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Project Title: Non-Invasive Biological Monitoring of Pesticides

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Previous PI: Charles Timchalk, PhD, DABT
Co-Investigator: Thomas J. Weber, PhD
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List of Terms and Abbreviations

HSG	Human Saliva Gland
NRC	National Research Council
PEST	Pesticide
PK	Pharmacokinetic
PBPK	Physiologically based pharmacokinetic
RfD	Reference Dose
SGEC	Saliva Gland Epithelial Cell
TEER	Transepithelial/Transendothelial Electrical Resistance
TCPy	Trichloropyridinol

Abstract.
Our overarching research strategy has been to establish a non-invasive biomonitoring capability to evaluate exposure to pesticides utilizing a sensitive, non-invasive, micro-analytical instrumentation for real-time analysis of biomarkers of exposure and response in saliva. Our previously completed research resulted in the development of pesticide sensor platforms, an *in vivo* animal model system for rapid characterization of saliva pesticide uptake and clearance, and an *in vivo* dosimetry model to predict systemic dose based upon a 'spot' saliva measurement. Since human exposure is rarely to single agents but rather to complex mixtures, there is a need to develop biomonitoring strategies capable of measuring multiple analytes. This is particularly true in agriculture where multiple pesticides are routinely utilized on crops. Hence there is a need to extend the strategy to other important pesticides; however, a major limitation is the inability to *a priori* identify which chemicals are adequately cleared in saliva, hampering our ability to easily develop a multiplex screening platform. To address this challenge, this recently completed project evaluated the hypothesis ***that chemical uptake and clearance in saliva can readily be predicted based upon limited in vitro experiments which are integrated into a pharmacokinetic model.*** To test this hypothesis this project exploited a previously developed *in vivo* rat model for salivary gland uptake and clearance and established *in vitro* cell and sub-cellular based approaches to evaluate salivary gland uptake and clearance. More specifically, a serous-acinar Transwell® model system was developed as an *in vitro* screening platform to prioritize chemicals for non-invasive biomonitoring through salivary clearance mechanisms. Rat primary serous-acinar cells express both α-amylase and aquaporin-5 proteins and develop significant tight junctions at post-confluence - a feature necessary for chemical transport studies *in vitro*. This model system provides a useful *in vitro* screening platform to support the non-invasive monitoring using human saliva and provide guidance for development of advanced *in vitro* screening platforms utilizing primary human salivary gland epithelial cells. Secondly, computational modeling approaches have been developed that couple *in vivo* and *in vitro* experiments to predict salivary uptake and clearance of xenobiotics and provides additional insight on species-dependent differences in partitioning that are of key importance for extrapolation. Specifically, a cellular transport computational model was developed using experimentally derived transport parameters based upon the *in vitro* serous-acinar cell system. Furthermore, the cellular transport computational model was integrated into an existing physiologically based pharmacokinetic (PBPK) model to enable accurate simulation of target metabolite concentrations in saliva of rats that were exposed to selected pesticides *in vivo*. Overall, this approach demonstrates the utility of a combination experimental and computational approach to predict chemical transport in saliva potentially increasing the utility of salivary biomonitoring in the future. The development of a real-time saliva analysis coupled to a predictive pharmacokinetic model represents a significant advancement over current biomonitoring strategies. This model system represents the next generation of biomonitoring tools and approaches that can be utilized to assess worker exposure to insecticides under a wide range of occupational situations.

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Section 1.

Executive Highlight

In articulating a vision for Toxicity Testing in the 21st Century, the National Research Council (NRC) noted that exposure science will play a critical role in a new risk-based framework. In this context, biomonitoring is a key component that quantitatively associates an internal dose with a measurable effect. It has also been suggested that epidemiology studies which accurately assess chemical exposures along with biological effects will have the most meaningful interpretation and thus maximal impact. However, a major impediment in conducting quantitative biomonitoring within epidemiology studies is the lack of rapid, field deployable, quantitative technologies that measure chemical exposure/response biomarkers using minimally invasive biological fluids (i.e. saliva). Due to the complex nature of environmental exposures and biological systems, measurement of a single biomarker may not provide adequate quantitative assessment of exposure. However, a major limitation to advancing multiplex biomonitoring in saliva is the inability to identify a broader range of chemicals that are readily cleared in saliva and quantifiable. Hence, this project has undertaken development, validation, and refinement of a novel *in vitro* cell based screening assay capable of identifying candidate analytes for saliva biomonitoring. Furthermore, the pharmacokinetic results from the *in vitro* assays have been utilized to develop a cellular/PBPK computational model to facilitate quantifying dosimetry in both animal model systems and humans. This novel approach will enable a more accurate prediction of exposures and can readily be employed to assess dosimetry to pesticides in support of biomonitoring and epidemiology studies.

Significant (Key) Findings. The primary objective(s) of this project has focused on three key objectives: **(A)** to develop an *in vitro* cell based system capable of evaluating cellular uptake and clearance of xenobiotics; **(B)** to develop an *in vitro* computational model capable of simulating *in vitro* uptake and clearance; and **(C)** to integrate *in vitro* and *in vivo* data into computational models, to enable prediction of *in vivo* pharmacokinetics and estimate systemic dosimetry and biological response based upon a saliva measurement.

(A). The development of the *in vitro* cell based system under this objective established a rat salivary gland epithelial cell (SGEC) assay to evaluate vectoral transport of pesticides from the basolateral to apical (lumen) side of a Transwell® cell culture system. This system is capable of quantifying transport (both uptake and efflux) and evaluating mechanisms of this transport including transport protein interactions, and non-linear pharmacokinetics for a broad range of chemicals and drugs.

(**B**). An *in vitro* cell transport model was developed to simulate transport rates from SGEC experiments under both non-physiological and physiological conditions. Compound transport was modeled using a modified Fick's Law of Diffusion (function: concentration, surface area, membrane thickness and diffusion constant). The model was successfully parameterized and validated using a variety of kinetic- and dose-dependent transport experiments in a Transwell® *in vitro* SGEC system (Objective **A**). In these experiments, pesticide concentrations in basolateral and apical compartments were concurrently determined. Cells demonstrated a capacity to metabolize pesticides, and target analytes were modeled using diffusion, suggesting that passive transfer is the primary mechanism of transport for these chemicals. Similar results have been measured *in vivo*. Diffusion coefficients were consistent across concentrations tested, suggesting no dose-dependent differences in transport (at concentrations tested).

(**C**). To validate the overarching approach based upon *in vitro* and *in vivo* data the cellular transport model (Objective **B**) was integrated into a physiologically based pharmacokinetic (PBPK) model to predict analyte transport in the cellular experiments (*in vitro*) and analyte concentrations in saliva of rats (*in vivo*). The integrated (*in vitro*/*in vivo*) models were subsequently utilized to simulate occupational exposure to pesticides (as single chemicals or mixtures) at relevant occupational exposure concentrations. To our knowledge, this is the first time this approach has been applied to salivary transport. Validation of the approach was confirmed based upon the computational models adequately simulating both *in vivo* and *in vitro* experimental results.

Translation of Findings.

The findings of this study demonstrate that an *in vitro* salivary cell model system can be exploited to identify ideal chemical candidates for saliva biomonitoring. In the current project an *in vitro*/*in vivo* computational model was also utilized to quantitatively evaluate the relationship between *in vitro* salivary gland cell uptake and clearance and *in vivo* saliva and blood analyte concentrations to enable a quantitative prediction of occupational exposure based upon saliva. It is anticipated that the technology developed under this project will be exploited to identify a broad range of candidate chemicals for saliva biomonitoring, and computational models will enable risk assessors to evaluate the potential impact of occupational exposures on worker health. The *in vitro* cell system and computational tool that has been developed under this project can be readily adapted to accommodate many important occupational chemical exposures forming the foundation for evaluation of worker exposures, dosimetry and by extrapolation, risk.

Outcomes/Impact. There are a number of important outcomes and impacts of this research on occupational safety and health. This project has resulted in the development and laboratory based validation of a novel *in vitro* rat Salivary Gland Epithelial Cell (SGEC) assay that is capable of quickly screening a broad range of chemicals and drugs for salivary gland uptake and clearance. Hence, it is now feasible to rapidly identify those occupational chemicals that are ideal candidates for saliva biomonitoring. Prior approaches involved the use of *in vivo* rodent models, which are not ideally suited as a screening tool. Based on the new approaches developed under this project, we anticipate that it will now be possible to significantly increase the number of candidate chemicals for saliva biomonitoring. This novel platform enables a more rapid and accurate prediction of saliva clearance and can readily be employed to further assess dosimetry to pesticides in support of biomonitoring and epidemiology studies. Secondly, the findings also demonstrate the utility of quantitative computational modeling approaches (*in vitro*/*in vivo*) to assess the impact of exposures on occupational risk. This strategy has not historically been the approach that has been utilized by risk assessors, but does offer a more biologically based approach that can be utilized to evaluate worker exposure and risk. Although more research is warranted, it is clear from the finding of this project that a risk assessment approach that incorporates biomonitoring coupled with computational dosimetry modeling represents a cost-effective quantitative strategy for evaluating occupational risk associated chemical exposures.

Section 2.

Scientific Report.

A. SPECIFIC AIMS: This research project tested the hypothesis that ***chemical uptake and clearance in saliva can readily be predicted for chemicals based upon limited in vitro experiments which are integrated into a pharmacokinetic model.*** To test this hypothesis, this project exploited a previously developed *in vivo* rat model for salivary gland uptake and clearance and developed additional *in vitro* cell and sub-cellular based approaches. The following Specific Aims were evaluated:

1. ***To experimentally determine salivary gland uptake and efflux for pesticides with differing chemical, physical and biological properties using in vitro cell and sub-cellular based assays.***
2. ***To develop computational pharmacokinetic models capable of simulating and predicting pesticide uptake and clearance in saliva.***
3. ***To experimentally validate in vivo saliva uptake and clearance of pesticides in a rat model.***

B. STUDIES AND RESULTS: The primary objective(s) of this project has focused on three key objectives: **Objective (A)** to develop an *in vitro* cell based assay to quantify vectoral transport of pesticides; **(B)** to establish an *in vitro* saliva uptake and clearance model to obtain key model parameter estimates to enable accurate simulation of *in vitro* experimental results under non-physiological and physiological cell culture conditions; and **(C)** to develop and utilize computational dosimetry biological response models to enable prediction of systemic dose and biological response based upon a saliva measurement.

***Objective (A)*:** We have established and validated our *in vitro* salivary cell system and Transwell® chamber-based assay that is capable of predicting transport of chemicals from blood into saliva based upon transcellular

or paracellular transport (**Fig A-1**). This cell system has been exploited to evaluate vectoral transport of selected pesticides from basolateral to apical (lumen) side of a Transwell® cell culture system (**Fig A-2**). Initial experiments have focused on evaluating chemical pesticides where cellular uptake and clearance are primarily a diffusional based process; whereas, subsequent studies have evaluated classes of pesticides where transport (both uptake and efflux) may be associated with non-linear pharmacokinetics. These results have been further utilized to develop the computational models under **Aim 2**.

Initial efforts on this project focused on an evaluation of 3-cell systems: Par-C10 cells (immortalized rat cells), Human Salivary Gland (HSG) cells and rat primary submandibular Salivary Gland Epithelial Cells (SGEC) as potential cell systems for this project. A major challenge was establishing these cells in a Transwell® culture where tight junctions between cells were definitively established. This is critically important since any "leakiness" in the system will adversely impact experiments. Therefore after careful evaluation, we focused on exploiting SGEC system that was developed in our laboratory since our validation studies demonstrated that these cells in culture form extremely good tight junctions and maintain critical phenotypic characteristics of salivary cells. The SGEC isolation and phenotypic characterization is further described in **Fig A-3**, and the experiments presented in **Fig A-4** provide the critical validation of the establishment and maintenance of tight junction formation in SGEC and included time-course evaluation, multiple markers of tight junction formation (i.e. TEER vs. Lucifer Yellow) and comparison across cell sub-clone populations.

SGECs were evaluated for polarity to assess compatibility of *in vitro* cells to serous acinar cells *in vivo*. Antibodies capable of binding only to apical surfaces, or primarily basolateral surfaces, were utilized to address this question. The primary antibody, rat CRB3 (Crumbs apical polarity complex) was chosen to fluorescently tag (with Alexa488 secondary antibody) the apical surface of SGEC. In addition, DAPI nuclear stain was used as a positive control and for spatial reference. The reconstructed, cross-sectional laser scanning confocal microscopic images demonstrate apical localization of CRB3, indicating a polarized state under these conditions (**Fig A-5**).

Initial transport studies with the SGEC system have established appropriate positive and negative control response (altering tight junctions), *in vitro* cellular cytotoxicity evaluation to establish pesticide dosage range, time-course of tight junction maintenance (over 10 days) and initial experiments demonstrating bi-directional chemical diffusion (**Fig A-6, A-7**). Overall, these experiments established the *in vitro* cellular conditions that were utilized throughout all remaining *in vitro* studies to evaluate both passive and active transport process. Additional time-course, concentration, metabolism, cytotoxicity and tight junction formation based evaluations of pesticides have been conducted with SGEC cell and will be more fully discussed under **Objective (B)**.

***Objective (B)*:** The *in vitro* cell transport model (**Fig B-1**) was developed to simulate transport rates from SGEC experiments. Compound transport was modeled using a modified Fick's Law of Diffusion (**Fig B-2**) (function: concentration, surface area, membrane thickness, and diffusion constant). The model was parameterized with chlorpyrifos and TCPy concentrations (0, 2, 5, 24 hr) in basolateral and apical compartments following exposure to chlorpyrifos (70 µM) and TCPy (3 µM, **Fig B-3**). Fitted parameters were validated with kinetic (0, 1, 2, and 3 hr, 125 µM, **Fig B-4**) and dose-dependent (25, 50, and 100 µg/mL, 4 hr, **Fig B-5**) experiments using chlorpyrifos as an initial test pesticide in a Transwell® *in vitro* SGEC system (experimental results from **Objective A**). The *in vivo* transcellular or paracellular diffusion of unbound chemicals in plasma to saliva has been computationally modeled using compartmental and physiologically based approaches. Of key importance for determining the plasma:saliva partitioning was the utilization of a modified Schmitt algorithm that calculates partitioning based upon the tissue composition, pH, chemical pKa and plasma protein-binding. Although in the rat *in vivo* model system saliva/blood concentration ratios did not change over varying experimental conditions, however normal differences in saliva pH values for humans (range 5.6-7.9) have been noted in the literature which may significantly impact partitioning. Model simulations and a sensitivity analysis was conducted to further evaluate the impact of variable pKa (4→10) or protein-binding fractions (0.1→0.9) on the saliva:blood partitioning coefficient. A summary of model parameters for both rat and human are presented in **Table B-1,** and the sensitivity analysis is presented in **Table B-2**. For this

analysis, the model is highly sensitive to changes in plasma protein-binding and pKa for both humans and rats. Hence, these parameters are of critical importance and can substantially impact partitioning. To further illustrate this point in the current model simulation (**Fig B-6**), the analyte concentration in saliva (based upon partitioning) is directly reflective of the concentration that is not bound to plasma proteins in blood. Likewise, as shown in **Fig B-7,** the pH of blood and saliva are also key parameters modulating salivary clearance of compounds particularly where the chemical pKa is comparable to the pH of biological fluids. Hence, due to variation in rat and human pH values, the Schmitt algorithm has been utilized successfully to calculate plasma:saliva partitioning. This modification enables extrapolation of the blood to saliva partitioning coefficient to humans over a range of reported human salivary pH levels.

To facilitate *in vitro* to *in vivo* extrapolation, *in vitro* transport of the chlorpyrifos metabolite 3,5,6-trichloro-2-pyridinol (TCPy) was measured using non-physiological and physiological transport experiments with the serous-acinar chemical transport assay. Under non-physiological conditions (**Fig B-8a,b)** TCPy demonstrated transport to equivalent concentrations at equilibrium, suggesting a passive diffusion transport mechanism. In addition, TCPy demonstrated dose-dependent times to reach equilibrium, suggesting that protein binding is an important determinant in TCPy cellular transport. After optimizing the permeation coefficient (0.41 cm/hr), the cellular transport computational model reasonably simulated non-physiological transport experiments and observed behavior. Under physiological conditions (**Fig B-9a,b**) TCPy transport was much slower than those from the non-physiological experiments. The cellular transport computational model accurately simulated physiological transport experiments after re-optimizing the permeation coefficient (1.14 cm/hr). In this study, we observed that by utilizing a cellular system that is more representative of physiology in terms of protein levels, we were able to more accurately predict actual TCPy levels observed in rat saliva (see **Objective C**).

In vitro transport was also assessed with 2,4-D, a commonly used, broad-leaf selective herbicide. Under non-physiological conditions, 2,4-D demonstrated transport to equilibrium across the monolayer resulting in equivalent concentrations in both compartments at 24 hours (**Fig 8-10**) after dosing (10 or 100 µg/ml), demonstrating slower transport than TCPy. 2,4-D was also highly bound to proteins under physiological conditions (**Fig B-11a**), which resulted in much slower transport compared to 2,4-D transport in non-physiological conditions (**Fig B-11b**).

Objective (C): *In vivo* salivary uptake and clearance of chlorpyrifos and TCPy were determined in a rat model (**Fig C-1**). Utilizing parameters measured in the physiological experiment (**Fig B-9**), a modified PBPK model (**Fig C-2**) was able to accurately predict TCPy levels in blood and saliva of rats (**Fig C-3a,b**). The model has likewise been used to simulate analyte concentrations in blood and saliva based upon simulation at a Reference Dose (RfD) of 3 µg/kg/day (**Fig C-4**). These results demonstrate an ability to link *in vitro* experimental results into a cell/PBPK computational modeling framework capable of predicting dosimetry based upon an evaluation of saliva.

C. SIGNIFICANCE: This project has focused on the development and validation of an *in vitro* cell based model system using rat saliva gland epithelial cells to determine the extent and rate of xenobiotic uptake and clearance in saliva. In addition, by coupling kinetic parameter estimates determined in the *in vitro* experiments results into a PBPK modeling framework, we are now capable of predicting saliva clearance of xenobiotics in both animal model systems and in humans that are occupationally exposed to pesticides. This novel approach will enable a more accurate prediction of exposures and can readily be employed to assess dosimetry to pesticides in support of biomonitoring and epidemiology studies.

D. PLANS: Results of this project are now being used to extend the current research by focusing on other chemical agents that could be important candidates for saliva biomonitoring. The *in vitro* cell system and the computational dosimetry model that have been developed can now be exploited to evaluate a broader range of chemicals that have different physical and chemical properties to help identify candidate agents that would have a high probability of being detected in saliva.

Publications:
Peer-reviewed publications (credited to this project).

1. Timchalk, C. Weber, T. J. and **Smith, J. N.** (2017). The need for non- or minimally-invasive biomonitoring strategies and the development of pharmacokinetic and pharmacodynamics models for quantification. *Current Opinion in Toxicol.* (*E-Pub*) *doi: 10.1016/j.cotox.2017.03.003.*
2. **Smith, J. N.**, Carver, Z. A., Weber, T. J. and Timchalk, C. (2017). Predicting transport of 3,5,6-trichloro-2-pyridinol (TCPy) into saliva using a combination cellular and computational approach. *Tox. Sci.*, (*E-Pub*) *doi: 10.1093/toxsci/kfx055*.
3. Weber, T. J., **Smith, J. N.** Carver, Z. A. and Timchalk, C. (2017). Non-invasive human biomonitoring through saliva clearance mechanism: development of an *in vitro* platform. *J. Exp. Sci. and Environ. Epidemiol.* **27:** 72-77. *doi:10.1038/jes.2015.74*.
4. Timchalk, C., Weber, T. J. and **Smith, J. N.** (2015). Computational strategy for quantifying human pesticide exposure based upon a saliva measurement. *Front. Pharmacol.,* **6:**115 1-10 *doi: 10.3389/ fphar.2015.00115*.

Presentations (credited to this project):

1. **Smith J. N.** (2017). Better Understanding Exposures to PAHs and Pesticides. ATSDR investigator initiated research translation activity. April 20, Agency for Toxic Substances and Disease Registry (ATSDR), Atlanta, GA.
2. Timchalk, C., Carver, Z. A., Weber, T. J., and **Smith, J. N.** (2017). Implications of protein binding in predicting pesticide salivary transport using a combination experimental and computational approach. *Society of Toxicology Annual Meeting*, March 12-16th, Baltimore, MD.
3. Weber, T. J., **Smith, J. N.**, Carver, Z. A., and Timchalk, C. (2017). Rapid feasibility screening to microsensor platforms for non-invasive human biomonitoring. *Society of Toxicology Annual Meeting*, March 12-16th, Baltimore, MD.
4. **Smith J. N.** (2017). Understanding the Dose that Makes the Poison Using an Experimental and Computational Approach. Invited Seminar, February 1, Oregon State University, Corvallis, OR.
5. Timchalk, C., Carver, Z. A., Weber, T. J., and **Smith, J. N.** (2016). Extrapolating salivary acinar cell *in vitro* pesticide transport to whole animals using computational modeling. *Society of Toxicology Annual Meeting*, March 13-17th, New Orleans, LA. (Awarded Top 10 Abstract in Society of Toxicology (SOT) Risk Assessment Specialty Section).
6. Carver, Z. A., Timchalk, C., **Smith, J. N.**, and Weber, T. J. (2016). 2,4-Dichlorophenoxyacetic acid (2,4-D) transport across an *in vitro* salivary acinar cell system: A novel approach to biomonitoring. *Society of Toxicology Annual Meeting*, March 13-17th, New Orleans, LA.
7. Weber, T. J., **Smith, J. N.**, Timchalk, C., and Carver, Z.A. (2016). Non-invasive human biomonitoring: Development of a traswell assay to support exposure science assessment and health impacts. *Society of Toxicology Annual Meeting*, March 13-17th, New Orleans, LA.
8. Timchalk, C., Weber, T. J., and **Smith, J. N.** (2015). Development of a computational model describing and extrapolating salivary acinar cell *in vitro* pesticide transport. *Society of Toxicology Annual Meeting*, March 22-26th, San Diego, CA.
9. Weber, T. J., **Smith, J. N.**, and Timchalk, C. (2015). Development of an *in vitro* screening assay for non-invasive biomonitoring. *Society of Toxicology Annual Meeting*, March 22-26th, San Diego, CA.

Relevant Figures and Tables (in order of discussion):

Objective A:

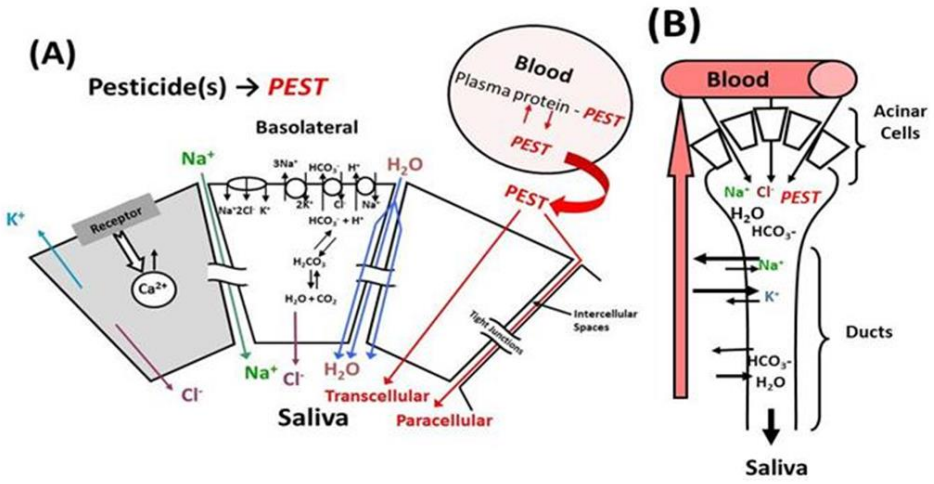
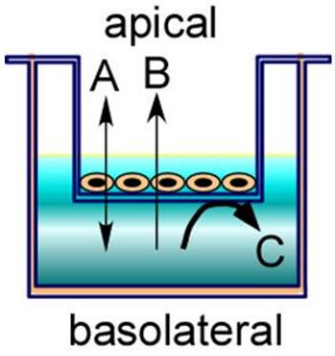


Figure A-1. *Schematic model illustrating* ***(A)*** *acinar cell and* ***(B)*** *salivary duct function associated with saliva formation. Note:* ***PEST*** *is used as a general notation for pesticides.*



- Isolate salivary gland epithelial cells
- Determine epithelial type
  - serous vs mucous acinar
- Determine tight junction formation
  - Transepithelial electrical resistance
  - Lucifer Yellow passage (<2%/hr)
- Determine chemical transport
  - Chlorpyrifos initial test case
  - Hypothesis: passive diffusion

Figure A-2. *In vitro Transwell system for evaluation of pesticide transport in salivary gland epithelial cell (SGEC).*

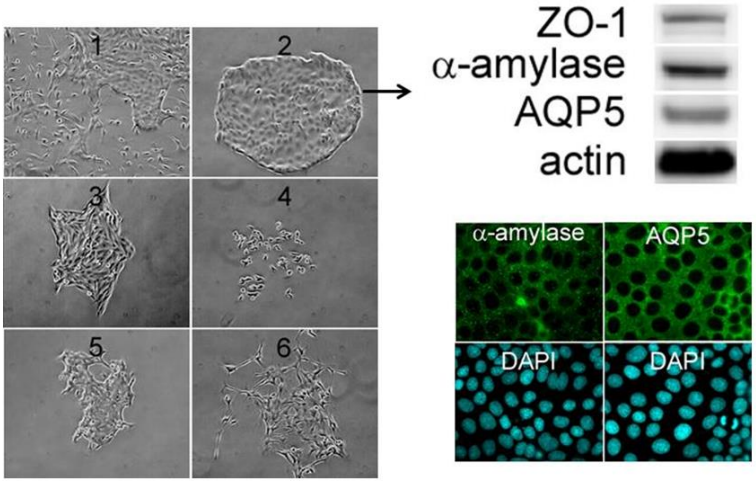


Figure A-3. *Isolation of salivary gland epithelial cells (SGEC). Images show crude cell mixture following digestion of rat submaxillary gland with collagenase/trypsin (panel 1). Seeding cells at low density enabled distinction of multiple cell types propogating as foci (panels 2-6). Cells with obviuos epithelial characteristics were subclones and screened for acinar cell markers (alpha amylase, aquaporin 5) and zono occludins-1 (ZO-1) as a tight junction marker.*

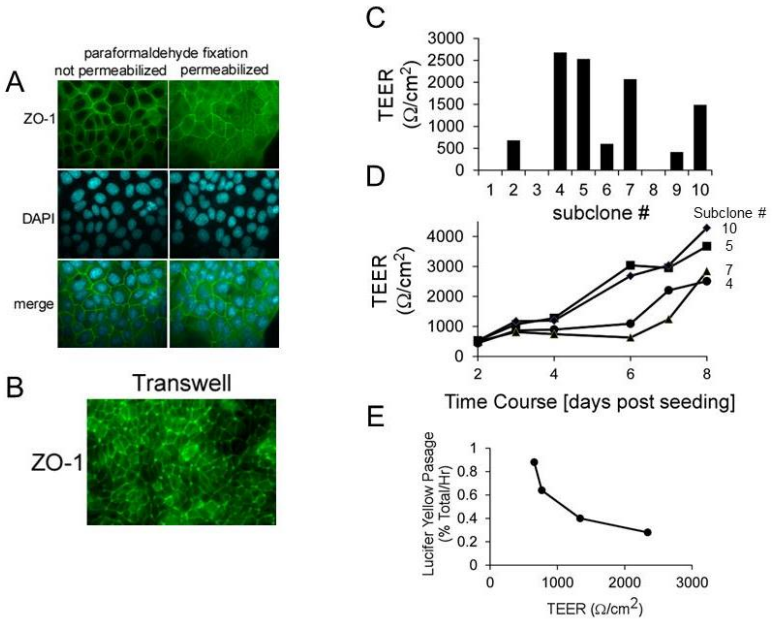


Figure A-4. *Tight junction formation. Panel A&B: Epithelial subclones show ZO-1 localization at points of cell-cell contact on standard tissue culture dishses (A) and in transwell inserts (B). Panel C: TEER values for 10 different epithelial subclones that were seeded in transwell inserts and maintained for 10 days . Panel D: Time course for tight junction formation in selected subclones. Panel E: Relationship between TEER and lucifer yellow passage across the transwell insert.*

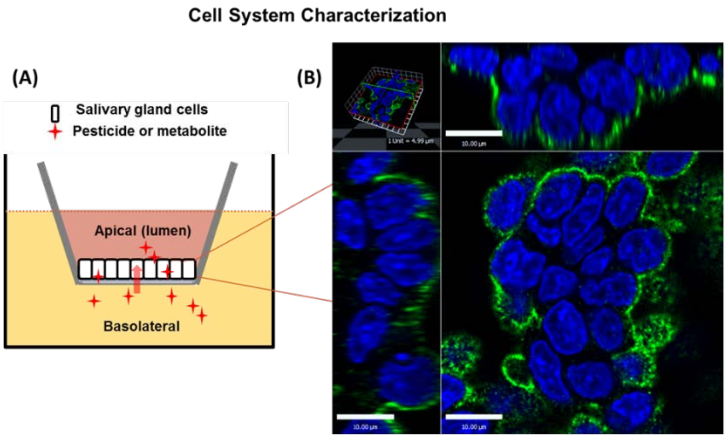


Figure A-5. *Transwell system with serous-acinar cells grown on the Transwell insert that separates the apical (saliva) and basolateral (plasma) compartments (A). Co-localization of DAPI (nuclear stain) shown in blue and Crumbs apical marker of polarity (CRB3) in green of SGEC grown on Nunc Tech II chamber slides 6 days post-seeding demonstrating polarity of cells (B). The cross-sectional y-z axis (vertical) and x-z axis (horizontal) identify CRB3 localization on the apical surface of SGEC.*

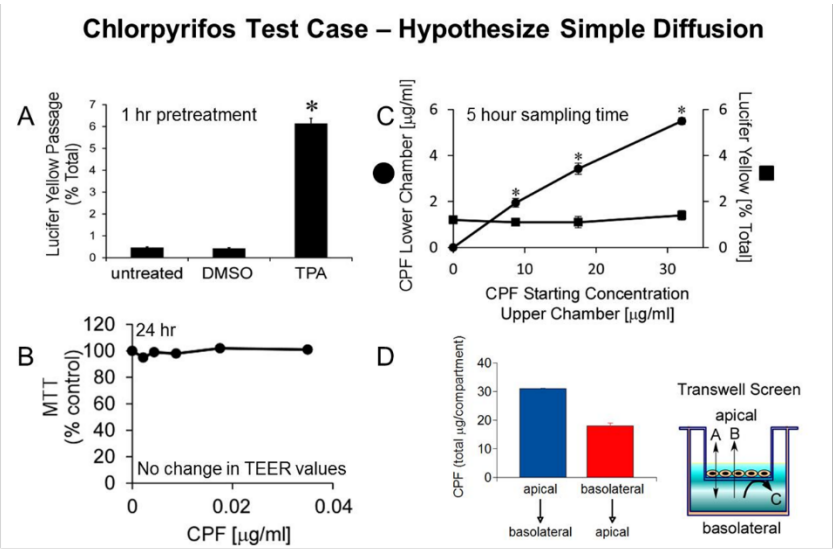


Figure A-6. *Chlorpyrifos passage across salivary gland epithelial cells in a transwell insert. Panel A: Treatment of salivary gland epithelial cells with phorbol ester (TPA) disrupts tight junctions as expected. This observation highlights a need to understand basic toxicology of chemicals being applied to the transwell assay since the receptor for TPA (i.e. protein kinase C) is frequently activated by stress* responsive *signaling. Panel B: Treatment of salivary gland epithelial cells with chlorpyrifos at concentrations used for transport studies does not induce overt toxicity. Panel C: Chlorpyrifos shows a clearly disproportional passage across the epithelial barrier as compared with lucifer yellow. Panel D: Chlorpyrifos exhibits bidirectional passage across the transwell insert, consistent with a diffusional process.*

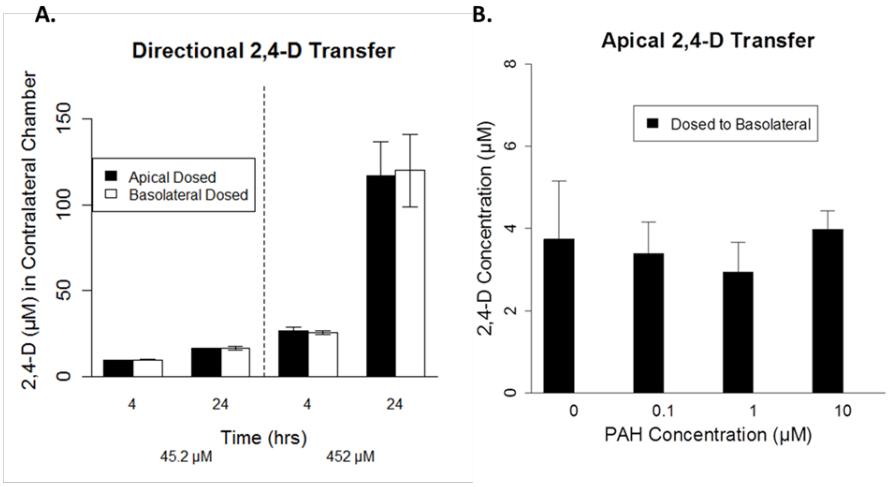


Figure A-7. *2,4-D was dosed [45.2 or 452 µM] to the apical or basolateral chamber and measured from the opposite chamber at 4 and 24 hours post-dose (A) and basolateral to apical transfer of 2,4-D [45.2 µM = 10 µg/ml] dosed in parallel with PAH [0, 0.1, 1, or 10 µM] in the basolateral and measured from the apical chamber at 4 hours post-dose (B).*

Objective B:

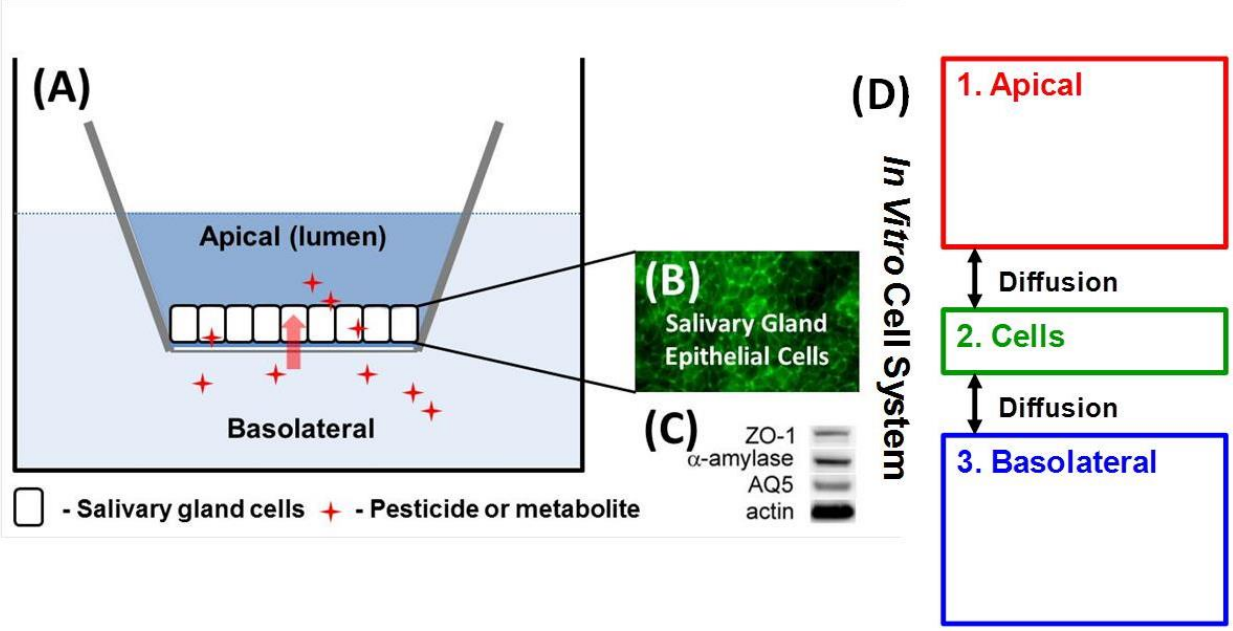


Figure B-1. *Schematic model illustrating* ***(A & B)*** *Transwell® with salivary gland epithelial cell (SGEC) system;* ***(C)*** *Western blot analysis showing expression of ZO-1, α-amylase and AQ5 proteins in SGEC lysates indicative of acinar cells; and* ***(D)*** *3-compartment model structure for calculating chemical transport in the in vitro system.*

$$\frac{dA_1}{dt} = -D_{12} \times SA_2 \times \frac{\left(C_1 - \frac{C_2}{PC_{21}}\right)}{Dis_2}$$

Figure B-2. The *in vitro* cell transport model., modified Fick's Law of Diffusion, where *t* is time (hr), *A* is the amount (nmol), *D* is the diffusion coefficient (cm^2/hr), *SA* is the surface area of cells (cm^2), *Dis* is thickness of the cell membrane (cm), *C* is concentration (nmol/mL), and *PC* is the partition coefficient.

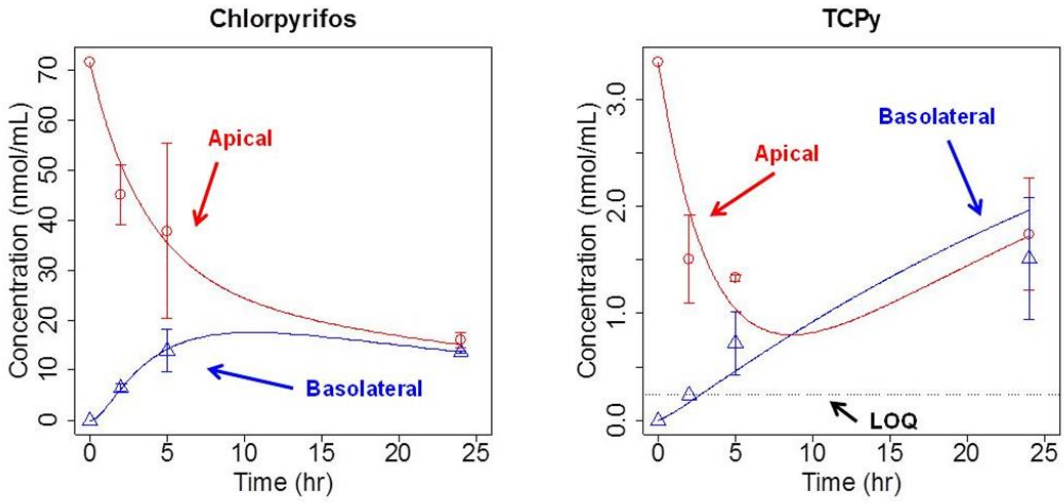


Figure B-3. *Concentrations of chlorpyrifos and TCPy in cell culture media of the apical (red) and basolateral chambers (blue) over time used to parameterize the model. Lines are model fits to the data.*

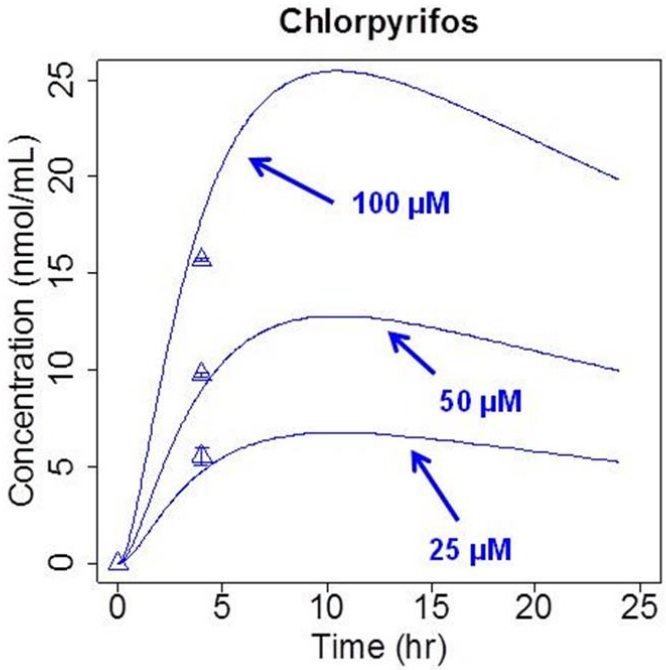


Figure B-4. *Concentrations of chlorpyrifos in cell culture media of the basolateral chamber (blue) after the apical chamber was spiked with 25, 50, or 100 µM chlorpyrifos. Lines are model simulations the data.*

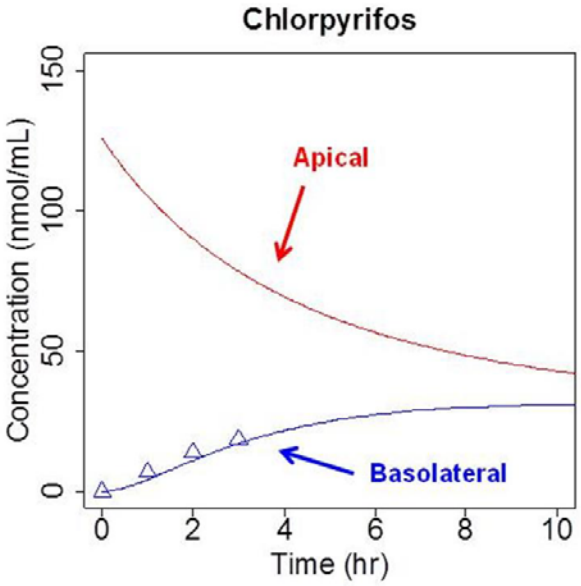


Figure B-5. *Concentrations of chlorpyrifos in cell culture media of the basolateral chambers (blue) after the apical chamber (red) was spiked with chlorpyrifos. Lines are model simulations of the data.*

Table B-1. Input chemical and tissue parameters used to simulate the saliva:blood partitioning for generic over a range of pKa and protein-binding values.

Parameter	Value	Source
Fraction unbound in plasma	0.1, 0.5 or 0.9	Fixed
pK_a	4, 7 or 10	Fixed
Log Kow @ nonionized	2	Fixed
Log Kow @ ionized	-1	Calculated from α
α	0.001	Schmitt 2008
Tissue Parameter	**Value (rat/human)**	**Source**
Plasma		
Fraction protein	0.073	Hold *et al.*, 1995; Ritschel and Tompson, 1983
Fraction water	0.915	Hold *et al.*, 1995; Ritschel and Tompson, 1983
pH	7.4	Smith *et al.*, 2010
Saliva		
Fraction cells	0	Estimated
Fraction protein	0.003	Hold *et al.*, 1995; Ritschel and Tompson, 1983
Fraction water	0.98	Hold *et al.*, 1995; Ritschel and Tompson, 1983
pH	8.9/6.7	Hold *et al.*, 1995; Ritschel and Tompson, 1983; and Smith *et al.*, 2010

Table B-2. Sensitivity analysis for selected parameters for generalized compound plasma:saliva partitioning coefficent. Absolute values for SC >0.5 suggest that model is highly sensitive to this parameter. Model simulations were run at 50% protein-binding and pKa=7.0, K_{ow} values from Table 1.

Sensitivity Coefficient (SC)				
Species	Frac. Unbound in Plasma	K_{ow} Nonionized	K_{ow} Ionized	pKa
Human	**2.0**	**$1.9x10^{-4}$**	**$-1.6x10^{-2}$**	**0.9**
Rat	**2.0**	**$2.2 x10^{-4}$**	**$3.9x10^{-3}$**	**-0.6**

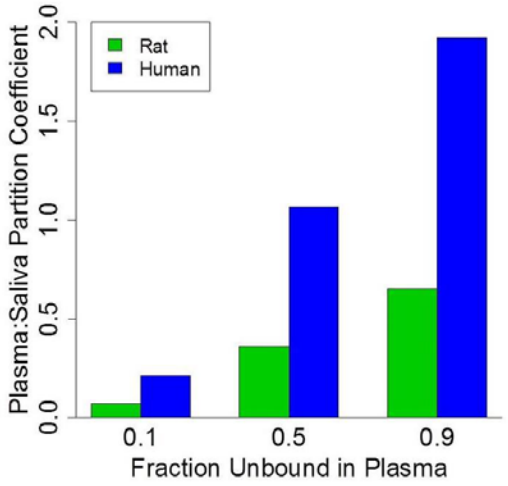


***Figure B-6**. PBPK model simulation of plasma:saliva partitioning coefficients for 3 generic compounds with varying plasma protein-binding.*

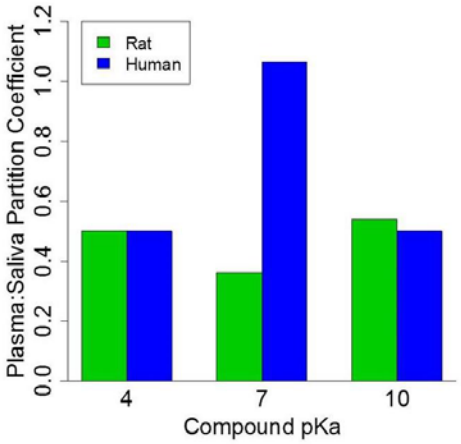


***Figure B-7.** PBPK model simulation of plasma:saliva partitioning coefficients for 3 generic compounds with varying pKa values. Protein binding set at 50%.*

Non-Physiological:

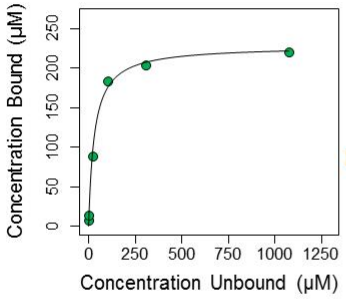


Figure B-8a. Concentrations TCPy bound and unbound to proteins in non-physiological cell culture medium.

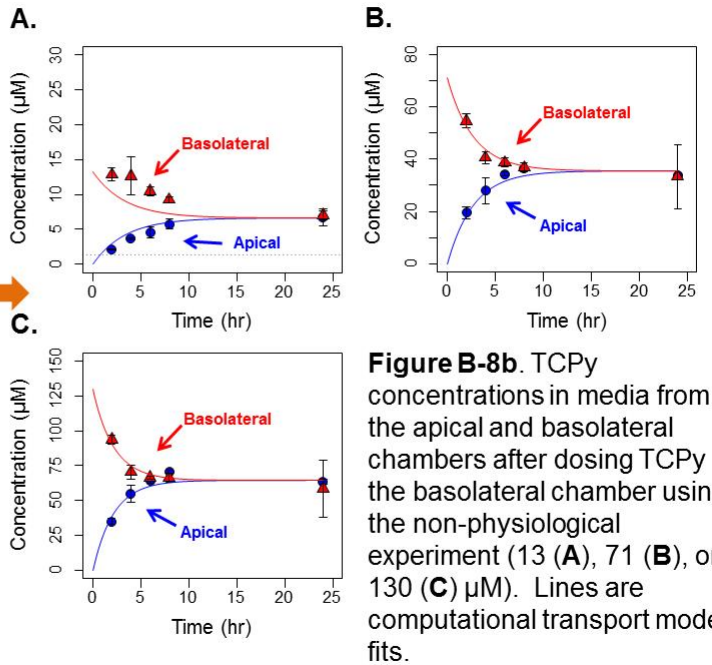


Figure B-8b. TCPy concentrations in media from the apical and basolateral chambers after dosing TCPy in the basolateral chamber using the non-physiological experiment (13 (**A**), 71 (**B**), or 130 (**C**) μM). Lines are computational transport model fits.

Physiological:

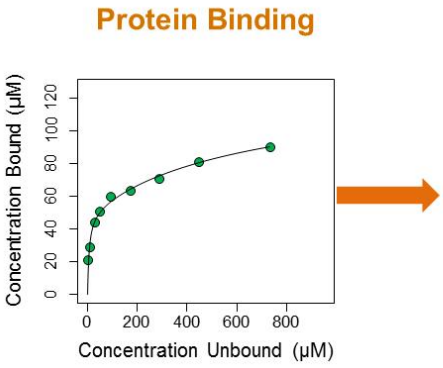


Figure B-9a. Concentrations TCPy bound and unbound to proteins in physiological cell culture medium.

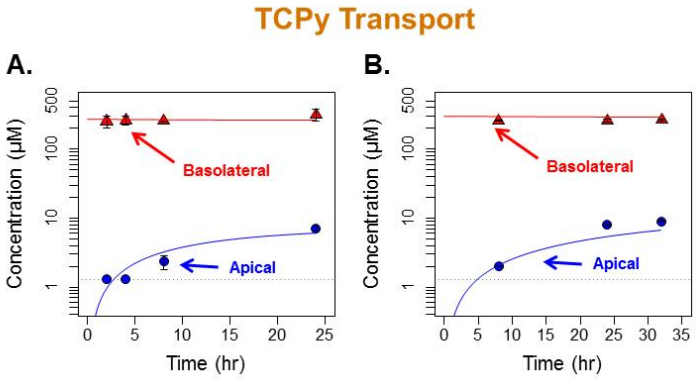


Figure B-9b. TCPy concentrations in media from the apical and basolateral chambers after dosing TCPy in the basolateral chamber during physiological transport experiments. Lines are computational transport model fits.

Non-Physiological:

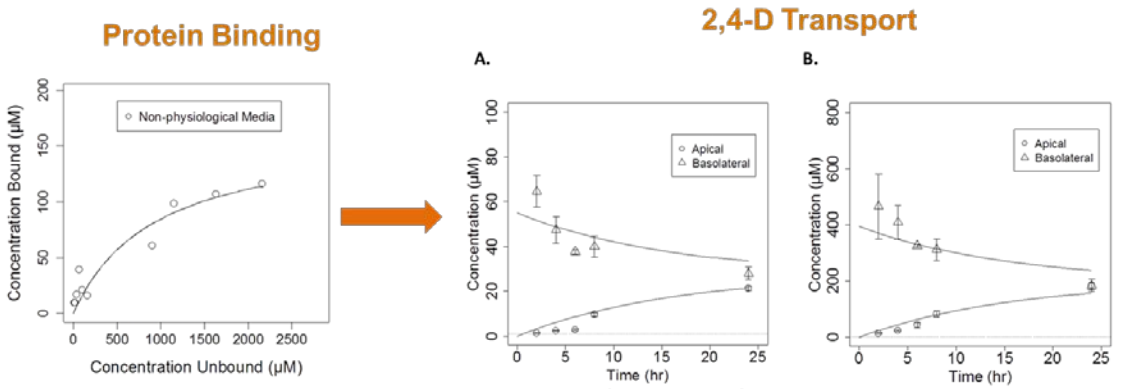


Figure B-10a. Concentrations of 2,4-D bound and unbound to proteins in non-physiological cell culture medium.

Figure B-10b. 2,4-D Concentrations in the cell culture medium from basolateral (triangles) and apical (circles) chambers with 45.2 μM (10 μg/ml) (A) or 452 μM (100 μg/ml) (B) of 2,4-D in the non-physiological experimental condition. Lines are computational model fits to the non-physiological data with a permeation coefficient of 0.04.

Physiological:

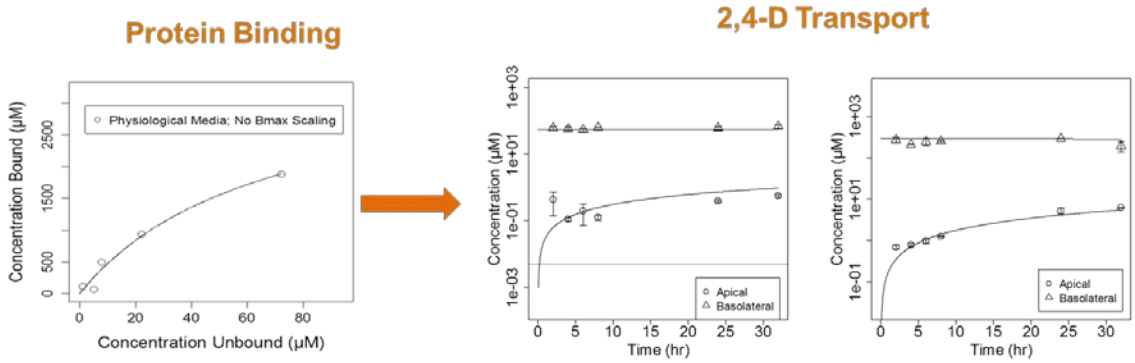


Figure B-11a. Concentrations of 2,4-D bound and unbound to proteins in physiological cell culture medium.

Figure B-11b. Concentrations of 2,4-D in cell culture medium from the basolateral (triangles) and apical (circles) chambers from 45.2 μM (10 μg/ml) (A) or 452 μM (100 μg/ml) (B) 2,4-D dosed to the basolateral chamber. Lines are computational model fits to the data; for clarity only the LOQ (dotted line) is shown for the 45.2 μM dose.

Objective C:

$$\text{Saliva TCP Clearance } Cl_s \text{ (mL min}^{-1}) = \frac{\text{Saliva TCP Conc. (}\mu\text{g mL}^{-1}) \text{ x Saliva Flow (mL min}^{-1})}{\text{Blood TCP Conc. (}\mu\text{g mL}^{-1})}$$

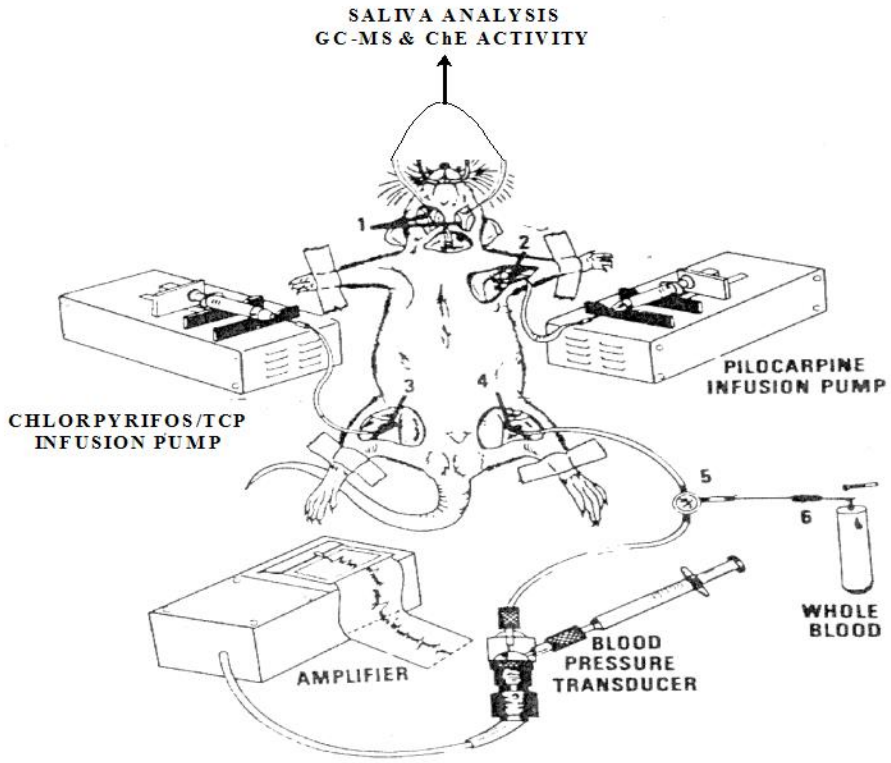


Figure C-1. Illustration of experimental approach for quantifying salivary clearance.

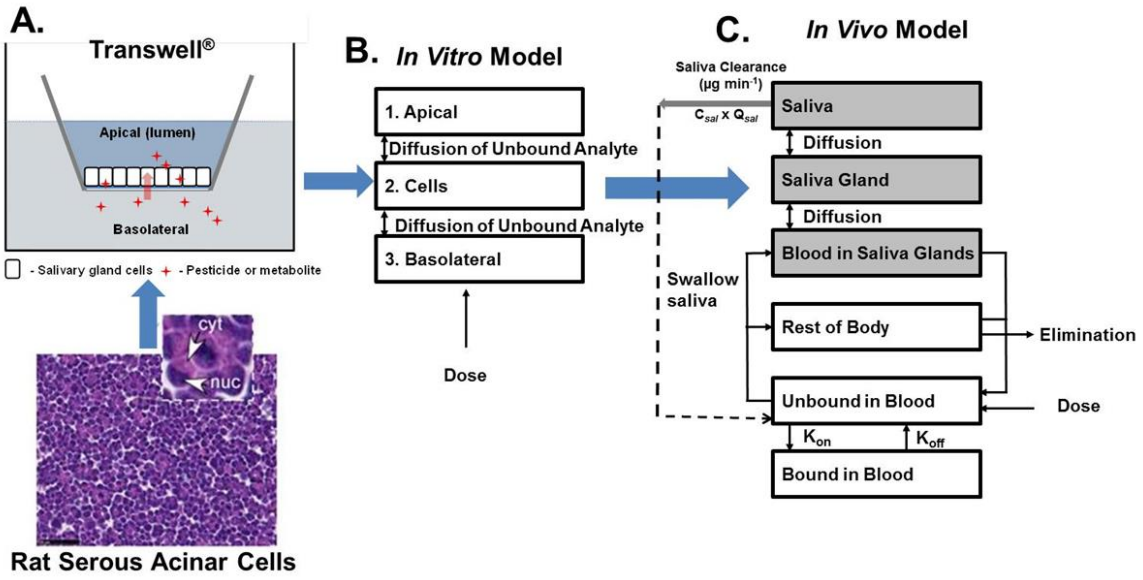


Fig C-2. Salivary Gland Clearance Model: **(A)** *In vitro* cell based assay to determine uptake and clearance; **(B)** *In vitro* computational model consisted of three compartments: the apical cell culture medium chamber, the mono layer of serous-acinar cells grown on Transwell inserts, and the basolateral cell culture medium chamber. Transport of TCPy not bound to proteins was modeled using modified Fick's Diffusion; **(C)** A modified physiologically based pharmacokinetic (PBPK) model. The shaded compartments are analogous to the cellular transport computational model.

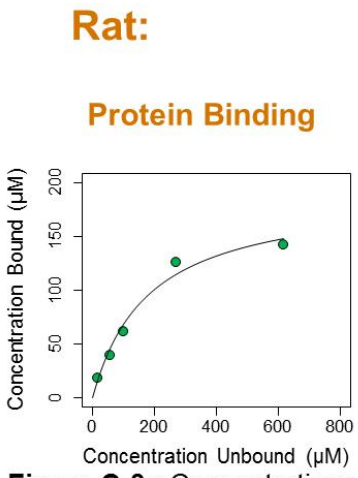


Figure C-3a Concentrations TCPy bound and unbound to proteins in rat plasma.

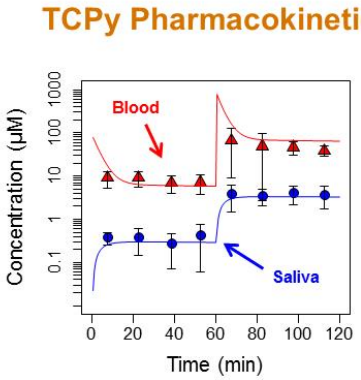


Figure C-3b. TCPy concentrations in blood and saliva of rats being progressively dosed with 1 mg/kg TCPy at 0 min and 10 mg/kg TCPy at 60 min. The line is the modified PBPK model simulation using the protein binding model.

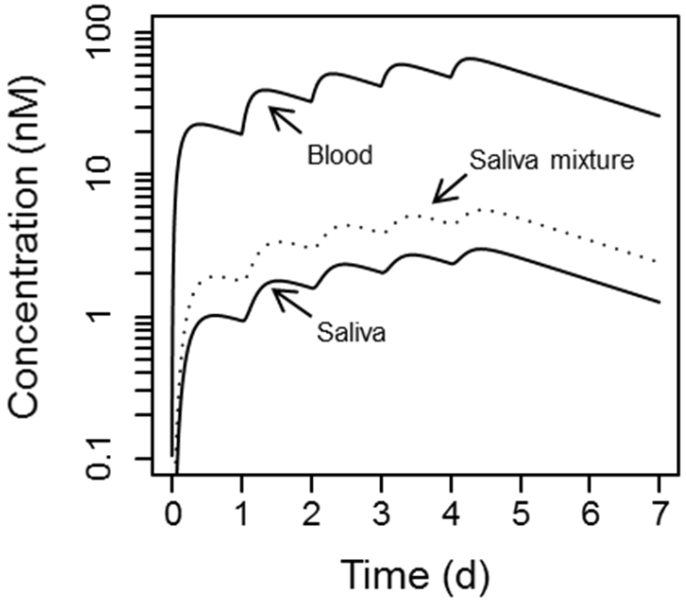


Figure C-4. Predicted TCPy concentrations in blood and saliva of humans exposed to 3 µg/kg/day of chlorpyrifos for 5 days using a modified PBPK model developed here. The dotted line represents the same simulation in a chemical mixture, where other chemicals could compete for binding sites with TCPy. To simulate this scenario, the number of binding sites were reduced by 50%. Note: concentration is on a logarithmic scale.