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Interlaboratory Validation Study of the Advanced Tissue Sciences' Skin^{2TM} Dermal Model And MTT Cytotoxicity Assay Kits

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Summary

Two factors of critical importance to the adoption of *in vitro* alternative assays are the predictive potential of the assay system and its intra- and interlaboratory reproducibility. Many *in vitro* alternatives to animal testing have been proposed, but only a few of them have been subjected to interlaboratory validation studies in order to assess their reproducibility and relevance to *in vivo* data. The study described in this paper was designed to assess the shippability, reproducibility and relevance to *in vivo* data of the Advanced Tissue Sciences skin^{2TM} Dermal Model substrate in concert with Advanced Tissue Sciences' MTT Cytotoxicity Assay Kits. This 3-dimensional Dermal Model, which consists of several layers of human, foreskin-derived, metabolically active fibroblasts grown to form a tissue equivalent on nylon mesh, was used as the substrate to test the toxicity of 12 chemicals proposed by the Commission of the European Communities (*i.e.*, toluene, n-hexane, 1-butanol, chloroform, sodium dodecyl sulfate, benzalkonium chloride, silver nitrate, tributyltin chloride, dibutyltin dichloride, 2-butoxyethyl acetate, acetaldehyde, and 2-methoxyethanol). Dermal Model kits and MTT Assay Kits were manufactured by Advanced tissue sciences (formerly Marrow-Tech) in the U.S. and shipped to Janssen Pharmaceutica in Belgium. Two runs of each of the twelve chemicals were performed in each lab one week apart and MTT-50 values were determined for each compound. The *in vitro* data were also compared with existing *in vivo* data and found to be highly correlative. Interlaboratory reproducibility was also excellent, demonstrating that the skin^2 human Dermal Model product remains stable during intercontinental shipment and that the MTT Assay Kits, also supplied by Advanced Tissue Sciences, provide a standardized method for assessing the toxicity of test agents.

Introduction

Several *in vitro* alternatives to animal testing are currently being studied worldwide in order to assess the toxicity of a number of chemical compounds, raw materials and formulated products. Few of these assay systems have been subjected to extensive validation studies in order to assess their intra- and interlaboratory reproducibility and relevance to existing *in vivo* data. A three-dimensional human skin model, developed by Advanced Tissue Sciences in the U.S., consists of several layers of actively dividing, metabolically active, neonatal, foreskin derived fibroblasts grown on nylon mesh. Fibroblasts growing within this three-dimensional framework secrete a number of known growth factors and extracellular matrix proteins (1). This substrate has been recently utilized to study the *in vitro* toxicity of a number of compounds including detergents, shampoos, cosmetics, alcohols, metals, antimicrobial preservatives and an ionophore using the Neutral Red (lysosomal-based) viability assay (2,3,4,5), the MTT (mitochondrial function) assay (3,4,5,6), prostaglandin E_2 (PGE_2) re-

lease (inflammatory mediator) assay (3,5), the lactate dehydrogenase (LDH) release (membrane integrity) assay (3,5) and [^{3}H]-thymidine incorporation (7) (a measure of test agent-induced inhibition of DNA synthesis). *In vivo: in vitro* comparative toxicity data gathered to date have proven very encouraging.

In the current study, skin^{2TM} Dermal Model substrate kits, together with standardized MTT Cytotoxicity Assay Kits were shipped to Janssen Pharmaceutica in Belgium by Advanced Tissue Sciences. Twelve chemicals, proposed by the Commission of the European Communities [CEC] for use in several *in vivo* and *in vitro* studies, were also shipped by Advanced Tissue Sciences to Janssen. Each laboratory (Advanced Tissue Sciences and Janssen) determined the MTT-50 values (i.e., the concentration of test agent which reduced the turnover of water-soluble, yellow MTT to a blue water-insoluble formazan precipitate to 50% of untreated control or vehicle control levels) for each test agent in two repeat assays. Rank-ordered comparisons of toxicity were prepared for each labortorys data and *in vivo: in vitro* correlations were assessed.

Materials and Methods

Test agents and growth media components: The following items were purchased from the indicated sources: Dulbecco's Modified Eagle's Medium [DMEM], phosphate buffered saline [PBS], L-glutamine, sodium pyruvate, nonessential amino acids and antibiotic/antimycotic from Gibco (Grand Island, NY, USA), fetal bovine serum [FBS] from Hyclone (Logan, UT, USA), MTT, isopropanol, n-hexane, 1-butanol, sodium dodecyl sulfate [SDS], benzalkonium chloride, silver nitrate and 2-methoxyethanol from Sigma Chemical Co. (St Louis, MO, USA), 2-butoxyethyl-acetate, dibutyltin dichloride and tributyltin chloride from Aldrich (Milwaukee, WI, USA) and chloroform, toluene and acetaldehyde from Fisher (Pittsburgh, PA, USA). All test chemicals were aliquotted at Advanced Tissue Sciences in La Jolla, CA and were mailed to Janssen in Belgium. Chloroform and n-hexane evaporated during shipment, so Janssen used n-hexane from Janssen Chimica (Beerse, Belgium) and chloroform from Merck (Darmstadt, Germany). In addition, for run #2, Janssen used 2-methoxyethanol and acetaldehyde from Janssen Chimica.

Sample proparation: All test chemicals were weighed before dilution in culture medium for use in the MTT assay. Two range-finding experiments were initially performed at Advanced Tissue Sciences in order to define a narrower range of test agent concentrations for a more accurate assessment of the MTT-50 endpoints. The chemical concentrations which were tested are listed in Table 1. Some chemicals, by virture of their insolubility in Assay Medium, required an initial dilution in the solvent DMSO, prior to subsequent dilution in Assay Medium. For these chemicals, DMSO was also added to the vehicle control.

Substrate preparation: Normal human fibroblasts, isolated from the dermis of freshly obtained neonatal foreskins, were propagated in DMEM containing 10% FBS, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin G sodium 100 μ/ml streptomycin sulfate and 0.25 μg/ml amphotericin B [DMEM complete], then seeded onto 8×8 cm nylon mesh pretreated with acetic acid-fetal bovine serum. When the cells became confluent and formed a multi-layered dermis tissue equivalent, the sheets of nylon mesh were cut with a laser (Texcel, Westfield, MA, USA) into 11×11 mm squares. These 11×11 mm squares (skin^{2TM} Dermal Model ZK1100 from Advanced Tissue Sciences) were used as the test substrate in the interlaboratory toxicity studies described in this paper.

MTT assay procedure: This procedure has been detailed elsewhere (3, 4). Briefly, 11×11 mm mesh squares containing fibroblasts were placed into 24-well plates and treated with 2 ml of five concentrations of test agents (two

replicates per concentration) diluted in DMEM complete containing 2% fetal bovine serum [Assay medium] for 20 hours at 37°C in 5% CO_2 (≥90% humidity). Two untreated control mesh squares were incubated with the appropriate vehicle control. After overnight incubation with test agents, the spent media were aspirated and replaced with 1 ml (per well) of Assay Medium containing 500 μg/ml of MTT. The cultures were incubated for 2 hours, then washed twice with 1 ml PBS. Blue formazan precipitate was extracted from the mitochondria using 2 ml isopropanol on a shaker platform at room temperature for 1 hour. Aliquots (200 μl) of the extracted MTT solutions were transferred to 96-well plates and the optical density at 540 nm (OD_{540}) was determined using a microplate reader zeroing to pretreated nylon mesh (without cells) which had been similarly treated with MTT. The mean OD_{540} of the duplicate vehicle control wells was set to represent 100% viability. Results for each concentration were plotted, as percentage of vehicle control, against the concentration of test agent (mM) on a log scale, and an MTT-50 value was determined directly from the graph for each chemical.

Results and Discussion

The intralaboratory reproducibility of the Dermal Model substrate was initially assessed using six titrations of SDS on six different lots of Dermal Model substrate. Figure 1 shows the intralaboratory reproducibility of the substrate, with error bars representing one standard deviation around the mean percent of untreated control at each concentration of SDS. The small error bars demonstrated the excellent intralaboratory reproducibility of the model.

The panel of twelve chemicals was tested by both laboratories following a standard protocol and using identical lots of both the Dermal Model substrate kits and of the MTT Cytotoxicity Assay Kits supplied by Advanced Tissue Sciences. Each lab was provided with a

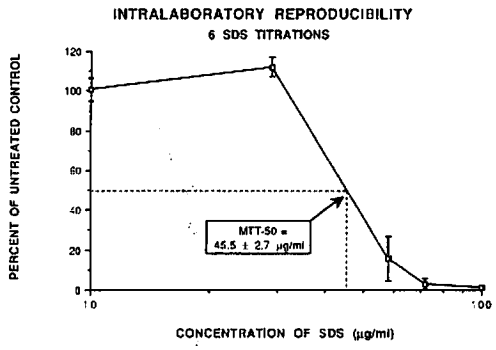

Fig. 1. Six different lots of Dermal Model were used on six different weeks. SDS was diluted in Assay Medium and assayed in quadruplicate at 0, 10, 29, 58, 72 and 101 μg/ml. Error bars represent one standard deviation around the mean percent of untreated control for each concentration. The mean MTT-50 value for the six runs was 45.5 +/-2.7 μg/ml (5.9 % C.V.).

recommended dilution range for determination of chemical-induced cytotoxicity (see Table 1 for dosing ranges and the use of appropriate vehicle controls). Both laboratories then determined the MTT-50 values for each chemical in millimolar (mM) units. The toxic effect of the test chemicals was assessed and ranked according to potency. Table 2 shows the MTT-50 values and relative toxicity for each of the twelve compounds in each laboratory. Figure 2 depicts a log: log linear regression scatterplot of the mean MTT-50 values obtained by each laboratory for nine of the twelve test chemicals in the two runs; the correlation coefficient (r^2) was found to be excellent (*i.e.*, 97%) with the MTT-50 endpoints spanning a concentration range of 6 log units.

The *in vitro* data were compared with existing *in vivo* rabbit skin irritation data generated by the Istituta di Ricerche Biomediche "Antoine Marxer" in Turin, Italy in 1987 and published by the Commission of the European Community (8). In this study, the skin irritation potential of these 12 chemicals was assessed using New Zealand white albino rabbits and an EC protocol which utilizes a Finn-chamber containing a nonadherent patch soaked in 0.5 ml of liquid test substance (diluted or undiluted) applied to one side of

Table 1. Chemical test concentrations

CHEMICAL	CONCENTRATIONS (μg/ml)
aqueous-soluble:	
SDS	10, 30, 50, 70, 100
benzalkonium chloride	1, 3, 5, 7, 10
silver nitrate	3, 10, 30, 50, 100
2-methoxyethanol	10000, 20000, 30000, 50000, 100000
acetaldehyde	10, 100, 500, 1000, 5000
aqueous-insoluble:	
n-hexane^{+}	500, 1000, 2000, 3000, 5000
chloroform^{+} (Run #1)	500, 1000, 2000, 3000, 5000
chloroform^{+} (Run #2)	1000, 5000, 7500, 10000, 20000
toluene^{+} (Run #1)	500, 1000, 2000, 3000, 5000
toluene^{+} (Run #2)	1000, 5000, 7500, 10000, 20000
1-butanol^{o}	1000, 5000, 7500, 10000, 15000
2-butoxyethyl acetate^{o}	1000, 3000, 5000, 7500, 10000
dibutyltin dichloride^{x}	1, 3, 10, 30, 100
tributyltin chloride^{x}	0.1, 0.5, 1, 5, 10

Aqueous-soluble chemicals were diluted to the designated concentrations directly in Assay Medium. Aqueous-insoluble materials were presolubilized in DMSO prior to subsequent dilution in Assay Medium to the designated concentrations. The vehicle controls for these chemicals received DMSO diluted in Assay Medium to a final concentration equal to that present in the highest concentration of chemical tested (i.e., $\times$=0.04%; +=2%; o=4% DMSO). The concentrations listed above were decided upon after two initial "range-finding experiments performed at Advanced Tissue Sciences.

the shaved skin surface on the dorso-lumbar area for 4 hours. The rabbits were examined for signs of erythema, eschar formation and edema after 1, 24, 48, 72 hours, 7 and 14 days. Using the EC guidelines, acetaldehyde, dibutyltin dichloride, tributyltin chloride, benzalkonium chloride and chloroform were classified as corrosive (*i.e.*, the structure of the rabbit skin at the site of contact was destroyed or changed irreversibly). Toluene and SDS were classified as irritating (*i.e.*, reversible inflammatory skin changes are produced at the site of contact); and 1-butanol, 2-methoxyethanol, 2-butoxyethylacetate and n-hexane were "nonirritating". (*Note*: The *in vivo* results using silver nitrate were inconclusive, but nonetheless showed a high degree of injury). Using the Dermal Model/MTT *in vitro* assay system, we also found acetaldehyde, dibutyltin dichloride, tributyltin chloride, benzalkonium chloride and SDS to be significantly more toxic than n-hexane, 1-butanol, 2-methoxyethanol and 2-butoxyethylacetate (Table 3). We disagree on the classification of toluene (irritating *in vivo*) and chloroform (corrosive *in vivo*), both of which we find to be relatively nontoxic. Five possible explanations of the discrepancies are: (1) The injury seen *in vivo* may be primarily due to an inflammatory-mediated response rather than to direct chemical-induced cytotoxicity, (2)

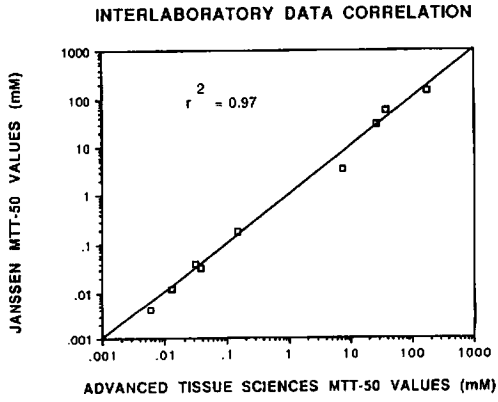

Fig. 2. The mean MTT-50 values of the two runs of nine of the 12 chemicals in each laboratory are plotted against each other in a linear regression over a range of 6 log units. (Results for n-hexane, 1-butanol and 2-methoxyethanol are not plotted, since their mean MTT-50 values were greater than the highest concentration in one or both laboratories; see Table 2). Excellent reproducibility was observed between the two labs.

Table 2. Interlaboratory toxicity data and ranking

CHEMICAL	ADVANCED TISSUE SCIENCES *RUN #1 MTT-50*	*RUN #2 MTT-50*	*MEAN MTT-50*
toluene	>54.3(10)	180(10)	180(10)
1-butanol	181(11)	181(11)	181(11)
2-methoxyethanol	>1310(12)	>(12)	>1310(12)
acetaldehyde	0.968(6)	14.0(6)	7.50(6)
SDS	0.144(5)	0.162(5)	0.153(5)
2-butoxyethylacetate	24.1(8)	28.5(7)	26.3(7)
dibutyltin dichloride	0.036(4)	0.040(4)	0.038(4)
tributyltin chloride	0.005(1)	0.006(1)	0.006(1)
benzalkonium chloride	0.011(2)	0.015(2)	0.013(2)
silver nitrate	0.031(3)	0.031(3)	0.031(3)
n-hexane	19.5(7)	46.4(9)	33.0(8)
chloroform	30.7(9)	43.3(8)	37.0(9)
CHEMICAL	JANSSEN *RUN #1 MTT-50*	*RUN #2 MTT-50*	*MEAN MTT-50*
toluene	>54.3(10)	142(10)	142(10)
1-butanol	>202(11)	>202(11)	>202(11)
2-methoxyethanol	1070(12)	>1310(12)	>1190(12)
acetaldehyde	1.80(6)	5.08(6)	3.44(6)
SDS	0.181(5)	0.195(5)	0.188(5)
2-butoxyethylacetate	32.8(8)	25.8(7)	29.3(7)
dibutyltin dichloride	0.020(3)	0.045(4)	0.033(3)
tributyltin chloride	0.003(1)	0.004(1)	0.004(1)
benzalkonium chloride	0.011(2)	0.012(2)	0.012(2)
silver nitrate	0.040(4)	0.036(3)	0.038(4)
n-hexane	7.94(7)	>58.0(9)	>33.0(8 or 9)
chloroform	>41.9(9)	55.0(8)	55.0(8 or 9)

MTT-50 values are expressed in millimolar units. Any MTT-50 value listed as "greater than x" implies that a 50% cell killing was not achieved at the highest concentration tested (x). Numbers in parentheses after the MTT-50 values represent the relative toxicity ranking for each of the twelve chemicals by each of the laboratories. A ranking of (1) is the most toxic; (12) is the least toxic.

Table 3. In vitro: in vivo dermal irritation correlation

TEST AGENT	ADV. TISS. SCI. MEAN MTT-50 (mM)	IN VIVO SKIN IRRITATION POTENTIAL[a]
tributyltin chloride	0.006(1)	corrosive
benzalkonium chloride	0.013(2)	corrosive
silver nitrate	0.031(3)	inconclusive (high injury)
dibutyltin dichloride	0.038(4)	corrosive
sodium dodecyl sulfate	0.153(5)	irritating
acetaldehyde	7.50(6)	corrosive
2-butoxyethylacetate	26.3(7)	nonirritating
n-hexane	33.0(8)	nonirritating
chloroform	37.0(9)	corrosive
toluene	180(10)	irritating
1-butanol	181(11)	nonirritating
2-methoxyethanol	>1310(12)	nonirritating

a=reference 8 used as source of *in vivo* rabbit skin irritation data (4 hour exposure; 0.5 ml soaked nonadherent patch; Finn chamber; shaved skin site).

the toxicity of these two compounds (if pH -dependent) may be buffered out by the tissue culture medium in which they have been diluted, (3) the DMSO solvent used to predilute these two chemicals may have somehow altered their toxicity-inducing potential (perhaps dilution in another solvent may be warranted), (4) the rabbit skin may simply be far more sensitive than the human tissue substrates and (5) the chemicals may have prematurely evaporated from the culture medium. Additionally, chloroform was classified as corrosive at 100% *in vivo* in the EC report, but was nonirritating at 25% (a big difference in a narrow dose range). Further studies, using different endpoints such as Prostaglandin E_2 release from the fibroblasts in the Dermal Model, may shed light on the mechanism of toxicity of these discordant materials.

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