EUSAAT

European Society for Alternatives to Animal Testing

EUSAAT2013 - Linz (Austria)
15-18 October 2012

NIHS National Institute of Health Sciences

JaCVAM

The JaCVAM validation study of the ROS in vitro photoxicity assay

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Berlin.de
Der Landestierschutzbeaufragte

Topics

I. In vitro photo-cytotoxicity testing in the 3T3 NRU PT (OECD TG 432)

II. Limitations of the 3T3 NRU PT??

III. The ROS chemical phototoxicity assay

IV. The JaCVAM ROS assay validation study of pharmaceuticals (ICH guideline S10)

Phototoxicity (photoirritation)

- toxic response, elicited after the first exposure of skin to certain chemicals and subsequent exposure to light
- toxic response, induced similarly by skin irradiation after systemic administration of a chemical substance

EXAMPLES:

in humans ➔ quinolones, NSAIDs, chlorpromazine, tiaprofenic acid (60%), perfume mix

in cattle ➔ hypericism after feeding on St. John's worth *(Johanniskraut)*

Definition of ACUTE PHOTOTOXICITY

(Acute Photoirritation)

has to be distinguished from:

- **Photoallergy**
- **Photomutagenicity**
- **Photocarcinogenicity**

Basic mechanism:

UV + visible light (300 - 750 nm)

+

chemicals, absorbing light (300 - 750 nm)

↓

activated chemical

Dose of light: irradiance (J/cm^2)

WAVEBANDS **ENERGY**

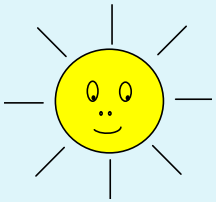
visible light➔ > 400 nm

UVA ➔ 320 - 400 nm ↑

UVB ➔ 280 - 320 nm ↑↑

UVC ➔ < 280 nm ↑↑↑

SUNBURN ➔ UVB

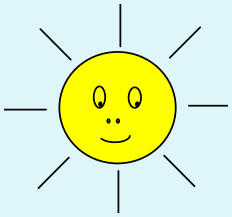


PHOTOTOXICITY TESTING ➔ UVA

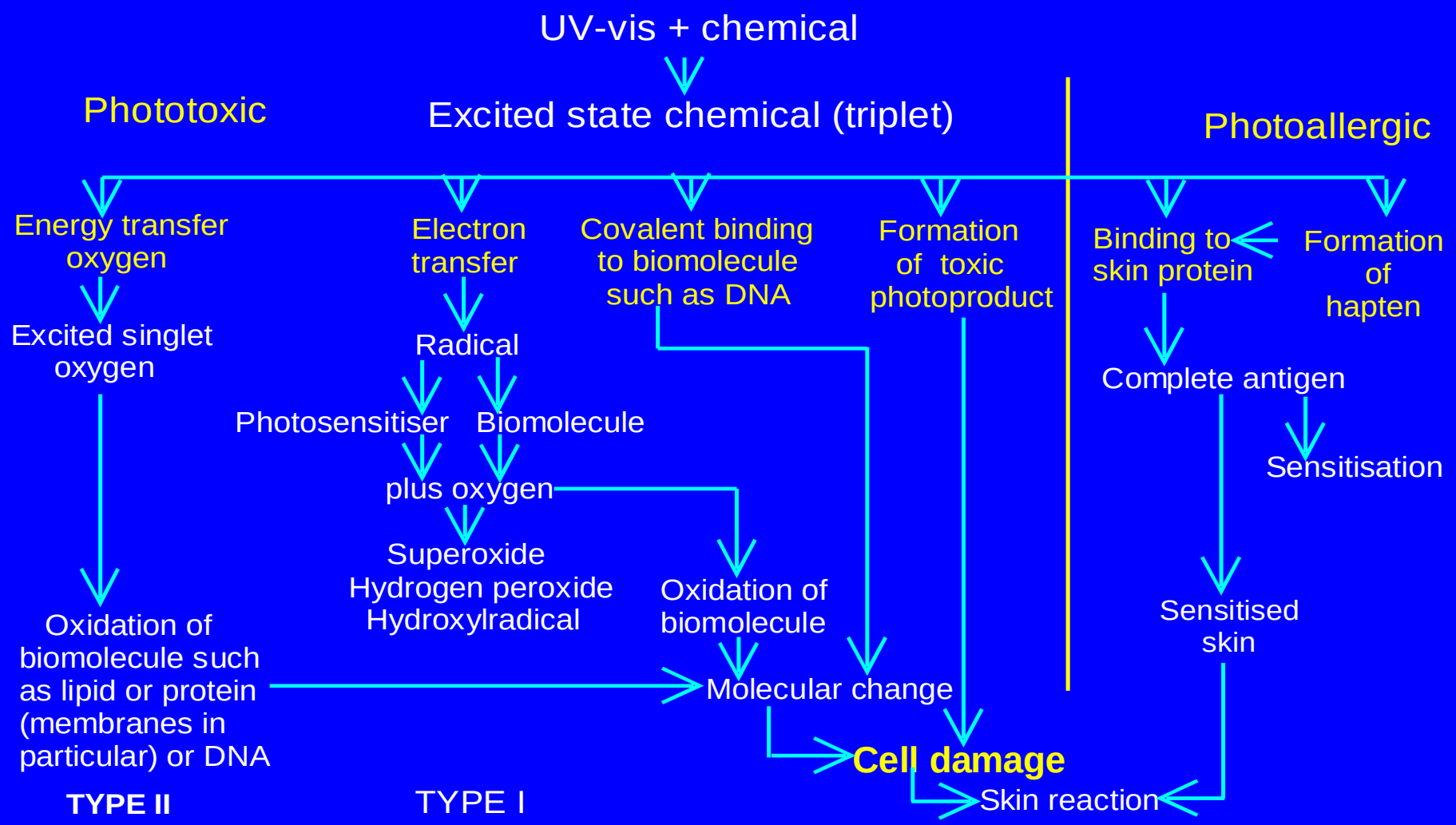
Light sources

emission spectra **UVA**
UVB

➔ solar spectrum simulator

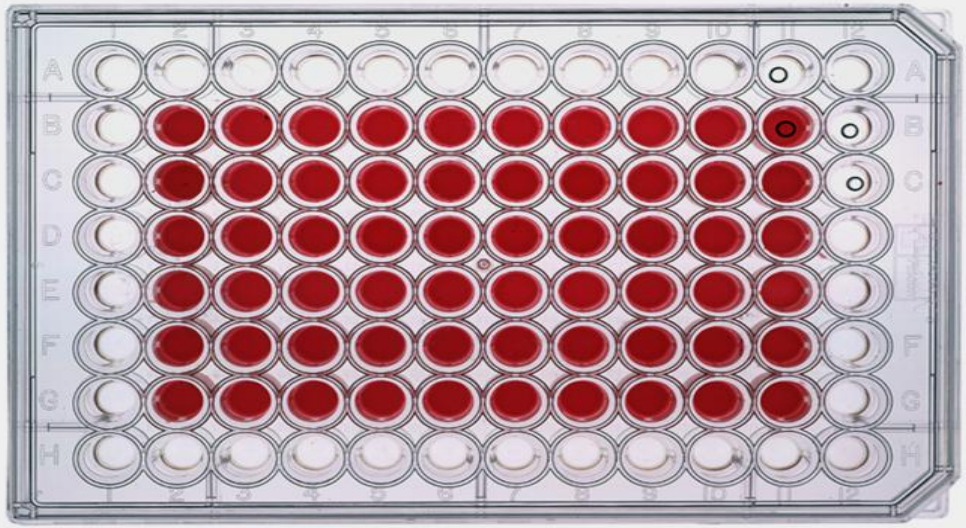


MECHANISMS OF PHOTOTOXICITY

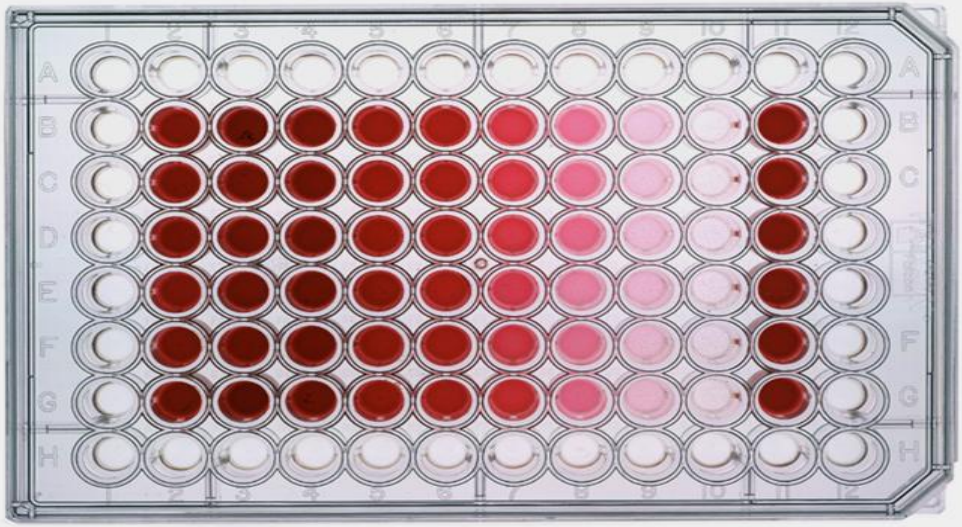


In vitro photo-cytoxicity testing

EXAMPLE: 3T3 Neutral Red Uptake Test Ketoprofen (phototoxic but no toxicity in the dark)



Ketoprofene -UV



Ketoprofene +UV

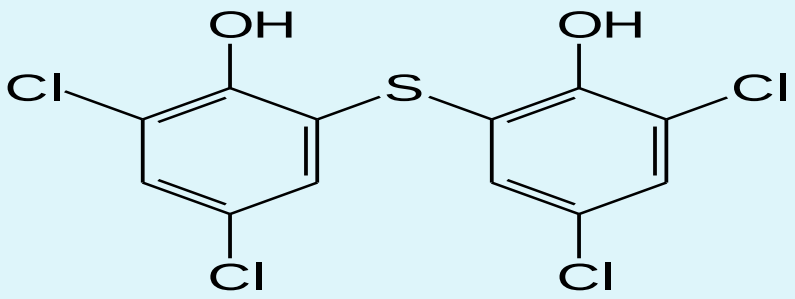
2 - 10 =
0 - 500 µg/ml

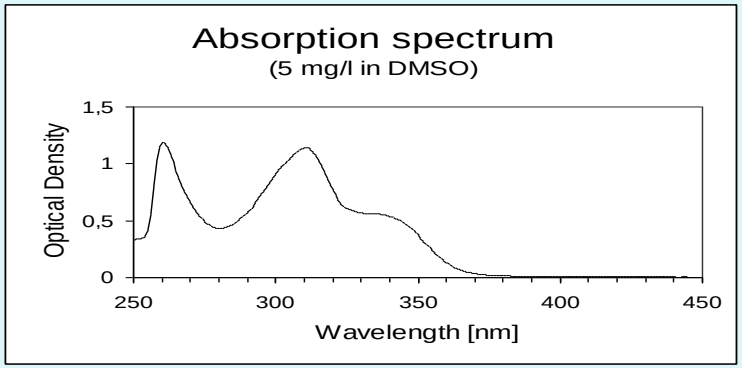
1 + 11 =
neg. contr.

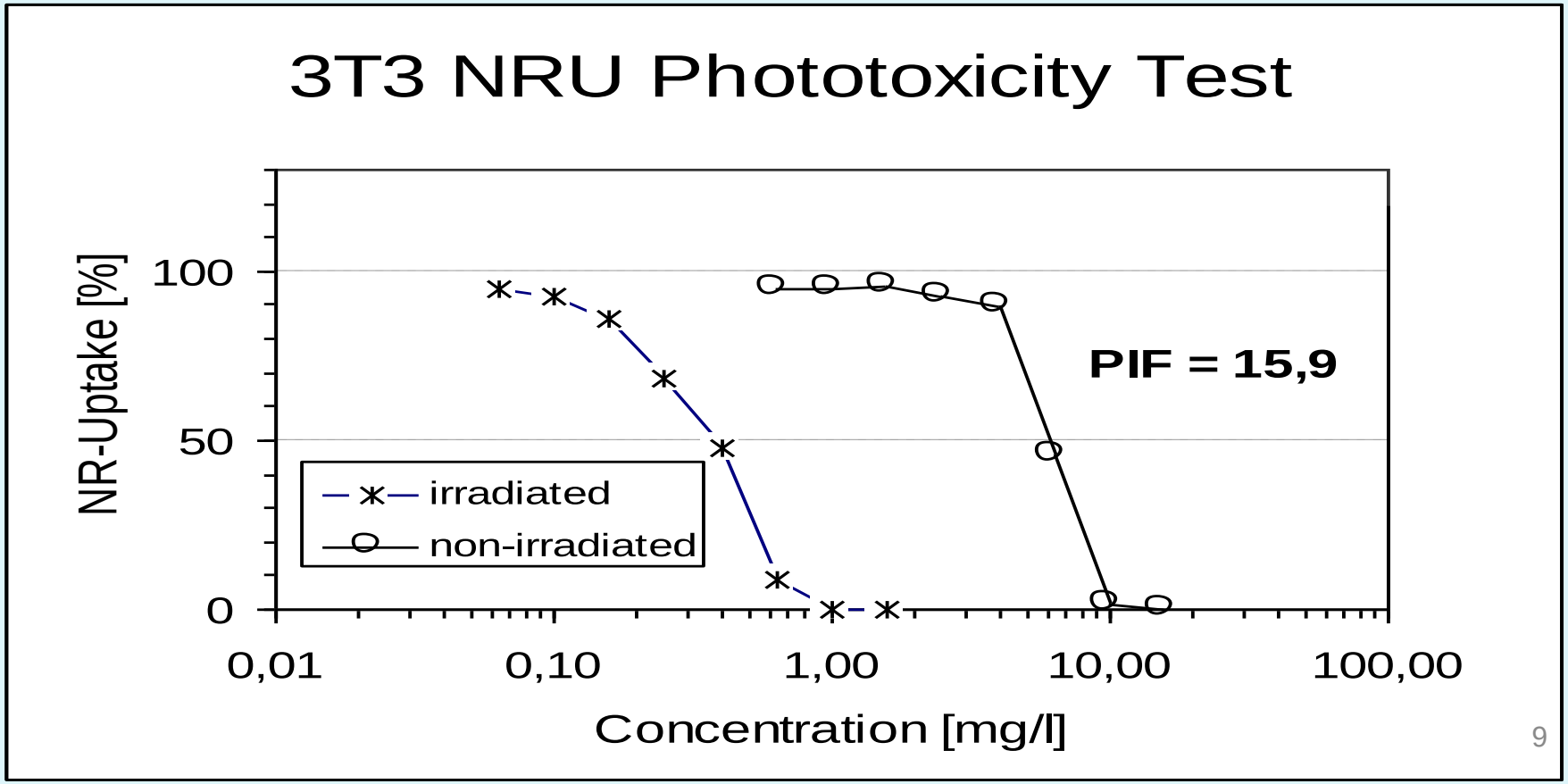
2 - 10 =
0 - 10 µg/ml

1 + 11 =
neg. contr.

EXAMPLE: BITHIONOL, phototoxic







VALIDATION of 3T3NRU in vitro phototoxicity test

SPONSORS

EU DG XI, ECVAM, ZEBET-BgVV, COLIPA

DESIGN of 3T3NRU-PT

3T3 monolayer ➔ 24h chemical ➔ 1h +UVA/-UVA ➔ 24h viability (NR Uptake)

Prediction model:
comparison of "dose-responses" obtained +UVA and -UVA exposure

CHEMICALS TESTED

(I)	**Prevalidation Study**	**11 PT**	**9 NPT**	**not blind**
(II)	**Validation Study**	**25 PT**	**5 NPT**	**blind trial**
(III)	**UV filter Study**	**10 PT**	**10 NPT**	**blind trial**

RESULTS

(II) VS	**sensitivity:**	**84%**	**specificity:**	**93%**
(III) UV-F	**sensitivity:**	**100%**	**specificity:**	**98%**

EU ➔ In the year 2000 for the first time in vitro toxicity test accepted by the EU into Annex V of Directive 67/548/EEC on the Classification, Packaging and Labelling of Dangerous Substances.

OECD ➔ 2004 worldwide acceptance of the 3T3NRU PT test

NIH JAPAN, TOKYO, December 2002 information on 3T3 NRU PT

OECD/OCDE **432**

Adopted :
13 April 2004

OECD GUIDELINE FOR TESTING OF CHEMICALS

***In Vitro* 3T3 NRU phototoxicity test**

1. Negative results obtained in the 3T3 NRU PT:

A negative result in the 3T3 tests, using a test compound at concentrations up to 1000µg/ml, provides sufficient evidence for the absence of adverse photo-biological effects of a test chemical.

Clinical testing in patients has been recommended by EU and US regulatory agencies (EMEA 2002, US FDA 2003),
If additional data on photo-genotoxic/photo-mutagenic potential and on photo-allergic potential are taken into account.

2. Positive results obtained in the 3T3 NRU PT:

2.1 safety assessment for cosmetic ingredients

If a positive result is obtained with a chemical to be used as ingredient in cosmetic formulations, additional photo-toxicity testing in a 3-D human skin model should be conducted to determine, if the chemical will penetrate into the skin.

2.2 safety assessment in drug development I

If a positive result is obtained with a new chemical in drug development, it has to be kept in mind that many classes of chemicals that are used in drugs have phototoxic potential and provide a positive result in the 3T3 NRU PT, e.g. NSAID, tetracyclins and quinolones.

Compounds tested in the 3T3 NRU PT test

43 compounds tested *in vitro*

30 compounds

NEGATIVE

(70 %)

13 compounds

POSITIVE

6 compounds tested *in vivo*

NEGATIVE

False positive results??

2.2 safety assessment in drug development II

In 2007 scientists from the European drug industry (EFPIA) reported that the 3T3 NRU PT test is "too sensitive", since it provides too many positive results, which may be "false positive" results.
Therefore it was recommended to conduct in vivo studies in experimental animals for conformation.

However, it was apparent in the discussion that in many instances determinations were not conducted according to the proper protocol of the 3T3 NRU PT test, which is available on the website of ECVAM as "INVITTOX protocol No. 78" (ECVAM 2008), which includes a list of reference chemicals.

Therefore, it is most important that the sensitive in vitro 3T3 NRU PT is established properly and provides correct positive and negative Results with established reference chemicals before new chemicals are being tested.

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journal homepage: www.elsevier.com/locate/yrtph

Workshop Report

The 3T3 neutral red uptake phototoxicity test: Practical experience and implications for phototoxicity testing – The report of an ECVAM–EFPIA workshop

Mara Ceridono [a], Pär Tellner [b,*], Daniel Bauer [c], João Barroso [a], Nathalie Alépée [d], Raffaella Corvi [a], Ann De Smedt [e], Mick D. Fellows [f], Neil K. Gibbs [g], Eckhard Heisler [h], Abigail Jacobs [i], Dagmar Jirova [j], David Jones [k], Helena Kandárová [l], Peter Kasper [m], Jacqueline Kinyamu Akunda [n], Cyrille Krul [o], Douglas Learn [p], Manfred Liebsch [q], Anthony M. Lynch [r], Wolfgang Muster [s], Kazuichi Nakamura [t], J. Frank Nash [u], Uwe Pfannenbecker [v], Gareth Phillips [w], Catherine Robles [x], Vera Rogiers [y], Femke Van De Water [z], Ulla Wändel Liminga [aa], Hans-Werner Vohr [ab], Olivier Wattrelos [ac], Julie Woods [ad], Valérie Zuang [a], Joachim Kreysa [a], Phil Wilcox [r]

It was concluded that the 3T3 NRU-PT identifies phototoxicological hazards with a 100% sensitivity, and thus is accepted as the tier one test that correctly identifies the absence of phototoxic potential. Consequently, positive results in the 3T3 NRU-PT often do not translate into a clinical phototoxicity risk. Possible ways to improve the practical use of this assay include: (i) adaptation of changed UV/vis-absorption criteria as a means to reduce the number of materials tested, (ii) reduction of the highest concentration to be tested, and (iii) consideration of modifying the threshold criteria for the prediction of a positive call in the test.

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

December 2012
EMA/CHMP/ICH/752211/2012
Committee for medicinal products for human use (CHMP)

ICH guideline S10
Guidance on photosafety evaluation of pharmaceuticals
Step 3

Transmission to CHMP	December 2012
Adoption by CHMP for release for consultation	December 2012
End of consultation (deadline for comments)	March 2013

3.2. Photoreactivity testing using chemical assays

If a drug developer chooses to assess photoreactivity, the assay should be qualified using pharmaceutical agents under appropriate conditions to demonstrate assay sensitivity. One such assay that is subject of a validation exercise is a ROS assay (e.g., Ref. 5). Preliminary data suggest that this assay has high sensitivity for predicting *in vivo* phototoxicants. However, it has a low specificity, generating a high percentage of false positive results. A negative result in this assay, conducted under the appropriate conditions for the particular assay, would indicate a very low probability of phototoxicity, whereas a positive result would only be a flag for follow-up assessment.

3.3. Phototoxicity testing using in vitro assays

The most widely used *in vitro* assay for phototoxicity is the "*in vitro* 3T3 Neutral Red Uptake Phototoxicity Test" (3T3 NRU-PT) for which a guideline (Ref. 6) is available. This is currently considered the most appropriate *in vitro* screen for soluble compounds that are not exclusively UVB absorbers.

Although the formal ECVAM validation exercise conducted on this assay indicated a sensitivity of 93% and a specificity of 84%, experience within the pharmaceutical industry suggests a much lower specificity (see Note 3). The original OECD protocol was not validated for pharmaceuticals specifically. Thus, some modifications to the original OECD protocol have been proposed to address the low specificity observed with drug substances (see 3T3 Workshop Report, Ref. 7, and Note 4). The sensitivity of the 3T3 NRU-PT remains unquestioned, and if a compound is negative in this assay it would have a very low probability of being phototoxic in humans. However, a positive result in the 3T3 NRU-PT should not be regarded as indicative of a likely clinical phototoxic risk, but rather a flag for follow-up assessment.

ECVAM INVTTOX Protocol No 78

3T3 NRU Phototoxicity Assay
INVITTOX n° 78

Phototoxicity

The cytotoxicity of the test compound to 3T3 cells is assessed by Neutral Red Uptake following exposure in the presence or absence of UVA light.

Objective

TYPE OF TESTING	:	screening, adjunct
LEVEL OF ASSESSMENT	:	toxic potential, toxic potency
PURPOSE OF TESTING	:	classification and labelling

The 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT) is designed to detect the phototoxicity induced by the combined action of a chemical and light by using an *in vitro* cytotoxicity assay with the Balb/c 3T3 mouse fibroblast cell line.
The test identifies compounds that act *in vivo* phototoxic after systemic application, as well as compounds, including UV filter chemicals, that act as photoirritants after topical application and distribution to the skin.

National Institute of Health Sciences

Japanese Center for the Validation of Alternative Methods (JaCVAM)
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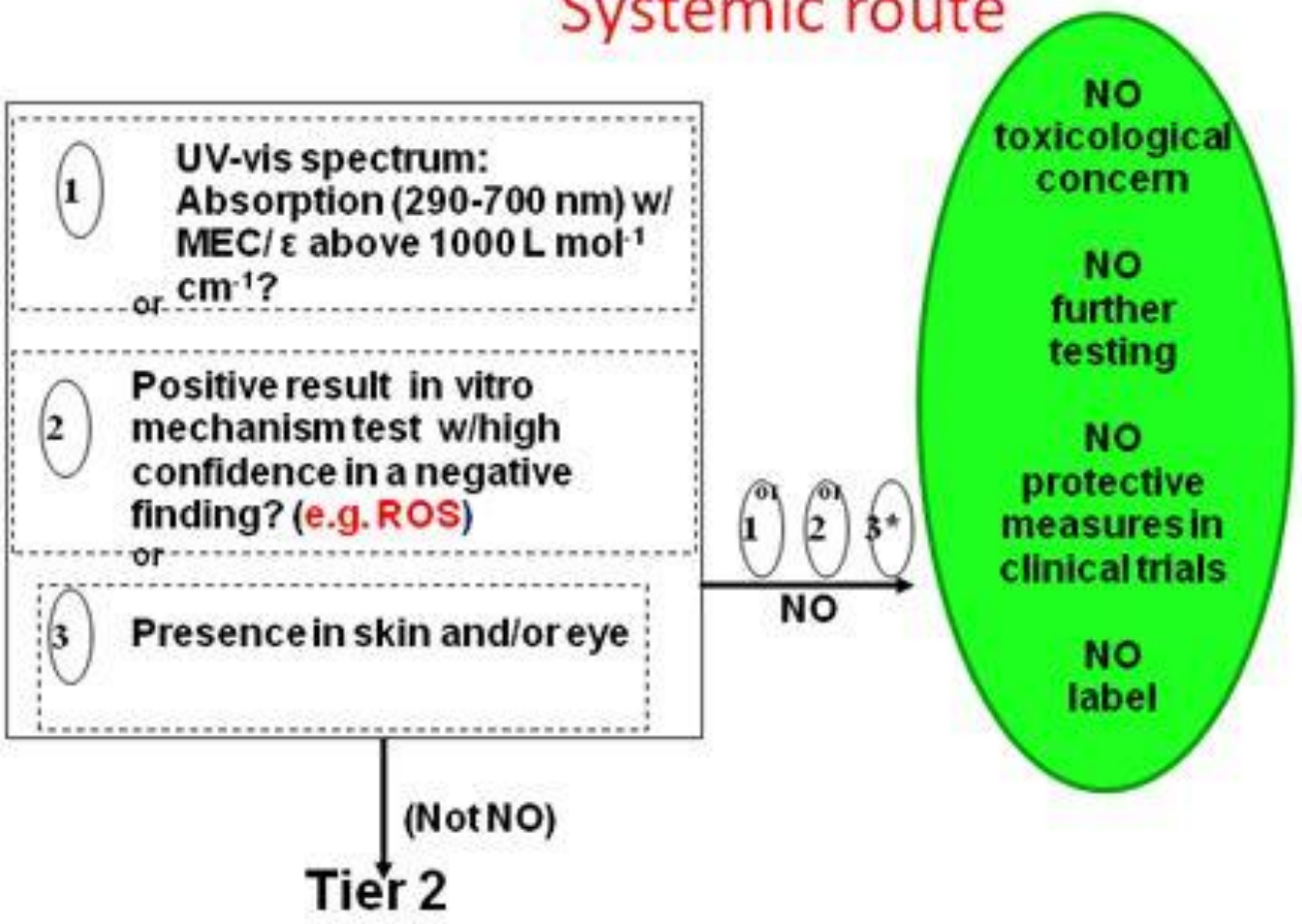
ICH update

August 21, 2013

Kazuhiro Hosoi

Fig.1 Photosafety Strategy: Tier 1 in ICH S10 guideline



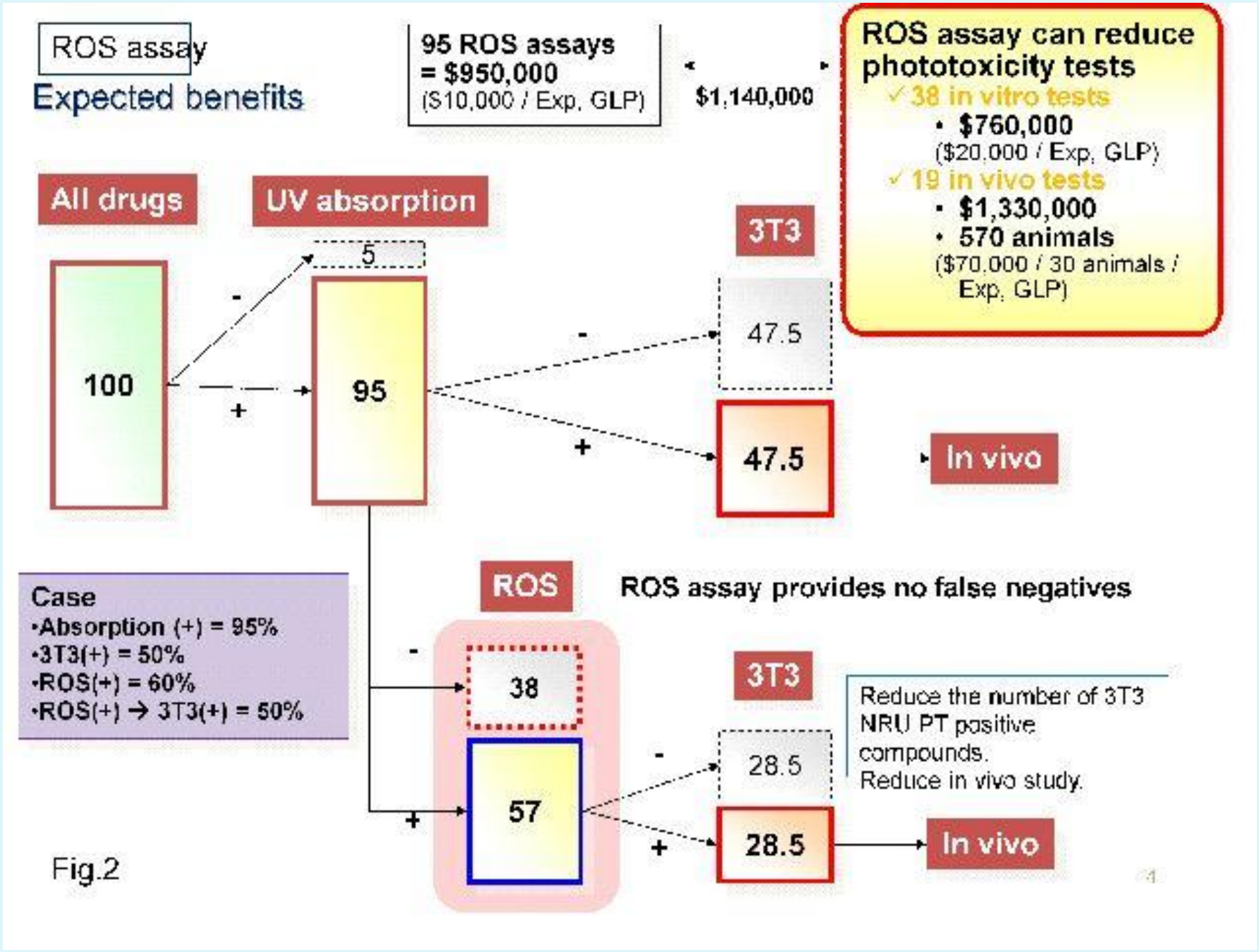

ROS assay
Expected benefits
95 ROS assays
= $950,000
($10,000 / Exp, GLP)
$1,140,000
ROS assay can reduce phototoxicity tests
✓ 38 in vitro tests
• $760,000
($20,000 / Exp, GLP)
✓ 19 in vivo tests
• $1,330,000
• 570 animals
($70,000 / 30 animals / Exp, GLP)
All drugs
UV absorption
3T3
100
5
95
47.5
47.5
In vivo
Case
•Absorption (+) = 95%
•3T3(+) = 50%
•ROS(+) = 60%
•ROS(+) → 3T3(+) = 50%
ROS
ROS assay provides no false negatives
38
57
3T3
28.5
28.5
In vivo
Reduce the number of 3T3 NRU PT positive compounds.
Reduce in vivo study.

Fig.2

Onoue et al.,
Pharmaceutical Research, 23 (1), 156-164 (2006)

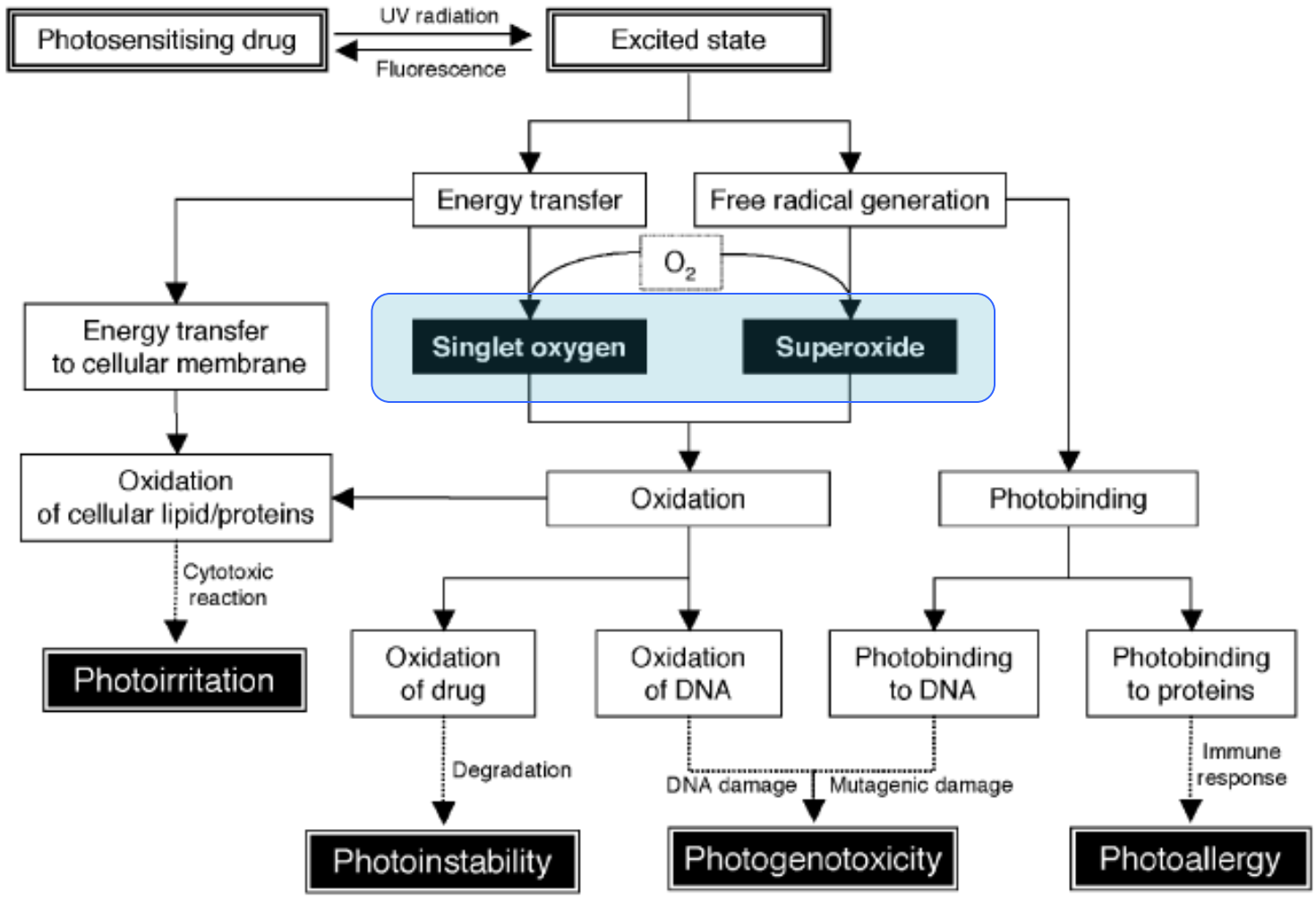
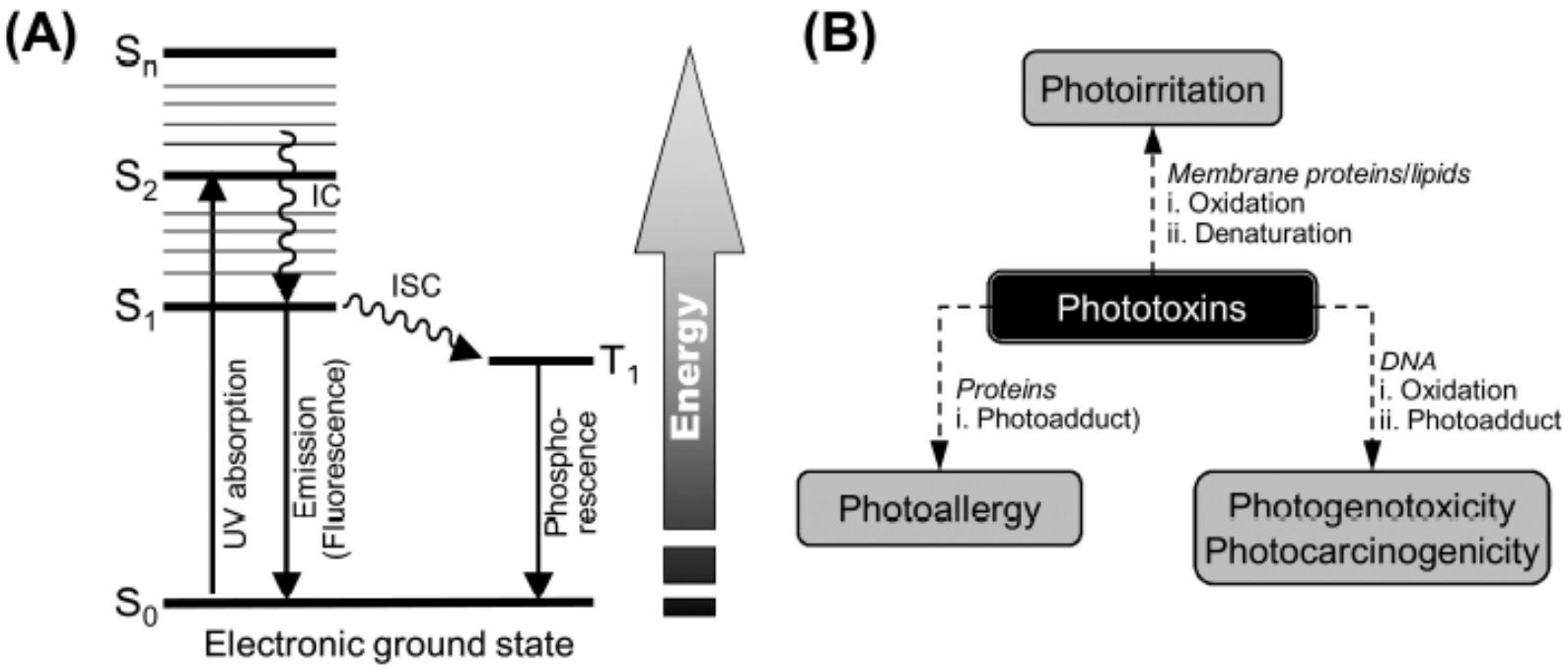


Fig. 5. Schematic representation of possible pathways for phototoxic responses induced by photosensitive drugs.

Rationale for ROS assay



(A) Jablonski diagram. S: singlet state; T: triplet state; IC: internal conversion; and ISC: intersystem crossing. Each line among singlet states indicates the excited vibrational states, and excited rotational states were not shown.
(B) Several phototoxic responses caused by photo-activated drugs.

ROS determination

1. Singlet oxygen ($^1O_2$);

by bleaching of p-nitrosodimethylaniline (RNO)

$^1O_2 + A \rightarrow [AO_2] \rightarrow AO_2$

$[AO_2] + RNO \rightarrow$ -RNO + Products

(A, $^1O_2$ acceptor, imidazole; RNO: nitroso compounds)

2. Superoxide anion (O_2^-)

by reduction of Nitroblue tetrazolium (NBT)

$O_2^- + NBT \rightarrow O_2$ + Nitroblue diformazan

Intra- and inter-laboratory precision of ROS assay

Atlas	Generation of reactive oxygen species					
	Singlet oxygen (Decrease of $A_{440\,nm}$ x 10^3)			Superoxide (Increase of $A_{560\,nm}$ x 10^3)		
	1	2	3	1	2	3
Intra-laboratory (*N*=9)						
Intra-day						
Quinine	553±14 (2.6)	438±11 (2.4)	366±5 (1.4)	424±31 (7.3)	305±12 (3.8)	306±27 (8.7)
Sulisobenzone	5±10	0±3	-2±1	-13±7	-12±3	-5±0
Inter-day						
Quinine	532±12 (2.2)	430±6 (1.4)	359±10 (2.7)	408±8 (2.1)	276±24 (8.8)	295±16 (5.4)
Sulisobenzone	2±5	1±3	0±2	-14±8	-11±3	-6±1
Inter-laboratory (*N*=9)						
Quinine	445±92 (20.6)			323±65 (20.1)		
Sulisobenzone	2±3			-11±5		

Mean±SD (CV, %)

Variations of each control value were sufficiently small to suggest good intra- and inter- laboratory reproducibility.

12

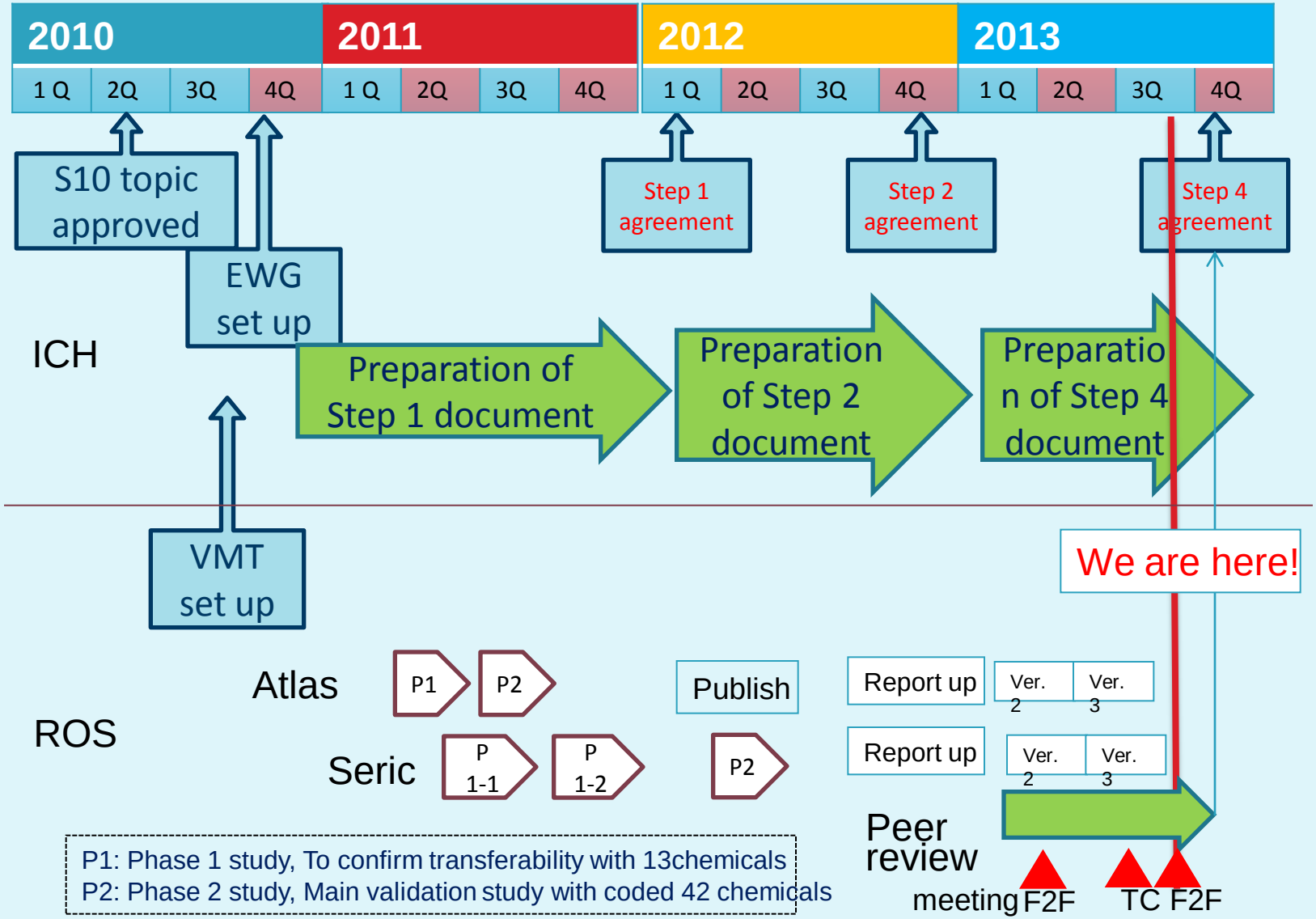
Photoreactive potential of 13 chemicals used 1st phase study

Compound	UV absorption		ROS assay			3T3-NRU PT	
	Wave length (MEC)	Ref.	Lab.A	Lab.B	Lab.C	Result	Ref.
5-Fluorouracil (5-FU)	– [290 (1837)]	Onoue et al. 2006	N	N	N	N	Kleinman et al. 2010
8-Methoxy psoralen (8-MOP)	303 (11818)	Onoue et al. 2006	P	P	P	P	Spielman et al. 1994
Amiodarone	303 (6209)	Onoue et al. 2006	P	P	P	P/E/N	Spielman et al. 1998
Chlorpromazine	307 (3831)	Onoue et al. 2006	P	P	P	P	Spielman et al. 1998
Diclofenac	– [290 (7753)]	Onoue et al. 2006	P	P	P	P	Onoue et al. 2010
Doxycycline	352 (8400)	Onoue et al. 2008	P	P	P	P	Spielman et al. 1998
Furosemide	331 (4978)	Onoue et al. 2006	P	P	P	P/N	Spielman et al. 1998
Ketoprofen	– [290 (4960)]	Onoue et al. 2006	P	P	P	P	Spielman et al. 1998
Levofloxacin	UVA/B (lamda max)	Kleinman et al. 2010	P	P	P	P	Kleinman et al. 2010
Norfloxacin	324 (13032)	Onoue et al. 2006	P	P	P	P	Spielman et al. 1998
Omeprazole	301 (15158)	Onoue et al. 2006	P	P	P	P/E	Kleinman et al. 2010
Quinine	331 (4451)	Onoue et al. 2006	P	P	P	P	Onoue et al. 2010
Sulisobenzone	320 (6600)	Onoue et al. 2008	N	N	N	N	Onoue et al. 2008

P: positive, N: negative, E: equivocal

– :If the peak/shoulder wavelengths were shorter than the lower limit fo UVB (290 nm), the absorbance at 290 nm was noted in parenthesis.

ICH S10 ROS Validation: Progress Flowchart



P1: Phase 1 study, To confirm transferability with 13chemicals
P2: Phase 2 study, Main validation study with coded 42 chemicals

Peer review panel for ROS assay validation

• William S. Stokes	Consultant to the NTP/NIEHS(Contractor)
• Horst Spielmann	Freie Universität Berlin
• Kim Bae Hwan	College of Natural Sciences, Keimyung University
• Ikuo Horii	DSRD Global consultant to Pfizer
• Yoshiki Tokura*	Dermatology, hamamatsu university school of medicine

The 1st peer review panel meeting was held on February 27-March 2, 2013.

Prof. Tokura was absent from the 1st peer review panel meeting.

Revised judgment criteria

Judgment[1)]	Concentration	SO (mean value of 3 wells)		SA (mean value of 3 wells)
Photoreactive	20 and/or 200 μM[2)]	≥25	and	≥70
		<25 and/or P[3)]	and	≥70
		≥25	and	<70 and/or P[3)]
Weakly photoreactive	20 and/or 200 μM[2)]	<25	and	≥20, <70
Non-photoreactive	20 and 200 μM[2)]	<25	and	<20
Inconclusive	The results do not meet the other criterion. [4)]			

Notes

1) It can be judged based on results of one experiment because the ROS assay shows good reproducibility in the validation studies.
2) It would be judged at 20 μM only when precipitation is observed at 200 μM.
3) Precipitation before irradiation.
4) When precipitation is observed at 20 and 200 μM before irradiation, the compound is regarded incompatible with the ROS assay.

(B) Performance of ROS assay to assess phototoxic potential Integrated judgment from the phase 2 results of atlas; Final judgment on the first assay results.

	Original Decision Criteria	*Revised Decision Criteria*	
		When the "±: Weakly photoreactive" chemicals were defined as "non-phototoxic" chemicals	When the "±: Weakly photoreactive" chemicals were defined as "phototoxic" chemicals
Sensitivity	100% (21/21)	100% (21/21)	100% (21/21)
Specificity	70.0% (7/10)	100% (18/18)	83.3% (15/18)
Positive predictivity	87.5% (21/24)	100% (21/21)	87.5% (21/24)
Negative predictivity	100% (7/7)	100% (18/18)	100% (15/15)
Accuracy	90.3% (28/31)	100% (39/39)	92.3% (36/39)

Activities in 2013

2013	Activities
Jan.	VMT submitted ver. 1 validation reports to the peer review panel.
Feb.	Peer review panel meeting (27 Feb. to 3 Mar.)
Mar. to Mid May	VMT prepared ver. 2 validation reports and revised proposed protocol, and submitted them to the peer review panel. #1: Introducing "Weakly photoreactive (SA: 20 - 70)" #2: "Negative at 20 µM": Non-photoreactive
Early June	ICH Brussels meeting Focused on "Negative **only** at 20 µM" After the meeting, additional data by Dr. Onoue #2: "Negative only at 20 µM": Probably non-photoreactive (follow-up studies would be needed)
Mid June	TC of Peer review panel
Late June	Comments from EWG on "Probably non-photoreactive" (would be misleading) #2: "Negative only at 20 µM": Inconclusive
End of July	VMT submitted ver. 3 Atlas validation report and revised proposed protocol. (Ver. 3 Seric validation report was submitted on Aug. 9.)

Four Big Issues

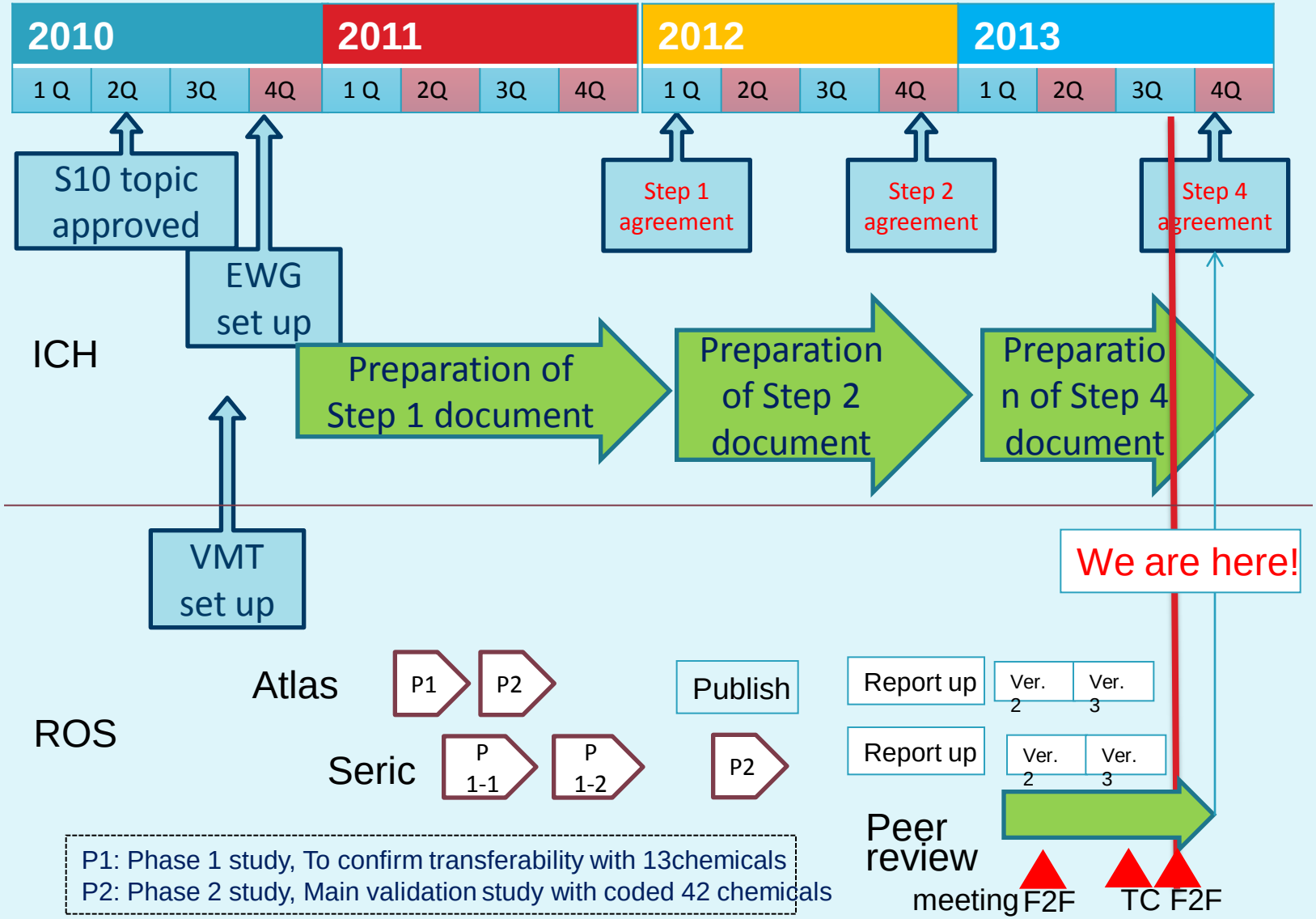
Regional Differences

Diagram Summary

Validation of ROS Assay

Ocular Administration of Pharmaceuticals

ICH S10 ROS Validation: Progress Flowchart



P1: Phase 1 study, To confirm transferability with 13chemicals
P2: Phase 2 study, Main validation study with coded 42 chemicals

rabbits are happy due to progress in *in vitro* phototoxicity testing !