Development of an Oral Photo-LLNA to Identify Photoallergens

M. Kirk[1], Y. Broomhead[1], G. DeGeorge[1], J. Piccotti[2], T. Kawabata[2]

[1]MB Research Laboratories, Spinnerstown, PA
[2]Pfizer Global Research & Development, Ann Arbor, MI

ABSTRACT

We previously modified a cytometry-based local lymph node assay (LLNA) to identify topically applied photoallergens, correctly classifying 13 photoallergens and non-photoallegens. Here we adapted the Photo-LLNA to determine the photoallergenic potential of orally administered compounds in the quinolone and quinoxalin antibiotic groups. CBA/J mice were administered drug by oral gavage for 3 consecutive days. Two sets of each group were dosed – one set irradiated (20 J/cm^2 UVA, Honle SOL 500 Solar Simulator), and one set left unirradiated. On day 6, the mice were injected with BromodeoxyUridine (BrdU, i.p.), a thymidine analog that incorporates into the DNA of proliferating cells. Auricular lymph nodes (LNC) were removed and processed for flow cytometric analysis. The Stimulation Index (SI) was determined by multiplying total LNC by the % proliferating LNC to calculate the total of proliferating LNC for each mouse. A positive response is indicated by a SI ≥3 for groups +UVA, with a SI <3 for unirradiated animals. Mice treated with olaquindox, enoxacin, and lomefloxacin all exhibited positive responses. Positive responses with significant ear swelling indicates possible (photo)irritation instead of (photo)allergenicity. As validated in our Flow Cytometry-based LLNA and dermal Photo-LLNA, additional endpoints were added to the Oral Photo-LLNA assay to help distinguish oral photoirritants from oral photoallergens. Olaquindox was tested in four separate assays, two of which resulted in significant ear swelling. In the last assay, immunophenotyping markers B220 (B cells) and CD3 (T cells), and immune activation markers (I-A^K, CD69) were used as endpoints to further distinguish sensitization and irritation. UVA treatment produced significant increase, compared to No-UVA and vehicle controls, in %B cells, B:T ratio, %I-A^K+ cells and %I-A^K/CD69+ cells, indicative of an allergenic response. Although more chemicals need to be tested, including a known photoirritant, this assay appears promising at identifying oral photoallergens.

INTRODUCTION

Photoreactions are serious concerns in dermatologic practices and pharmaceutical treatments. Photoreactions can be divided into two types: photoirritancy and photoallergy (photosensitization). Photoirritant responses lead to skin reactions similar to sunburn with or without severe edema. Photoallergy responses resemble hypersensitivity responses most often described as Delayed Type Hypersensitivity. These reactions can be quite serious, especially following more than one antigenic challenge.

Both photoirritancy and photoallergy can occur as a result of exposure to drugs or topically applied consumer products. The local lymph node assay (LLNA) has been proven to be a predictive test to detect sensitization responses to dermally applied substances. Two prominent non-radioactive methods have been adapted for use to investigate photo-dermal sensitizers. One method developed by Vohr et al., termed Integrated Model for the Differentiation of Skin Reaction (IMDS) uses a combination of measurements of ear irritation and draining lymph node cell proliferation to calculate a term deemed the Differentiation Index (DI) {1,2}. The sensitization test method developed by MB Research is termed Flow Cytometery Based LLNA (FC-LLNA) and measures total lymph node cell proliferation via cell number and BrdU incorporation to determine stimulation index (SI), as in the radiometric LLNA{3,4}. This method includes the additional quantitative endpoints of ear thickness and immunophenotyping to distinguish irritation and sensitization.

The challenge was to determine photoreactions in substances administered by the systemic (oral) route by adapting the topical dosing method. There are several known drug classes that may induce photoirritant and/or photoallergenic reactions – including antibiotics. Specifically, quinolones are a particular problem because they induce well-documented photoreactions when orally administered in humans. Furthermore, the extended classes of quinolone derivatives differ considerably in the extent of photoreactions in man and laboratory animals.

The IMDS method has shown some usefulness in determining photoirritation and photosensitization for systemically administered compounds {5,6}. Our FC-LLNA has the potential to develop a more predictive and sensitive systemic photo-LLNA assay. Our analysis method is very similar to the historic radioactive LLNA, and the additional immunophenotyping endpoints have the potential to effectively distinguish photosensitizers from photoirritants.

METHODS AND MATERIALS

Female CBA mice, 8-12 weeks old (n = 5/group), were used for these studies. Mice were dosed via oral gavage, once daily for three consecutive days (two groups per concentration of each test article). Two vehicle control groups of five mice each were treated with Methyl cellulose (MC), in the exact same manner. Following dose application, one matching set of each of the groups (i.e. one group for each test article concentration or vehicle control) was exposed to Solar Simulated Light (SSL, +UVA) irradiation (20 Joules UVA using a Honle Sol 500 solar simulator), while the second set of each group was not exposed to UVA irradiation ("No UVA"). On Day 6, the mice were injected with BrdU (i.p., 150 mg/kg) to be incorporated into proliferating lymph node cells (LNC). After 5 hours, the mice were euthanized and the auricular lymph nodes were collected on an individual animal basis. The nodes were then stored in FBS at 4℃. Lymph nodes were processed in plastic micr otubes by gentle agitation with a disposable pestle to yield a single-cell suspension in buffer. The cells were centrifuged, washed and resuspended in RPMI +10% FBS and kept overnight at 4℃.

Immunophenotyping: An aliquot of approximately 5 x 10^5 cells were stained with fluorescent-labeled B and T cell-specific antibodies (B220/CD3), or I-A^k and CD69 antibodies (Pharmingen/BD Biosciences) for analysis by flow cytometry.

Cell Proliferation: Aliquots of LNC were fixed by resuspending in 70% EtOH. Fixed cells were permeablized with 1N HCl, 0.5% TX-100, neutralized with 0.5M borate buffer, and stained with FITC-conjugated anti-BrdU antibody for cell proliferation analysis by flow cytometry (%BrdU positive). Additional aliquots were stained with PI and analyzed for LNC count via flow cytometry to calculate the total # cells per node set).

The stimulation index (SI) of a compound is the ratio of the total number of proliferating ($BrdU^+$) lymph node cells per mouse group to that in vehicle-treated groups. Test articles that yield a SI $\geq$ 3 are characterized as sensitizing substances. A test article is considered to be a photosensitizer if, in general, it results in an SI ≤3 +UVA and and SI <3 No UVA. Alternatively, a substance may be considered photosensitizing if both SI values are >3 and for at least one concentration, there is a >25% difference comparing a pair of +UVA and No UVA values. The total number of $BrdU^+$ LNC per mouse is calculated using the following equation:

(Total # cells per node set/mouse) X (% $BrdU^+$ LNC) = Total # $BrdU^+$ LNC/mouse

Flow cytometry was conducted using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with an Omnichrome 25 mW argon laser emitting at 488 nm at 15 mW of power. The plots generated in these experiments were analyzed using Cell Quest Software (version 3.3, Becton Dickinson Immunocytometry Systems, San Jose CA).

RESULTS

Figure 1: Flow Cytometry-Based LLNA Adapted for Oral Dosing and UVA Irradiation

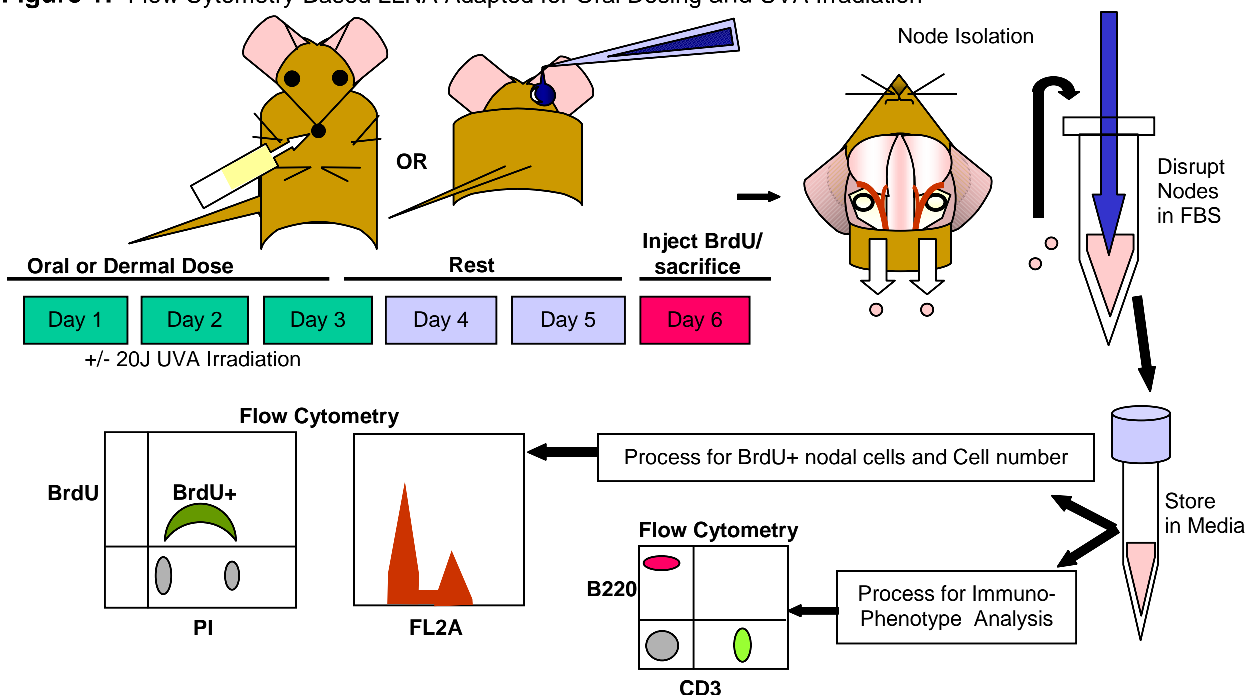
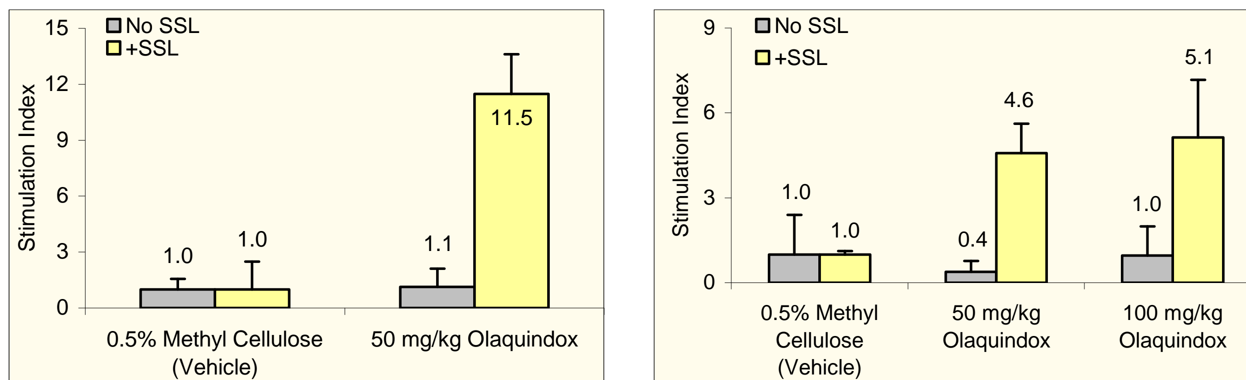


Figure 2: Initial Flow Cytometry-Based Oral Photo LLNA Experiment

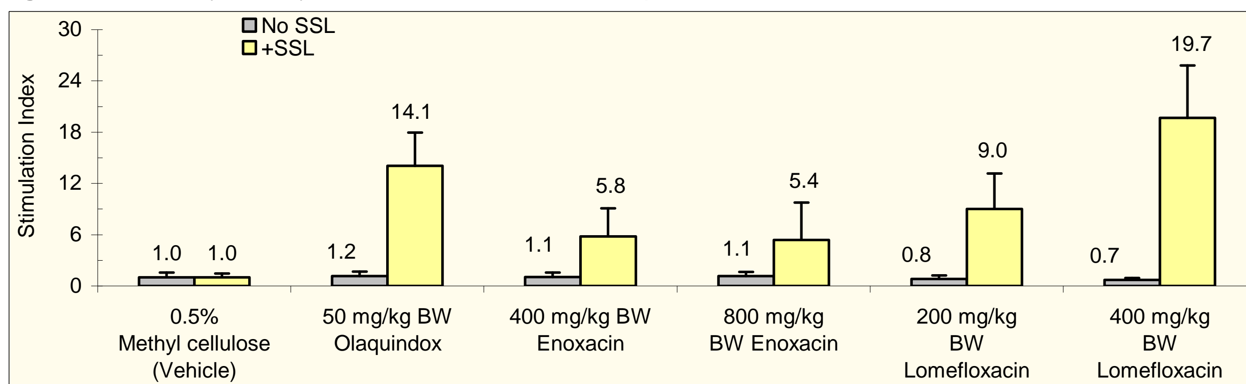


Ear Swelling (Irritation)

	0.5% Methyl Cellulose	50 mg/kg Olaquindox	0.5% Methyl Cellulose	50 mg/kg Olaquindox	100 mg/kg Olaquindox
+UVA	0.0%	9.5%	-4.8%	9.5%	26.3%
No UVA	0.0%	10.0%	0.0%	0.0%	5.0%

Olaquindox, orally dosed at 50 mg/kg and 100 mg/kg BW induces a positive SI only after UVA irradiation. Additionally, the 100 mg/kg dose induced irritation as detected by an increase in ear swelling.

Figure 3: Flow Cytometry-Based Oral Photo LLNA Experiment with Additional Chemicals

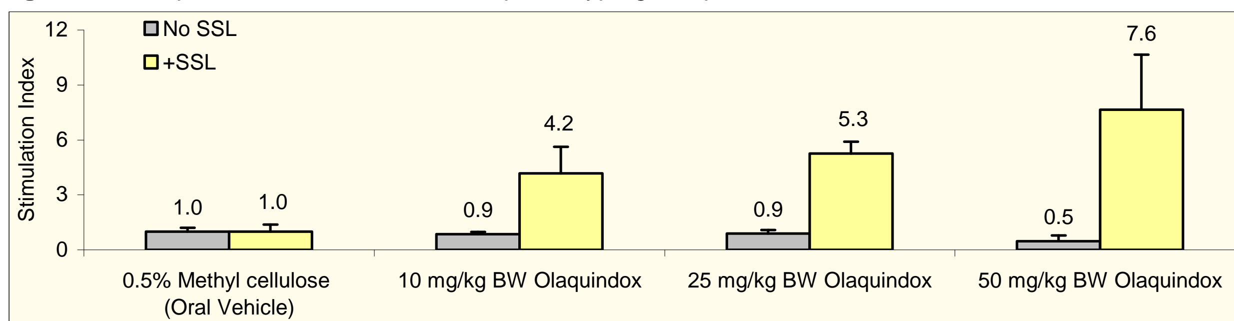


Ear Swelling (Irritation)

	0.5% Methyl Cellulose	50 mg/kg Olaquindox	400 mg/kg Enoxacin	800 mg/kg Enoxacin	200 mg/kg Lomefloxacin	400 mg/kg Lomefloxacin
+UVA	5.3%	26.3%	5.3%	0.0%	21.1%	25.0%
No UVA	5.3%	10.5%	5.3%	0.0%	0.0%	0.0%

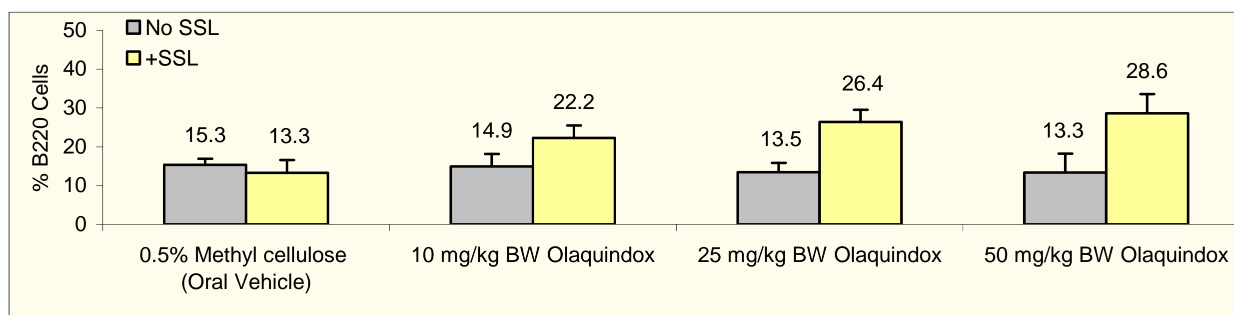
Oral dose of olaquindox, Enoxacin and Lomefloxacin all induce a positive SI following UVA irradiation. SI induced by Lomefloxacin appeared to be dose-related. Irritation was detected for both Olaquindox and Lomefloxacin.

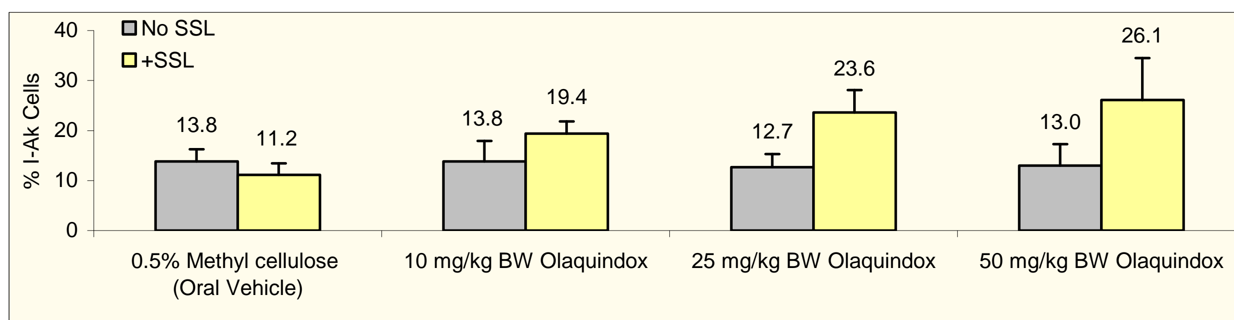
Figure 4: Olaquindox with Added Immunophenotyping Endpoints

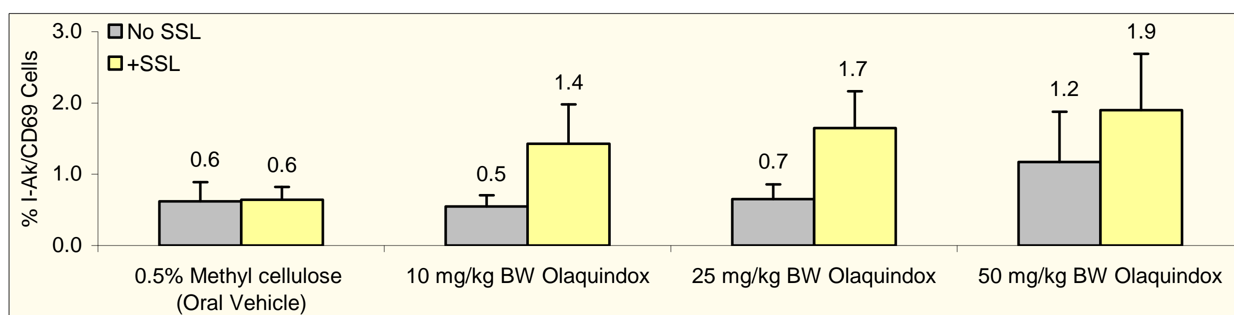


Ear Swelling (Irritation)

	0.5% Methyl Cellulose	10 mg/kg Olaquindox	25 mg/kg Olaquindox	50 mg/kg Olaquindox
+UVA	-5.0%	0.0%	5.3%	5.0%
No UVA	5.6%	0.0%	5.3%	11.1%







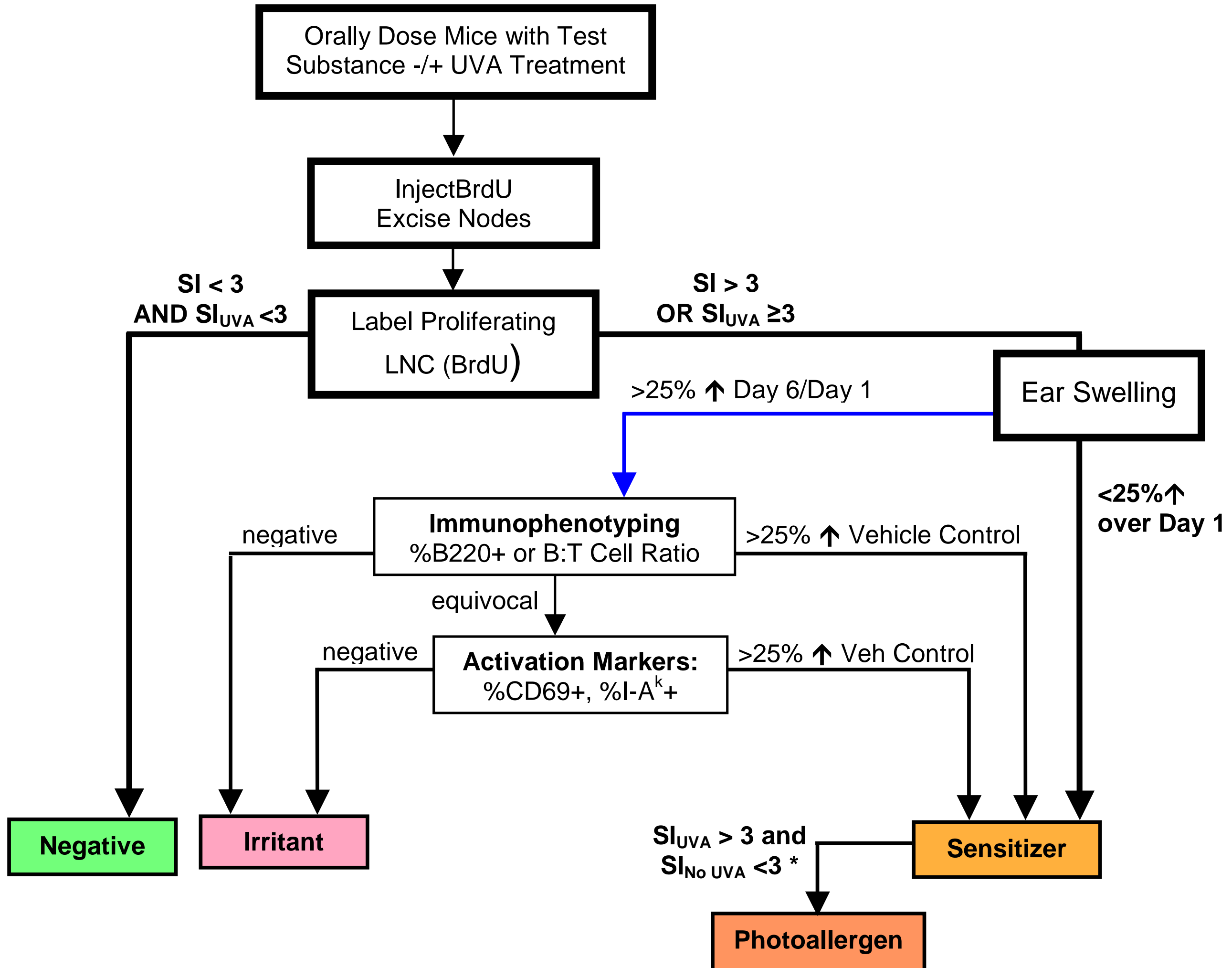
Oral dose of Olaquindox induced a dose-related increase of SI following UVA irradiation. Irritation was not detected in this assay, but has been shown for both the 50 and 100 mg/kg BW dose previously. Immunophenotyping analysis showed an increase of %B cells, B:T cells ratio and %IA^{K} cells only following irradiation, indicative of a photosensitizing response.

Figure 5: Summary of Quinolones Tested

	SI +UVA	SI No UVA	ES +UVA	ES No UVA
0.5% Methyl Cellulose	1.0	1.0	0.0%	0.0%
50 mg/kg Olaquindox	**11.5**	1.1	9.5%	10.0%
0.5% Methyl Cellulose	1.0	1.0	-4.8%	0.0%
50 mg/kg Olaquindox	**4.6**	0.4	9.5%	0.0%
100 mg/kg Olaquindox	**5.1**	1.0	**26.3%**	5.0%
0.5% Methyl Cellulose	1.0	1.0	5.3%	5.3%
50 mg/kg Olaquindox	**14.1**	1.2	**26.3%**	10.5%
400 mg/kg Enoxacin	**5.8**	1.1	5.3%	5.3%
800 mg/kg Enoxacin	**5.4**	1.1	0.0%	0.0%
200 mg/kg Lomefloxacin	**9.0**	0.8	21.1%	0.0%
400 mg/kg Lomefloxacin	**19.7**	0.7	**25.0%**	0.0%
0.5% Methyl Cellulose	1.0	1.0	-5.0%	5.6%
10 mg/kg Olaquindox	**4.2**	0.9	0.0%	0.0%
25 mg/kg Olaquindox	**5.3**	0.9	5.3%	5.3%
50 mg/kg Olaquindox	**7.6**	0.5	5.0%	11.1%

Summary of SI and Ear Swelling for the quinolones tested. Positive responses are in bold.

Figure 6: Sensitization decision tree adapted from FC-LLNA.



*Alternatively, a substance may be considered photosensitizing if both SI values are >3 and, for at least one concentration, there is a >25% difference comparing a pair of +UVA and No UVA values.

CONCLUSIONS

- The FC-LLNA has been successfully modified to identify oral photosensitizers. Oral treatment with the phototoxin Olaquindox in the Oral Photo-LLNA (OP-LLNA) results in a dose-related increase in stimulation index following UVA irradiation.
- The additional fluoroquinolones enoxacin and lomefloxacin orally dosed also induce a positive SI response in the FC-LLNA. Results from the previously published studyes by Vohr, et al., categorize enoxacin as a photoallergen and lomefloxacin as a photoirritant. In the results shown here, lomefloxacin induces increased ear swelling at 400 mg/kg doses, suggesting it may be both photosensitizing and photoirritating.
- Immunophenotyping in the OP-LLNA reveals a positive sensitizing response of Olaquindox only when coupled with UVA irradiation, thus identifying oxaquindox as a systemic (oral) photosentizer.
- Future studies require a positive photoirritant to be tested to illustrate the ability of the assay to distinguish between a photoirritant and photosensitizer.

REFERENCES

1. H. W. Vohr, B. Homey, H. C. Schuppe, and P. Kind. Detection of photoreactivity demonstrated in a modified local lymph node assay in mice. *Photodermatol Photoimmunol Photomed* 10 (2):57-64, 1994.
2. P. Ulrich, B. Homey, and H. W. Vohr. A modified murine local lymph node assay for the differentiation of contact photoallergy from phototoxicity by analysis of cytokine expression in skin-draining lymph node cells. *Toxicology* 125 (2-3):149-168, 1998.
3. T.L. Ripper, M.K. Reeder, D.R. Cerven, G.L. DeGeorge. Further Development and Validation of an Alternative Photo-Sensitization Assay Using Multiple Flow Cytometry-based Endpoints. American College of Toxicology Annual Meeting, 2004.
4. Reeder, MK., Cerven, DR., Gilotti, AC., DeGeorge, GL., Final Validation of a Flow Cytometry-based Local Lymph Node Assay with Immunophenotypic Endpoints
5. Blotz, L. Michel, A. Moysan, J. Blumel, L. Dubertret, H. J. Ahr, and H. W. Vohr. Analyses of cutaneous fluoroquinolones photoreactivity using the integrated model for the differentiation of skin reactions. *J Photochem Photobiol B* Biology 58 (2000):46-53, 2000.
6. N. J. Neumann, A. Blotz, G. Wasinska-Kempka, M. Rosenbruch, P. Lehmann, H. J. Ahr, and H. W. Vohr. Evaluation of phototoxic and photoallergic potentials of 13 compounds by different in vitro and in vivo methods. *J Photochem Photobiol B* 79 (1):25-34, 2005.