no SPMX effect occurs when human serum is used, although a limited number of assays with cell proliferation as end point used human serum. The suggested influence, however, is supported by several reports of different effects on cell proliferation between human and fetal bovine serum (McAlinden and Wilson, 2000; Soto and Sonnenschein, 1984; Soto et al., 1986). Further experimental analysis of the putative influence of serum type on the occurrence of an SPMX cell proliferation effect is required to evaluate this issue.

The frequency of SPMX is higher in transfected cell lines compared to wild-type cells with endogenous ERs and end points such as alkyl phosphatase and progesterone receptor production in mammalian cell lines and vitellogenin production in rainbow trout cells (Table 3). This is in agreement with the previously reported suggestion that the occurrence of SPMX depends on the targeted gene (Gehm et al., 1997). This may even explain the visible trend of a cell model significant difference, as all entries for U2OS, and seven out of nine for T47D were stably transfected reporter gene end points (Table 3). These results indirectly suggest an influence of clone characteristics on the SPMX effect occurrence.

Of the transfected mammalian cell lines, the stably transfected cells showed a higher SPMX incidence than the transiently transfected ones. The same difference was observed for transfected yeast cell lines. For instance, two seemingly comparable assays with 3xERE-TATA-LUC transiently transfected H293 cells were performed by different laboratories. While Kuiper et al. (1997) reported GEN SPMX, Hwang et al. (2006), however, did not. The differences might be accounted for by major differences between the two methods related to transfection method, ER source, and plasmid construction.

We found a significant dependence of the SPMX effect on the number of EREs in the plasmid construct of transfected cell lines. No SPMX was described for models with only one ERE upstream of the CAT reporter gene. The plasmids of the Luc and GFP reporter genes all have two or more ERE’s. Several authors reported increased E2 induced response with multiple EREs (Catherino and Jordan, 1995; Ponglikitmongkol et al., 1990; Sathyas, 1997; Tyulmenkov et al., 2000). In addition, both the number of EREs and their spacing, proximity to the promoter and the promoter nature, are suggested to influence the occurrence of synergistic estrogenic responses (Sathyas et al., 1997). Synergism could be related to SPMX responses, and exposure media contain many compounds of which their possible role in inducing estrogenic responses is not known. To our knowledge, there are currently no publications addressing the multiple ERE synergistic effect with ligands other than E2. Nonetheless, the dependence of SPMX effect on the number of EREs and on the nature of the promoter revealed in our meta-analysis may indicate that other compounds like GEN and BPA can induce this ERE transcriptional synergism as well. In that case, the observed SPMX effects in specific transfected cell lines indicate a stronger transcriptional synergism induced by GEN and BPA compared to that induced by E2. It is not clear why the compounds did not induce SPMX in COS-1 cell line. It is important to note that for these cells, only two sources were available, both from the same laboratory, so the variation in conditions is limited. In both papers, the use of two consensus EREs with the tk promoter is reported. Both characteristics are associated here with a lower likeliness for SPMX responses.

This meta-analysis confirms the inconsistent occurrence of SPMX effects, which could be related to a number of specific assay characteristics: (1) the type of serum used to supplement the exposure medium, (2) the end point used to quantify the estrogenic potency (endogenous or transfected), (3) the number of EREs, and (4) the nature of the promoter. It is important to understand the mechanism behind this phenomenon because in vitro assays for estrogenicity are used extensively to characterize and quantify the estrogenic potency of compounds, mixtures, and environmental extracts. To our knowledge, the influence of significantly different maximum responses of compounds in a mixture to the mixture response has not been addressed. For the quantification of the estrogenic potency of mixtures, concentration addition is assumed (Jonker et al., 2005), but when some compounds induce SPMX, this assumption is not valid. In addition, the estrogenic equivalency factors are derived from the EC50 value of the compounds of interest compared to that of E2. A significantly higher maximum will invalidate the equipotent assumption and thus derive a miscalculation of the compounds potency. The estrogenic potency of complex mixtures such as environmental extracts is determined by diluting the extract to a response, which falls into the linear part of the standard dose-response curve. The consequences of the SPMX phenomenon could result in overestimation of the estrogenic potency if the SPMX is the consequence of a molecular artefact of the cell model. But perhaps the SPMX effect only occurs at high concentrations and not at concentrations that could occur in blood. To be able to decide how to interpret and deal with the occurrence of SPMX in certain cell models, it is important to understand its underlying mechanism. Is it an artefact or a mechanism that is relevant for the in vivo situation as well? This cannot be resolved by further analysis of already reported results but requires dedicated additional research to unravel this intriguing phenomenon.
SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

ACKNOWLEDGMENTS

We are grateful for the input of M. A. Davidson, MSc, Dr J. Aarts, and Dr T. Udelhoven on earlier versions of the manuscript.

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