# Safety Assessment of (*E/Z*)-Endoxifen and (*E/Z*)-Endoxifen Gel in Toxicology Screening Assays

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## ABSTRACT

In comparison to oral or intravenous administration, direct topical application of breast cancer drugs may increase local delivery and reduce systemic absorption, thereby increasing efficacy and reducing systemic toxicity. Bacterial mutagenicity, *in vitro* clastogenicity, *in vitro* photo-toxicity, and *in vivo* dermal sensitization studies were performed to evaluate the safety of a gel formulation of endoxifen being developed for breast cancer prevention. (*E/Z*)-endoxifen was not mutagenic in four strains of S. typhimurium or in *E. coli* WP2 uvrA, with or without metabolic activation; positive control articles were mutagenic in all tester strains. (*E/Z*)-endoxifen did not induce structural chromosomal aberrations in Chinese Hamster Ovary cells, with or without metabolic activation; two positive control articles did demonstrate significant clastogenicity. (*E/Z*)-endoxifen was not phototoxic in the neutral red uptake assay in BALB/c 3T3 fibroblasts; the positive control article did induce significant phototoxicity. Dermal sensitization was evaluated in female Hartley guinea pigs (modified Buehler method) using four study groups [vehicle control gel; 0.5% (*E/Z*)-endoxifen gel; 1.0% (*E/Z*)-endoxifen gel; and positive control (2,4-dinitrochlorobenzene; DNCB)]. DNCB induced well-defined to severe erythema in all animals. Possible evidence of dermal sensitization in endoxifen-treated groups was seen at 24 h after the first challenge (very slight to well-defined erythema in 5/10 and 4/10 animals in low and high dose groups versus very slight edema in 1/10 vehicle controls). After rechallenge, very slight edema was seen at 24 h in 4/10 vehicle control animals and in 4/10 animals in groups receiving low or high doses of (*E/Z*)-endoxifen gel. These data demonstrate that: (1) (*E/Z*)-endoxifen is not mutagenic in a bacterial (Ames test) test battery that is used widely for mutagenicity evaluations; (2) (*E/Z*)-endoxifen does not induce chromosome aberrations in a mammalian cell system that is commonly used to identify clastogenic agents; and (3) (*E/Z*)-endoxifen is not phototoxic in a standard *in vitro* assay. The possible weak sensitizing activity of (*E/Z*)-endoxifen gel appears to be caused by a component of the gel vehicle rather than by (E/Z)-endoxifen itself.

## INTRODUCTION

Endoxifen (4-hydroxy-N-desmethyltamoxifen) is a tamoxifen metabolite produced by sequential action of cytochromes P4503A4 (CYP3A4) and CYP2D6. As a result of its high affinity binding to estrogen receptor α (ERα), endoxifen demonstrates antiestrogenic activity comparable to that of the well-studied tamoxifen metabolite, 4-hydroxytamoxifen. Two stereoisomers of endoxifen, (*Z*)-endoxifen and (*E*)-endoxifen, are produced during P450-mediated metabolism of tamoxifen; (*Z*)-endoxifen is the more potent of the two isomers.

### Tamoxifen and Endoxifen in Breast Cancer Prevention and Therapy

Tamoxifen is a standard first-line therapy for women with ER+ breast cancer. Clinical data also demonstrate tamoxifen efficacy in breast cancer prevention. However, patient genotype (particularly the presence of CYP2D6 polymorphisms) is an important determinant of tamoxifen efficacy against breast cancer: clinical studies demonstrate that tamoxifen is significantly less active in women with CYP2D6 polymorphisms that reduce their ability to metabolize tamoxifen. Concomitant exposure to inhibitors of CYP2D6 (e.g., serotonin reuptake inhibitors such as paroxitene) may also reduce tamoxifen activity. On this basis, tamoxifen may be considered to be a prodrug that requires CYP-mediated biotransformation to one or more active metabolites.

After oral administration of tamoxifen, plasma levels of endoxifen in patients with functional CYP2D6 are as much as 6-fold greater than plasma levels of 4-hydroxytamoxifen. Considering the comparable antiestrogenic potencies of endoxifen and 4-hydroxytamoxifen and the higher plasma levels of endoxifen seen after oral administration of tamoxifen, endoxifen appears to be responsible for much of tamoxifen's pharmacologic activity.

To obviate reductions in tamoxifen efficacy that may result from its reduced metabolism in women with CYP2D6 polymorphisms and/or exposure to CYP2D6 inhibitors, (*E/Z*)-endoxifen, a mixture of the two geometric isomers of endoxifen, is being developed for possible use in breast cancer prevention and therapy.

### Transdermal SERMS in Breast Cancer Prevention and Therapy

Recent evidence suggests that administration of tamoxifen or other selective estrogen receptor modulators (SERMs) by direct topical application to the breast may both increase efficacy and decrease systemic toxicity. Preclinical studies demonstrate that levels of endoxifen in the mammary gland are significantly higher in rats receiving local topical administration of an endoxifen gel than in rats receiving oral exposure to tamoxifen.

In a randomized Phase II presurgical trial of women with ductal carcinoma in situ, topical administration of gel containing 4-hydroxytamoxifen to the skin of the breast demonstrated antiproliferative effects in the breast that were similar to those seen with oral administration of tamoxifen. Importantly, local topical administration of 4-hydroxytamoxifen induced less systemic toxicity than did oral administration.

## RATIONALE

This study is a component of a preclinical program to characterize the toxicity, pharmacokinetics (PK), and metabolism of a gel formulation of endoxifen designed for topical application to the skin of the breast for use in breast cancer prevention and therapy.

The results of genetic toxicology assays, phototoxicity screening assays, and *in vivo* dermal sensitization studies provide critical evidence to support "Go/No Go" decisions in the preclinical development of topical formulations of drugs being developed for cancer prevention or therapy. Clearly, positive or equivocal evidence of genetic toxicity would be considered sufficient to discontinue development of any agent for the purposes of cancer chemoprevention. Similarly, evidence of phototoxicity or dermal sensitization would be considered major negative factors in the development of an agent for topical administration when the agent is orally bioavailable and can be administered systemically.

## STUDY GOALS

1. Assess the possible genetic toxicity of (*E/Z*)-endoxifen using bacterial mutagenesis assays (Ames tests) and mammalian cell clastogenicity assays (structural chromosomal aberration assay).
2. Assess the possible phototoxicity of (*E/Z*)-endoxifen using the *in vitro* neutral red uptake assay for phototoxicity screening.
3. Assess the possible dermal sensitizing activity of (*E/Z*)-endoxifen in the modified Buehler assay in guinea pigs.

## BACTERIAL MUTAGENESIS ASSAYS

### *Test Systems and Positive Control*

Potential mutagenic activity was evaluated in bacterial reverse mutation assays (Ames tests) performed in four tester strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and one strain of *Escherichia coli* (WP2 *uvrA*).

**Table 1: Bacterial Reverse Mutation Assays (Ames Tests)**

| Strain | Mutation Site | Additional Mutations | | | Positive Controls | | Type of Mutation Detected |
|---|---|---|---|---|---|---|---|
| | | LPS | Repair | R-Factor | + S9 | - S9 | |
| TA98 | *HisD3052* | rfa | *uvrB* | pKM 101 | 2-AA, 2-AF | Daunomycin | Frameshift |
| TA100 | *HisG46* | rfa | *uvrB* | pKM 101 | 2-AA, 2-AF | MMS | Substitution |
| TA1535 | *HisG46* | rfa | *uvrB* | None | 2-AA | $NaN_3$ | Substitution |
| TA1537 | *HisC3076* | rfa | *uvrB* | None | 2-AA | ICR-191 | Frameshift |
| WP2 *uvrA* | *TrpE* | None | *uvrA* | None | 2-AA | 4-NQO | Substitution |

### *Assay Design*

Range-finding and definitive assays were performed in triplicate in all tester strains both with and without an exogenous metabolic activation system (Aroclor 1254-induced rat liver S9 mixture). The range of (*E/Z*)-endoxifen doses used in range-finding assays was 0.05 to 5.0 mg/plate. Based on solubility and cytotoxicity, doses used in definitive assays were 0.01, 0.025, 0.05, and 0.1 mg/plate. Positive control articles were included in each assay; the vehicle (negative control) was dimethylsulfoxide (DMSO).

Positive evidence of mutagenicity was defined as a dose-related increase in number of revertant colonies in any strain (± S9 mix), with minimum increase of ≥ 2-fold in strains TA98, TA100 and WP2 *uvrA* and a minimum increase of ≥ 3-fold in strains TA1535 and TA1537.

### RESULTS

- Responses to positive control articles in all tester strains met previously defined criteria for study validity and were within the range of historical controls.
- In range-finding assays, (*E/Z*)-endoxifen was found to be insoluble at doses ≥ 1 mg/plate, and was cytotoxic at dose levels ≥ 0.5 mg/plate. Based on these results, definitive assays were conducted using dose levels ≤ 0.1 mg/plate.
- No evidence of endoxifen mutagenicity was identified in either range-finding or definitive assays (± S9 mix) performed in any tester strain. In every study, the number of revertants seen in cultures treated with (*E/Z*)-endoxifen was comparable to that seen in vehicle controls.

## *IN VITRO* STRUCTURAL CHROMOSOMAL ABERRATION

### *Test System*

The potential clastogenic activity of (*E/Z*)-endoxifen was evaluated *in vitro* using the structural chromosomal aberration (SCA) assay in Chinese Hamster Ovary (CHO-WBL) cells.

### *Assay Design and Positive Control*

Assays were performed both with and without S9 mix. Solutions of (*E-Z*)-endoxifen were prepared in DMSO; concentrations tested (in media) were 0 (control), 1.25, 2.5, 5.0, 10.0, 35.0, 100, 250, and 500 µg/mL. Cyclophosphamide (CP; 10 µg/mL) was used as the positive control article for experiments including S9 mix. Mitomycin C (MMC; 0.5 µg/mL) was used as the positive control article for experiments without S9 mix.

Cells were exposed to test or control articles for 3 hr (with or without S9) or 24 hr (without S9). Colcemid was added at 22 hr, and cells were harvested for cytogenetic analysis at 24 hr. Cells were rinsed with PBS, trypsinized, and an aliquot from each culture was counted to determine viability; only cultures with > 50% viability were processed further. Cells suspensions were centrifuged, swollen with KCl, fixed, dropped onto slides, dried, and stained with 5% Giemsa solution. Scoring was performed in blinded fashion: all slides were coded prior to scoring, and were evaluated for chromosomal aberrations by an investigator who was unaware of group identities.

Using duplicate cultures, approximately 150 metaphase cells per culture were evaluated to determine the number of cells demonstrating chromosomal aberrations. Percent polyploidy [presence of >2 paired (homologous) sets of chromosomes] and endoreduplication (replication of the nuclear genome in the absence of cell division) were also determined. Gaps were recorded but were not included in calculated frequencies of cells demonstrating aberrations.

Positive evidence of clastogenicity was defined as a statistically significant increase in the number of cells in a group treated with (*E/Z*)-endoxifen that demonstrate ≥ 1 aberrations versus the number of number of cells in the vehicle control group that demonstrate ≥ 1 aberrations.

### RESULTS

- Assay validity was demonstrated by statistically significant increases in the number of cells demonstrating chromosomal aberrations in all groups exposed to a positive control article (with or without S9). The aberration frequency in cells treated with vehicle only was < 1%.
- When compared to cultures exposed to vehicle only under the same conditions for the same period of time, no statistically significant increases in the number of CHO cells demonstrating chromosomal aberrations were seen in cultures exposed to (E/Z)-endoxifen: with S9 for 3 hours, without S9 for 3 hours, or without S9 for 24 hours.
- The data demonstrate that (*E/Z*)-endoxifen is not clastogenic (either with or without metabolic activation) in a mammalian cell test system that is widely used to identify agents that induce chromosomal aberrations.

## *IN VITRO* PHOTOTOXICITY ASSAYS

### *Test System*

The potential phototoxic activity of (*E/Z*)-endoxifen was evaluated in vitro using the Neutral Red Uptake (NRU) Assay in BALB/c 3T3 mouse fibroblasts. In this assay, NRU is compared in cultures of 3T3 cells exposed to serial dilutions of test article or a positive control article versus control cells exposed to vehicle only. Parallel cultures are exposed to ultraviolet radiation (UVR) or receive no UVR exposure.

The concentration of test article or positive control article that causes a 50% reduction in NRU ($IC_{50}$) serves as a quantitative measure of cytotoxicity. The phototoxic potential of a test article is determined by comparing $IC_{50}$ values in groups treated with the test article (with and without UVR exposure) versus cells treated with vehicle only (with and without UVR exposure).

Positive evidence of phototoxic potential was defined as a Photoirritancy Factor (PIF; calculated as the ratio of $IC_{50}$ values with and without UVR exposure) of > 5 and a Mean Photo Effect (MPE; comparisons of agent + UVR versus agent – UVR across the dose range) > 0.15.

### *Assay Design and Positive Control*

- A preliminary range-finding (solubility) assay was performed to identify concentrations of (*E/Z*)-endoxifen that are appropriate for use in definitive studies
- Two simultaneous but discrete definitive phototoxicity assays were performed to evaluate the possible phototoxicity of (*E/Z*)-endoxifen. Endoxifen concentrations used in these studies were: 0.100, 0.178, 0.316, 0.562, 1.00, 1.78, 3.16, and 5.62 µg/mL. The positive control article, promethazine, was used at a concentration range from 0.100 to 178 µg/mL.
- In the two definitive assays, selected groups of cells were also exposed to UVA (5 J/cm$^2$) + UVB (22 mJ/cm$^2$) from a xenon arc solar simulator equipped with a Schott WG 320 filter.

**Table 2: Results of Definitive *in vitro* Phototoxicity Bioassay**

| Test Article | $IC_{50}$ (µg/mL) –UVR | $IC_{50}$ (µg/mL) +UVR | PIF[a] | MPE[b] | Phototoxic Potential? | UVR Survival (%) | Average $OD_{540}$ |
|---|---|---|---|---|---|---|---|
| Promethazine | 127.471 | 1.730 | 73.700 | 0.509 | YES | 82 | 0.985 |
| (E/Z)- Endoxifen (Assay 1) | 4.628 | 2.814 | 1.646 | 0.028 | NO | 78 | 0.943 |
| (E/Z)- Endoxifen (Assay 2) | 4.384 | 3.424 | 1.282 | 0.032 | NO | 87 | 0.982 |

[a] criterion for a positive response is PIF > 5
[b] criterion for a positive response is MPE > 0.15

### RESULTS

The validity of the phototoxicity assay was demonstrated by: a) mean percent UVR survival was comparable to the OECD minimum criterion for assay acceptance, b) mean percent $OD_{540}$ exceeded the OECD minimum criterion for assay acceptance, c) the positive control article, promethazine, demonstrated a clear photoxic response, and c) responses in two independent studies with (E/Z)-endoxifen were highly reproducible.

## DERMAL SENSITIZATION ASSAY

### *Animal Welfare*

Prior to the initiation of experimentation, the dermal sensitization protocol was reviewed and approved by the IIT Research Institute Animal Care and Use Committee. Other studies were performed using *in vitro* models and did not require IACUC approval. The dermal sensitization study was performed in full compliance with NIH Guidelines for the Care and Use of Laboratory Animals.

### *Assay Design*

Negative control and treatment groups (Table 1, Groups 1-3) consisted of 10 female Hartley guinea pigs. The positive control group included 6 female Hartley guinea pigs.

**Table 3: Study Design for Dermal Sensitization Study of (*E/Z*)-Endoxifen Gel in Guinea Pigs**

| Group No. | Group Identifier | Test or Control Article | No. of Animals |
|---|---|---|---|
| 1 | Vehicle Control | Vehicle Gel | 10 |
| 2 | Low Dose | 0.5% *E/Z*-Endoxifen Gel | 10 |
| 3 | High Dose | 1.0% *E/Z*-Endoxifen Gel | 10 |
| 4 | Positive Control | 0.05% dinitrochlorobenzene | 6 |

**Induction Phase:** Animals received topical doses of test or control article weekly for 3 weeks.

**Challenge Phase:** Single dose administered topically two weeks after the final induction dose

**Rechallenge Phase:** Single dose one week after challenge dose (Groups 1 to 3 only).

**Endpoints:** Erythema and edema scoring (Draize criteria).

### *Experimental Animals*

Female Hartley guinea pigs (approximately 6 weeks at arrival), from Charles River Laboratories, Saint Constant, Quebec. Guinea pigs were quarantined for 2 weeks prior to study start.

### *Animal Husbandry*

**Housing:** During quarantine, pair-housed in polycarbonate "shoebox" cages with hardwood bedding. Housed individually during the experimental period.

**Basal Diet:** Certified Guinea Pig Diet #2040C (Harlan/Teklad, Madison, WI)

**Drinking Water:** City of Chicago, supplied by automatic watering system

**Animal Room Light Cycle:** 12 hours light/12 hours dark per day

### *Test and Control Articles*

Vehicle gel (0% endoxifen) and (*E/Z*)-endoxifen gels containing 0.5% (low dose) or 1.0% (high dose) (*E/Z*)-endoxifen were supplied by the National Cancer Institute. DNCB was purchased from Sigma-Aldrich.

### *Test and Control Article Storage and Administration*

Test and control articles were stored at controlled room temperature. Gel formulations were administered topically at a volume

### RESULTS

- Clear evidence of dermal sensitization was seen in the positive control group, demonstrating the validity of the study.
  - At 24 hours after the challenge dose of DNCB, 4/6 animals demonstrated erythema scores of 3 (moderate to severe); 2/6 animals had erythema scores of 2 (well-defined).
  - At 48 hours after the challenge dose of DNCB, 5/6 animals demonstrated erythema scores of 2.
- Possible evidence of dermal sensitization was seen both in animals exposed to the test article and in animals exposed to the negative control article.
  - After the first challenge dose, 1/10 animals in the negative control group had an erythema score of 1 at 24 hours; 5/10 animals in the 0.5% group and 4/10 animals in the 1% group demonstrated erythema scores of 1 or 2 at 24 hours, and 2/10 and 1/10 animals in these groups demonstrated erythema scores of 1 or 2. None of these positive responses was statistically significant.
  - After rechallenge at 24 hours, erythema scores of 1 were seen in 4/10 animals each in the vehicle, 0.5%, and 1.0% groups; erythema scores of 2 were seen in 1/10 animals each in the vehicle and 0.5% groups. At 48 hours, erythema scores of 1 were seen in 1/10 animals in the vehicle and 0.5% groups. Erythema scores of 2 were seen in 2/10 animals in the 0.5% group. None of these differences from control were statistically significant.

## CONCLUSIONS

1. (*E/Z*)-endoxifen demonstrated no evidence of genotoxic activity when evaluated in bacterial mutagenesis assays (Ames tests) or when evaluated in structural chromosome aberration assays in mammalian cells. The results of these genetic toxicology studies strongly suggest that (*E/Z*)-endoxifen is not genotoxic.
2. (*E/Z*)-endoxifen demonstrated no evidence of phototoxic activity in a widely used *in vitro* screening system in mammalian cells. The results of these studies strongly suggest that endoxifen is not phototoxic.
3. The results of dermal sensitization studies in guinea pigs demonstrated mild sensitizing or irritant activity of endoxifen gel. However, mild sensitization or irritation was also seen in animals treated with gel vehicle only. These data suggest that the observed sensitization or irritation is likely the result of the activity of the gel vehicle or one of its components, rather than endoxifen itself.
4. The results of four toxicity screening assays failed to provide a "No Go" signal for further preclinical development of (*E/Z*)-endoxifen.

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