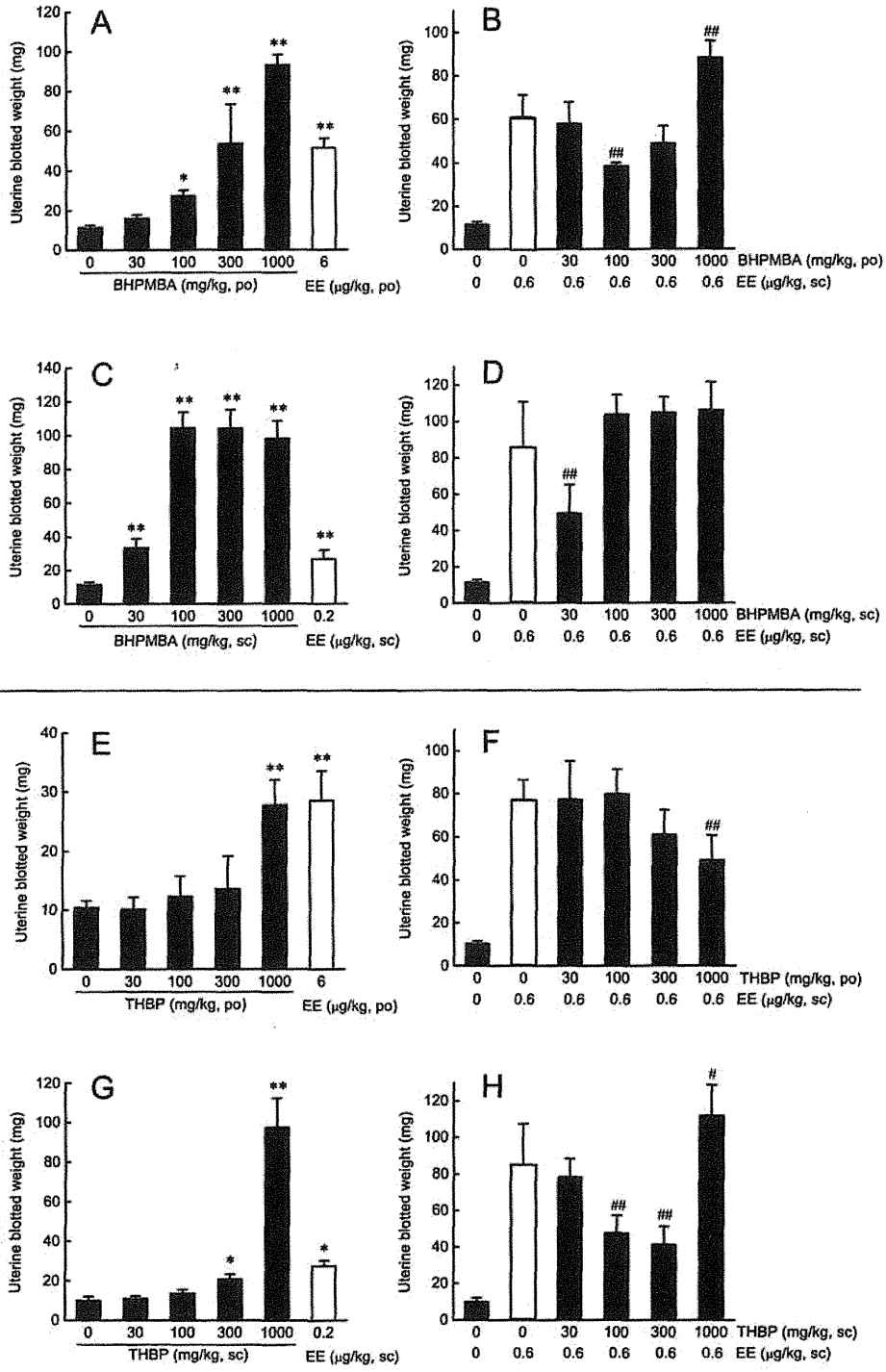

Fig. 3. A bar graph representation of the dose-response characteristics of uterotrophic effect of BHPMBA and THBP. A) agonistic effect by po, B) antagonistic effect by po, C) agonistic effect by sc, and D) antagonistic effect by sc of BHPMBA. E) agonistic effect by po, F) antagonistic effect by po, G) agonistic effect by sc, and H) antagonistic effect by sc of THBP. Sensitive responses were seen by sc route of exposure. U-shaped dose response was monitored by antagonist studies (B, D and H). The mean blotted uterine weight is presented with S.D. *: $p < 0.05$, **: $p < 0.01$ from vehicle control, and #: $P < 0.05$, ##: $P < 0.01$ from the group of vehicle plus EE.

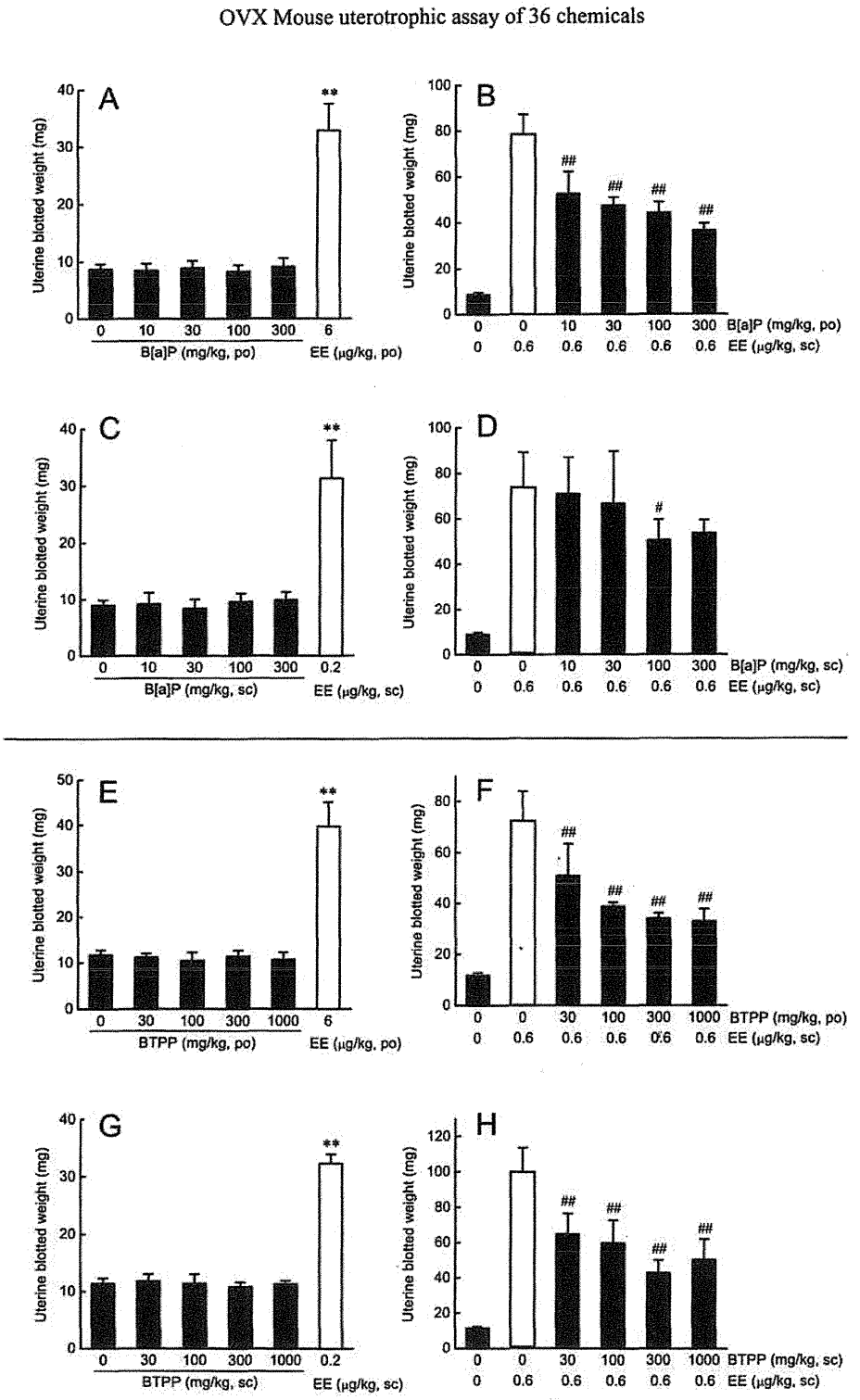

Fig. 4. A bar graph representation of the dose-response characteristics of uterotrophic effect of B[a]P and BTPP. A) agonistic effect by po, B) antagonistic effect by po, C) agonistic effect by sc, and D) antagonistic effect by sc of B[a]P. E) agonistic effect by po, F) antagonistic effect by po, G) agonistic effect by sc, and H) antagonistic effect by sc of BTPP. Antagonistic effect alone was induced by these chemicals. B[a]P was more effective by po, whereas BTPP showed equal response to both routes of exposure. The mean blotted uterine weight is presented with S.D. #:P < 0.05, ##:P < 0.01 from the group of vehicle plus EE.

nistic chemicals, including BPA and genistein, did not induce U-shaped dose-response because the UT effects at maximal dose (limited by MTD) were smaller than the reference EE (Group 8). We conducted a preliminary study on BPA to determine whether a U-shaped dose response curve can be seen by reducing the amount of reference EE to a level lower than the UT effect of the maximal dose. This attempt, however, failed to induce U-shaped dose response. Instead, an additive agonistic response was obtained; the uterine weight started to increase monotonously from the UT level of reduced reference EE (data not shown). There may be an absolute condition for a chemical to induce U-shaped dose response in an antagonistic study.

ANB showed weak agonistic activiy by sc and weak antagonistic activity by both routes of exposure (chemical No.6). In sc study, antagonistic tendency emerged below the dose of agonistic effect.

Next four chemicals of the Table 3 (chemical No.7~10) were agonistic only by the sc route. Among them, only PDD lacks supportive *in silico/in vitro* data (cf. Supplement Table 1). However, a related compound, diphenyl-p-phenylenediamine, has been reported to be estrogenic (Yamasaki *et al.*, 2003a).

The next 11 chemicals in Table 3 (Chemicals No.11~21) only showed antagonistic effects. Among them, polycyclic aromatic hydrocarbons (PAHs), B[a]P, DB[a]A and B[a]A, are known, aside from their mutagenic and carcinogenic activities, to provoke aryl hydrocarbon receptor (AhR) signaling. In general, AhR agonists are reported to act as antiestrogens, not by competing at the ligand binding domain of the ERα, but by down-regulating ERα signaling (Chen *et al.*, 2001) and/or increasing the metabolism of estradiol (Badawi *et al.*, 2000). AhR agonists are also reported to inhibit various growth factors and modulate crosstalk between AhR and ERα pathways (Safe, 1999), and to increase proteosomal degradation of the ER receptor (Wormke *et al.*, 2003) (Ohtake *et al.*, 2011). On the other hand, there are conflicting reports indicating that the hydroxylated metabolites of B[a]P are estrogenic *in vitro* (Fertuck *et al.*, 2001) and both B[a]P and B[a]A are estrogenic in the immature rat uterotrophic assay after three days of sc (Kummer *et al.*, 2008). The discrepancy might be related to the difference between the immature and adult OVX assay, known to have different hepatic metabolism and/or to the length of the exposure periods (3 days versus 7 days). Further studies in AhR knock out and/or Cyp1a1 knockout mice might clarify the molecular basis of (anti)estrogenic effects of PAH. By the *in vitro* reporter assay (Supplement Table 1), B[a]P and MGB were antiestrogenic, and FBZ was estrogenic. Col was selected as a candidate for the uterotrophic assay by QSAR and by ER alpha reporter assay. Therefore, Col might have interacted with ER alpha. However, its well known anti-mitogenic effect (given above human pharmacological doses) against the UT response might not be excluded. The remaining of the 11 chemicals were predicted to interact with ERα by *in silico* screening.

The last 15 chemicals in Table 3 (Chemical No. 22 ~ 36) were negative for estrogenic and antiestrogenic effects by both routes of exposure.

As a conclusion, we consider that the OVX mouse UT assay is an effective screening test method for the detection of estrogenic agonistic and antagonistic properties of a chemical, and may be comparable to the rat system. The advantage of mouse over rats are two fold: the smaller amount of test chemicals and laboratory space needed and the possibility to use transgenic and/or knockout mice, such as AhR/Cyp1a1 knock out and humanized mice, such as for cytochrome P450 (Scheer *et al.*, 2008) and for steroid and xenobiotic receptor (Igarashi *et al.*, 2012) for further mechanistic studies including that of U-shaped dose response.

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