

The Potential and Suitability of 2-Methoxyestradiol in Cancer Therapy

To the Editor: We disagree with the conclusions in a recent article by Sutherland et al. (1) stating that 2-methoxyestradiol (2ME2) is an unsuitable antitumor agent that lacks efficacy and has estrogen receptor–dependent and estrogen receptor–independent adverse effects. Although the data reported in this article are interesting, it is inappropriate to conclude that 2ME2 is unsuitable as an antitumor agent. The weight of evidence from numerous *in vivo* tumor models, in addition to clinical experience with 2ME2, indicates it is a promising anticancer agent and deserves further evaluation.

Sutherland's conclusion about preclinical efficacy was based on *in vivo* studies in which doses of 2ME2 from 15 to 150 mg/kg, in their hands, had no antitumor activity in immunodeficient mice bearing either MDA-MB-435 or MCF7 tumors. These data are contradicted by numerous published reports indicating antitumor activity of 2ME2 in multiple preclinical models, including previous reports showing antitumor activity at these doses in an apparently identical tumor model (2).

The *in vivo* antitumor and antiangiogenic activity of 2ME2 has been well described and, as Sutherland et al. indicate, administration of 2ME2 is known to reduce tumor volumes and inhibit tumor progression in diverse tumor models. Additional reports to strengthen this statement continue to appear in the literature (3–5). Notwithstanding this large body of literature, the authors conclude that 2ME2 lacks efficacy and cite three articles to support this statement (6–8). However, two of the cited articles used marginal or subtherapeutic doses of 2ME2 (6, 7) whereas the third article failed to show antitumor activity not only with 2ME2 but also with TNP-470 and paclitaxel (8). Therefore, the majority of the published scientific literature clearly shows that administration of 2ME2 in various tumor models results in dose-dependent activity.

The data presented by Sutherland et al. showing administration of 2ME2 has estrogenic-like effects are perplexing and not conclusive. Importantly, the 2ME2 used as the active pharmaceutical ingredient in all clinical studies, and in most recent work by EntreMed collaborators, has been qualified using validated analytic methods. Clinical-grade 2ME2 has been shown to be free of known estrogens (<0.01% for estradiol, estrone, or 2-hydroxyestradiol), which may be contaminants of other preparations of 2ME2. The 2ME2 used in the studies of Sutherland et al., which is from a commercial source (Sigma), is reported to have a >98% or >99% purity, but, more importantly, the use of validated methods to prove the absence of specific estrogenic contaminants is not guaranteed. Given the affinity of estradiol for its receptors, and the biological activity of estrogens, even a 0.1% contamination of estradiol could result in significant estrogenic effects *in vivo*. Unfortunately, it was unclear from the study of Sutherland et al. what analytic methods were used to qualify the presence or absence of potential estrogens in their 2ME2.

It has been reported that *in vivo* metabolism of 2ME2 can produce small amounts of compounds, such as 2-hydroxyestradiol, that have weak estrogenic activity (9). However, the cumulative clinical experience to date (170 patients treated for up to 3 years with daily oral 2ME2) has not identified any clinically significant adverse events that would implicate 2ME2 or a metabolite as an estrogen receptor agonist. Of particular relevance, a phase 1 clinical study in refractory metastatic breast cancer has been conducted at Indiana University (10). Thirty-one patients were treated with 2ME2 and 19 were estrogen receptor positive. 2ME2 was well tolerated with minimal toxicity and no maximum tolerated dose was identified. No alterations in hormone levels were shown and seven patients had stable disease for at least 4 months with the median time to failure of 55 days for all patients. One patient who was estrogen receptor positive had a minor response lasting 9 months.

2ME2 has been orally administered to cancer patients since 2000. As a prerequisite for the initiation of clinical trials with 2ME2, preclinical pharmacology and toxicology studies supported the antitumor and antiangiogenic activities and safety of this agent. Recent development activities with 2ME2 have identified a new nanocrystal colloidal dispersion formulation that improves on the low bioavailability of micronized 2ME2 formulations that have been used in previous preclinical studies (11). The improved bioavailability of this 2ME2 formulation results in enhanced antitumor effects and no new toxicities in preclinical studies. This new formulation has recently been introduced into the clinic.

In summary, contrary to the conclusions of Sutherland et al., we believe that based on the preclinical antitumor efficacy and excellent safety profile of 2ME2, further clinical development of this molecule is warranted.

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In Response: We thank Dr. Sidor and colleagues for their comments on our recent paper describing studies that lead to the conclusion that 2-methoxyestradiol (2MEO) lacks anti-tumor activity and has estrogen agonist activity, raising obvious concerns about the suitability of this agent for clinical development and especially for breast cancer (1). Two questions were raised by their correspondence. Are the conclusions that were reached in our study invalid due to trace contamination of 2MEO by estradiol (E2) or related estrogenic substances? How should the lack of antitumor activity observed in our experiments be interpreted, having regard to the number of publications reporting preclinical efficacy in diverse tumor models?

We reported evidence of binding of 2MEO to [^3H]E2-binding sites in a preparation of rat uterine cytosol (2) commonly used to detect estrogen receptor (ER) binding. This finding was extended by demonstrating that 2MEO, acting through an ICI 182,780 mechanism, increased expression of estrogen-responsive genes, including *cyclin D1*, which is essential for proliferation of the ER-positive breast tumor cell line MCF7. Sidor and colleagues from EntreMed, a company developing 2MEO for a variety of tumor indications, have pointed out that they and their collaborators use a pharmaceutical-grade 2MEO that has <0.01% E2. The potential for trace amounts of E2 to account for the estrogenicity detected in our experiments was considered at the time of the study, which commenced in 2001. The material purchased from Steraloids (batch W242) was confirmed to be 2MEO by ^1H nuclear magnetic resonance analysis (Varian Inova 400 MHz). Electrospray ionization-mass spectrometry (Quattro II triple quadrupole mass spectrometer, Micromass UK, Manchester, United Kingdom) gave a base peak of m/z 301 in the negative ionization mode consistent with that of 2MEO (M_r 302). Moreover, reverse-phase high-performance liquid chromatography gave a single peak with purity determined at 99.6% using diode array detection at 220 nm. As the fluorescence yield of E2 is greater than that of 2MEO and there was no peak detected at the retention time of E2, the material was considered sufficiently pure to draw conclusions that related to the pharmacology of 2MEO not confounded by trace contaminants. However, in response to the new information provided by Sidor and colleagues, we have undertaken further analysis of 2MEO (Steraloids, batch B01700, used in the experiments reported in Fig. 6 of ref. 1) by electrospray ionization-mass spectrometry conducted as described above. A limit of detection for E2 of 1 ng was determined when injected concurrently

with 8,000 ng 2MEO. Under these conditions, it was not possible to detect E2 (limit of detection $\sim 0.013\%$) in this batch of 2MEO. We measured the $-\log IC_{50}$ value of this batch of 2MEO for displacement of [^3H]E2 at 7.02 ± 0.04 (giving a K_d of 131 nmol/L), a value consistent with our previous estimates in rat uterine cytosol (1, 2), those of others in MCF7 cells (3), or on recombinant ER α (4) and $\sim 1/1,000$ that of E2 (0.13 nmol/L). In contrast, the maximum level of contamination of 2MEO by E2 of 1/8,000 clearly cannot account for the estrogenic activity that we have observed *in vitro*. There was no peak detectable at the m/z for estrone, but the sample was found to contain 0.25% (w/w) 2-hydroxy-E2, an E2 metabolite with significantly less estrogenicity than E2 but having 100 times the affinity of 2MEO for ER (2). Nevertheless, the 2-hydroxy-E2 level would need to be 1% to explain the extent of ER binding that we and others have repeatedly observed. We therefore conclude that it is 2MEO per se, and not the contaminants, that accounts for the observed estrogenicity. Sidor and colleagues refer to a key paper in the literature from EntreMed regarding estrogenic effects of 2MEO (4) purchased from Tetriconic (Madison, WI), which we presume to be the pharmaceutical supplier to which they refer. A careful analysis of this paper renders the discussion of trace contaminants redundant in considering the *in vivo* estrogenic potential of 2MEO in the microenvironment of the breast tumor. LaVallee et al. (4) reported binding data for 2MEO and E2 on recombinant ER α and ER β , indicating potency ratios for 2MEO/E2 of 500 and 3,200, respectively. Both the relative (to E2) and absolute affinity of 2MEO for recombinant ER α are similar to those we obtained. More importantly, their study showed unequivocally that 2MEO had estrogenic actions in sustaining the growth of cultured MCF7 cells, consistent with our findings on expression of growth-related genes. The estrogenicity of 2MEO was ascribed to its back conversion to E2 by MCF7 cells (4). Whereas we were unable to confirm such a back conversion, we repeatedly confirmed, with diverse approaches, that 2MEO had estrogen-like actions in MCF7 cells (1). In the broader context, the mechanism of such 2MEO-induced estrogenic actions is of little consequence to our overall conclusion. The estrogenic consequence of exposure to 2MEO is important. We show that this estrogenic activity is evident *in vivo* in a study of 2MEO administered to *nu/nu* mice inoculated with MCF7 cells in which 2MEO supported growth of this ER-dependent cell line and had uterotrophic effects despite the fact that the mice had not been ovariectomized. These outcomes are predictable from the *in vitro* observations of LaVallee et al.

The absence of estrogenic effects in the clinical studies cited by Sidor and colleagues needs to be interpreted with caution as the serum levels achieved in these studies are in the low nanomolar range, at which neither estrogenicity nor efficacy would be expected. Moreover, none of the outcomes referred to in their correspondence constitutes an objective therapeutic response. Whereas the new formulation is reported to achieve higher bioavailability without new toxicity issues, we await publication of more detailed PK/PD studies that would allow this formulation to be assessed.

Although we agree with Sidor and colleagues that the balance of the literature supports efficacy of 2MEO in a variety of preclinical models of solid tumor growth, our conclusions are based on the outcomes of six independent adequately powered and carefully conducted experiments using different tumor cell

lines (PC3, MDA-MB-435, MCF7), different sites of tumor cell inoculation (s.c or mammary fat pad), and different routes of 2MEO administration (i.p. or oral). In addition, there is considerable variance in the detail provided with many of the studies reporting efficacy. For example, to our knowledge, the studies that we have conducted are the only ones to have been carried out in a blinded manner. We consider this to be a particularly important aspect of the methodology when caliper-estimated tumor dimensions are the primary outcome. Our studies used both caliper measurement and postmortem tumor weight determinations. Interestingly, in a recent paper claiming preclinical efficacy of 2MEO in a model of human cervical cancer, an apparently large and significant difference in progressive tumor volume did not result in any difference in tumor weight (5). The latter study reported hepatotoxicity consistent with our unpublished observations and with the increases in liver weight reported in our article (1).

We reaffirm our conclusion that 2MEO itself is an ER agonist that will achieve significant ER occupancy at the 100 to 1,000 nmol/L concentrations required for antiproliferative effects on tumor and endothelial cells. Our view that the evaluation of 2MEO in breast cancer is inappropriate is reinforced by analysis of the purity of the 2MEO used in our studies and our confirmation of the ER binding affinity of this material.

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