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DDTSS Paper of the Year 2022:

Species-Specific Urothelial Toxicity With an Anti-HIV Noncatalytic Site Integrase Inhibitor (NCINI) Is Related to Unusual pH-Dependent Physicochemical Changes

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Conflict of Interest Statements

RR is an employee of ApconiX, an integrated toxicology and ion channel company that provides expert advice on non-clinical aspects of drug discovery and drug development to academia, industry, and not-for-profit organizations.

LABN is an employee of Magnolia Toxicology Consulting, providing expert advice on non-clinical aspects of drug discovery and drug development to academia, industry, and not-for-profit organizations.

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Tox Spotlight

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Research Article

Species-Specific Urothelial Toxicity With an Anti-HIV Noncatalytic Site Integrase Inhibitor (NCINI) Is Related to Unusual pH-Dependent Physicochemical Changes

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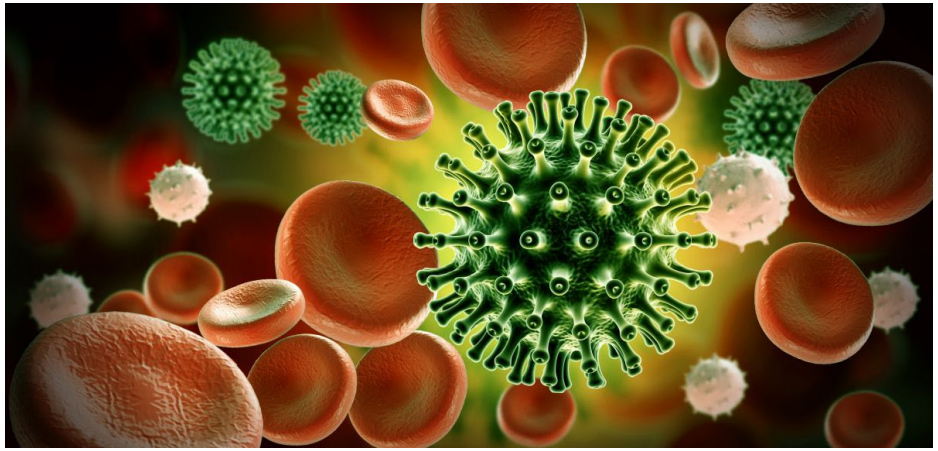
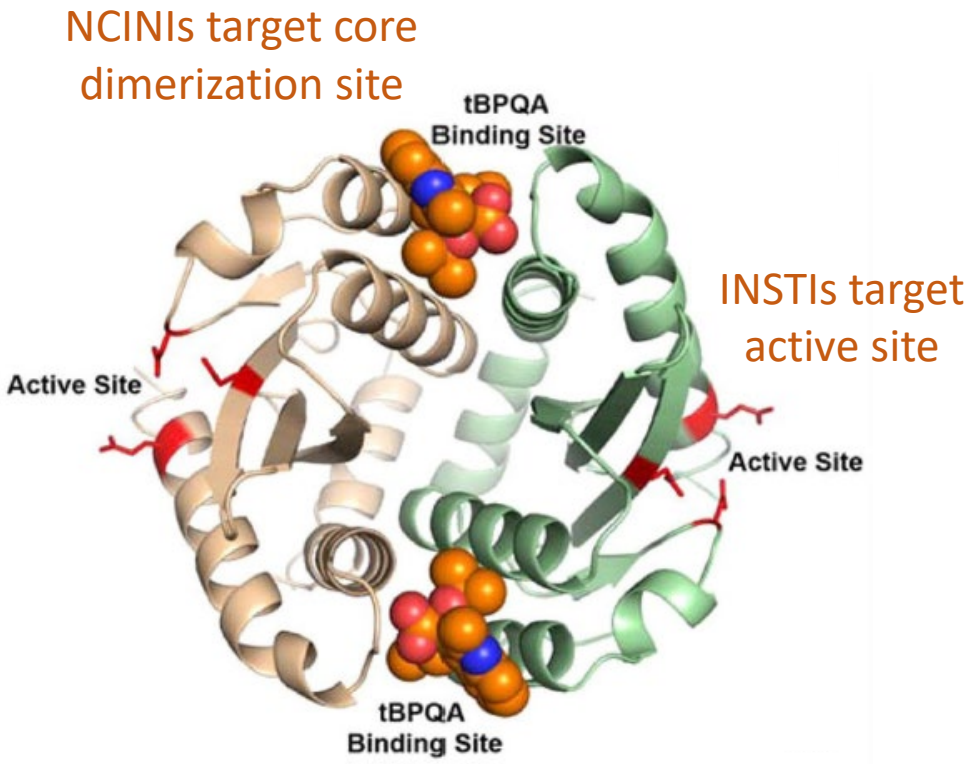
Human Immunodeficiency virus (HIV)-1

- Significant advances in treatment of HIV-1
- Resistance remains a challenge
- Need for new therapeutic drug classes
- HIV integrase catalyzes strand transfer reactions during integration of viral DNA
- The viral integrase enzyme comprises 3 domains: N-terminal, catalytic core, and C-terminal



Non-catalytic Site Integrase Inhibitors (NCINI)

- At the time, INSTIs represented a new therapeutic approach
- INSTIs target the catalytic domain, inhibiting strand transfer during viral DNA integration
- NCINI are novel allosteric inhibitors causing disruption of HIV-1 virion core maturation and assembly rendering it unable to replicate in target cells
- Interference with two distinct steps of integration through the same binding site = new antiviral paradigm



HIV-1 Integrase Enzyme Catalytic Core Domain

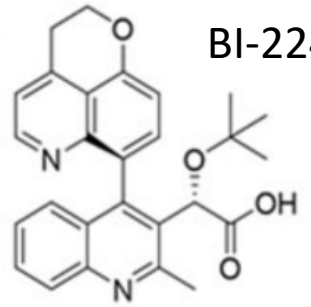
Tsiang et al., New class of HIV-1 integrase (IN) inhibitors with a dual mode of action, *J. Biol. Chem*, 287(25):21189-21203, 2012 (adapted from Figure 1)

GS-9695 and GS-9822

- GS-9695 and GS-9822 are next-generation NCINIs
- They have significantly improved potency compared with the previously described NCINI BI-224436 (Mitchell et al., 2017)
- GS-9695 progressed for early tox testing

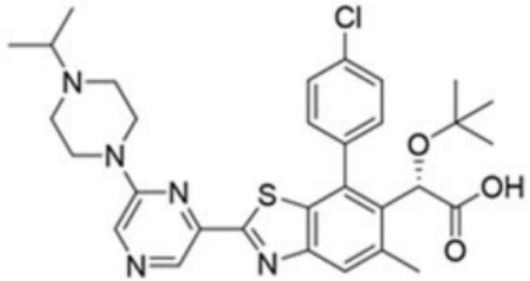
Note: GS-9695 and GS-9822 are zwitterions with pKa values of 4 and 7.8 (GS-9695) and 4.2 and 5.8 (GS-9822)

A

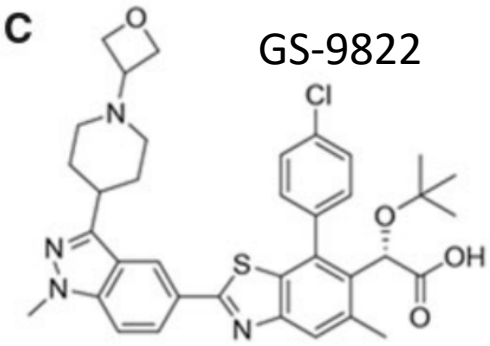


BI-224436

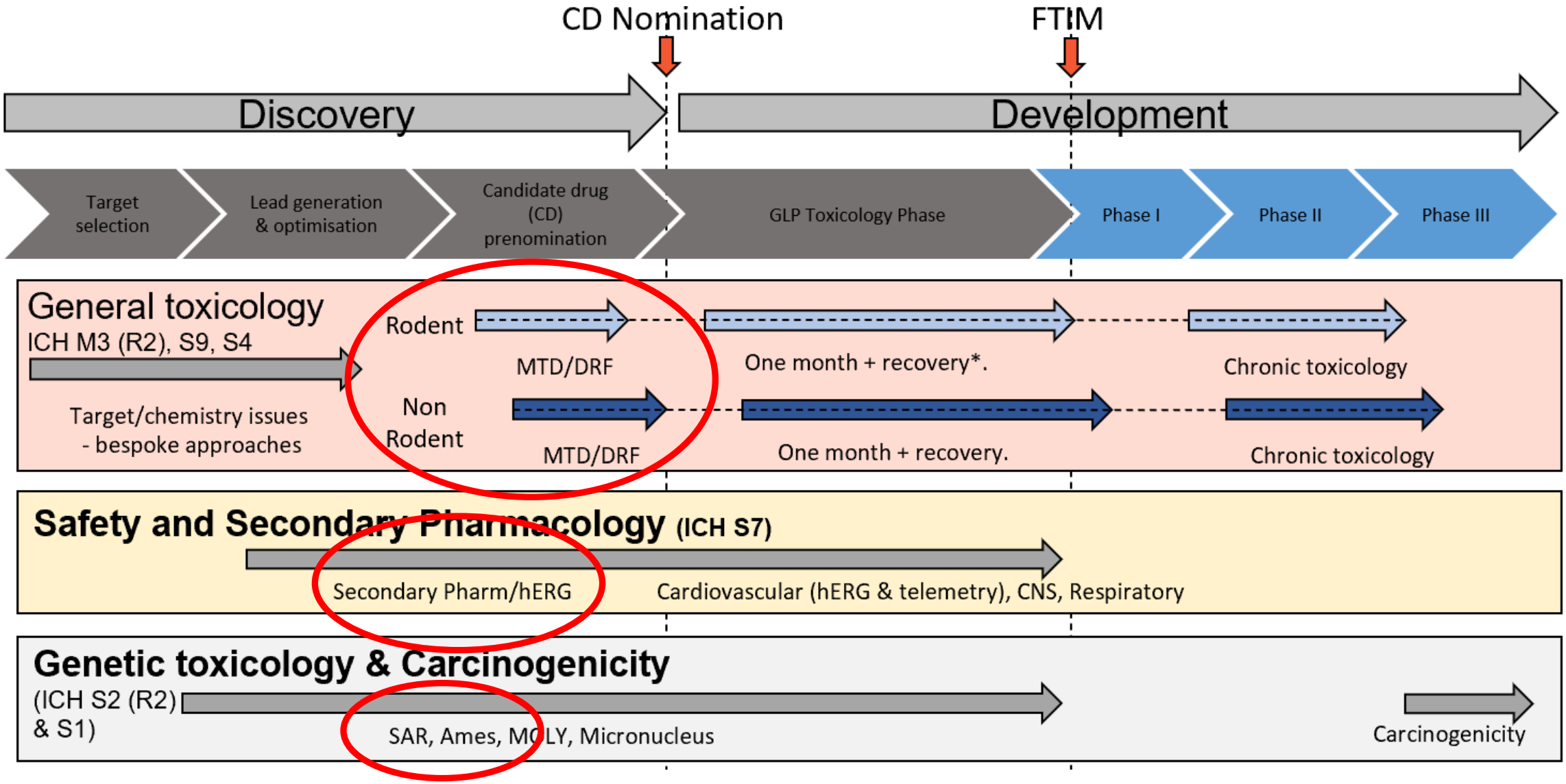
B GS-9695



C GS-9822

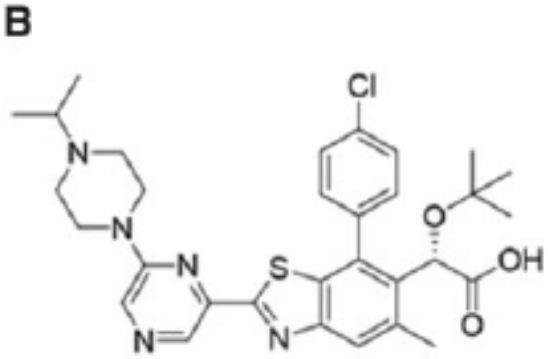


Early Tox Testing - Orientation!



See Walker, Iman and Roberts, 2018: Drug Discovery and development: Biomarkers of Neurotoxicity. EBM, 243, 1037-1045.

GS-9695

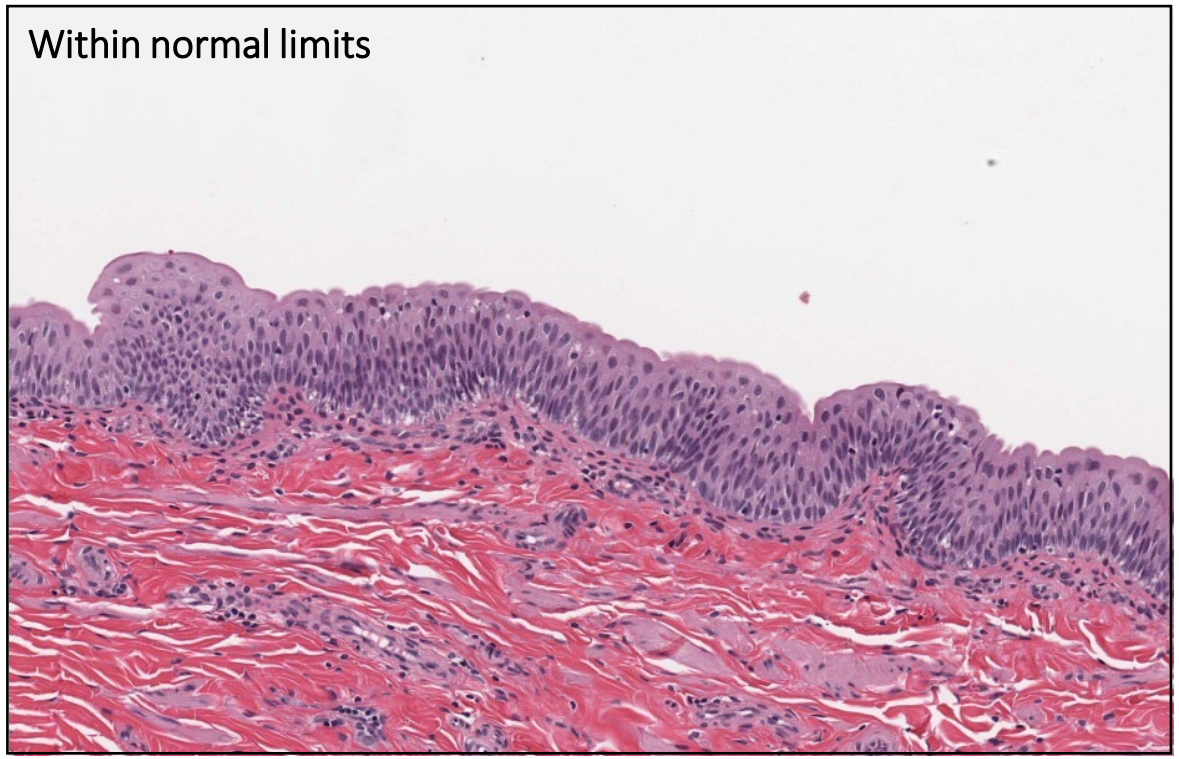


- Screening study data
  - hERG: negative up to the limit of solubility (10 uM)
  - Ames: negative
  - Molecular Target Screen (secondary pharmacology): minimal hits
- Selected for DRF studies to support nomination as a development candidate
- Species selected based on metabolic profile & PK
  - 14-Day Rat – no bladder effects
  - 7-Day NHP – urothelial toxicity in renal system (kidney, bladder, ureter)

GS-9695 7-Day Cynomolgus Monkey - Bladder

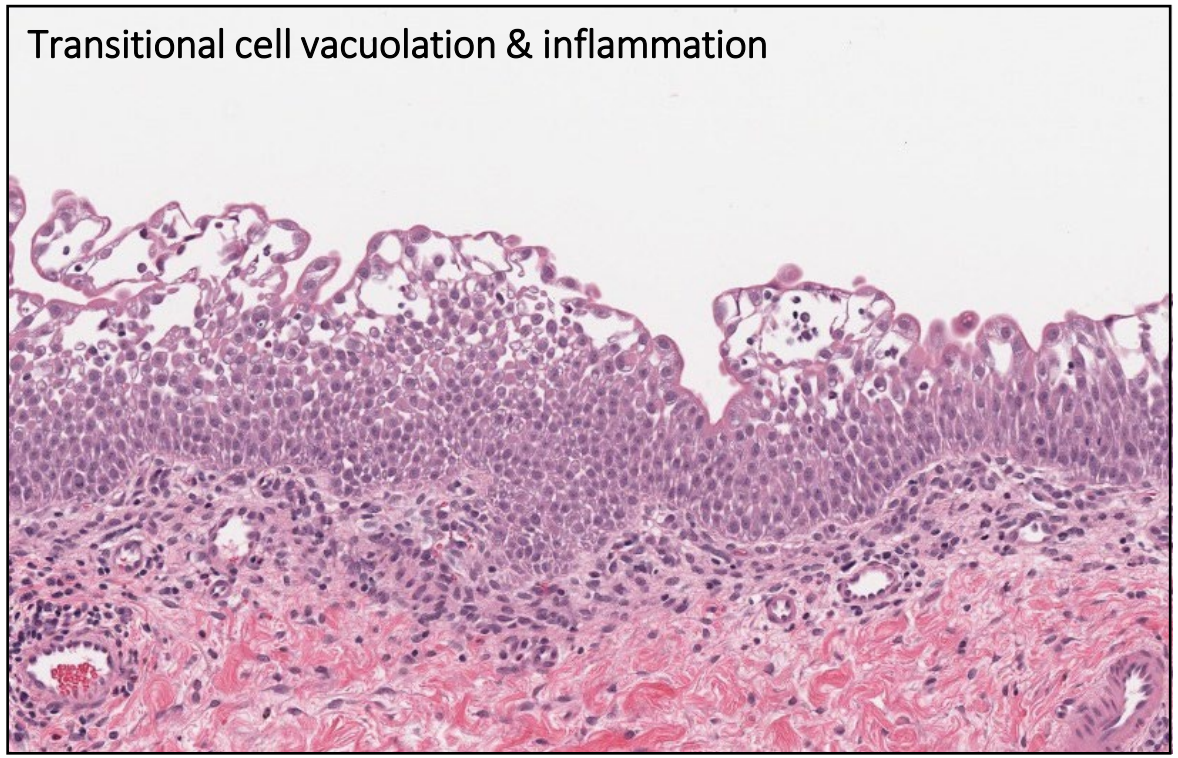
Vehicle

Within normal limits



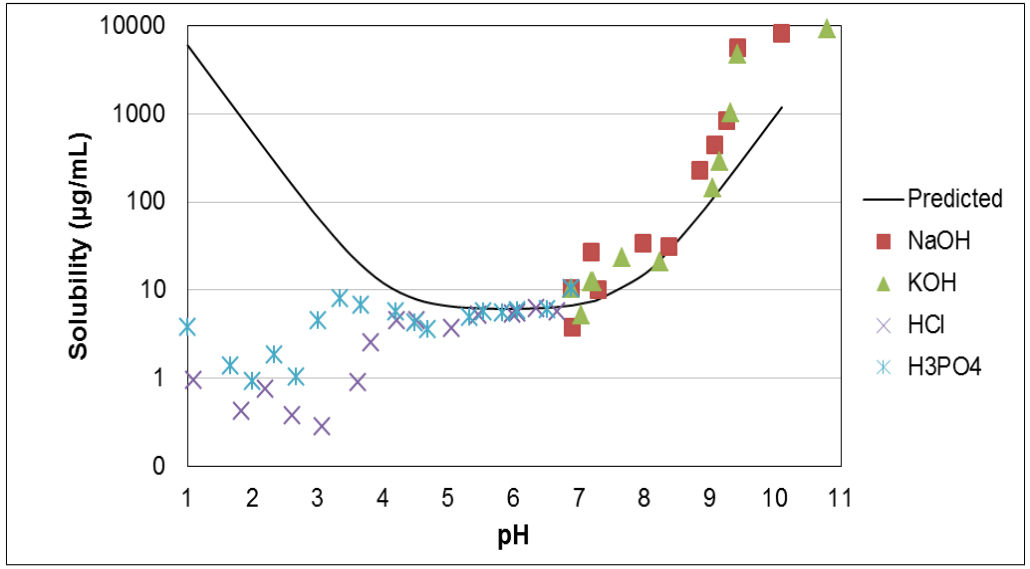
300 mg/kg/day

Transitional cell vacuolation & inflammation



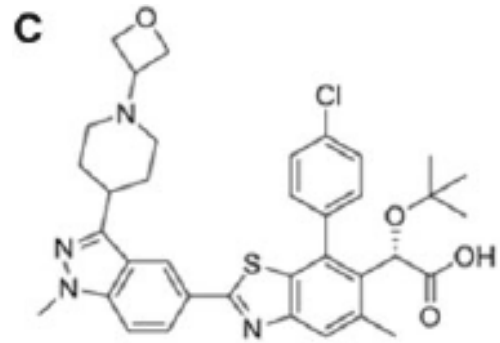
Initial Follow Up on GS-9695

- Question: Artifact of tissue processing?
- Question: Macrophage activation in NHP?
- Question: Crystallization in urine → irritation?
  - Low solubility, 2 pKa's (zwitterion; 4, 7.8)
  - Likely saturating a clearance pathway
  - Urine collection for concentration, crystals
    - Method - pan, cystocentesis
    - When? 8, 12, or 24 hr post dose? time course better?
- Repeat 7-day study, evaluate delays in harvesting tissue vs immediate and get EM; assess potential macrophage activation; measure urine concentration



Not artifact.
No apparent effect on macrophage activation.
No crystals observed but suggestive something unique was happening in urine/bladder

Back-Up Candidate: GS-9822



- Screening studies
  - hERG: negative up to the limit of solubility (10 uM)
  - Ames: negative
  - Molecular Target Screen: minimal hits
- Conduct DRFs
  - 14-Day Rat – no bladder effects
  - 7-Day NHP – same observation as with GS-9695

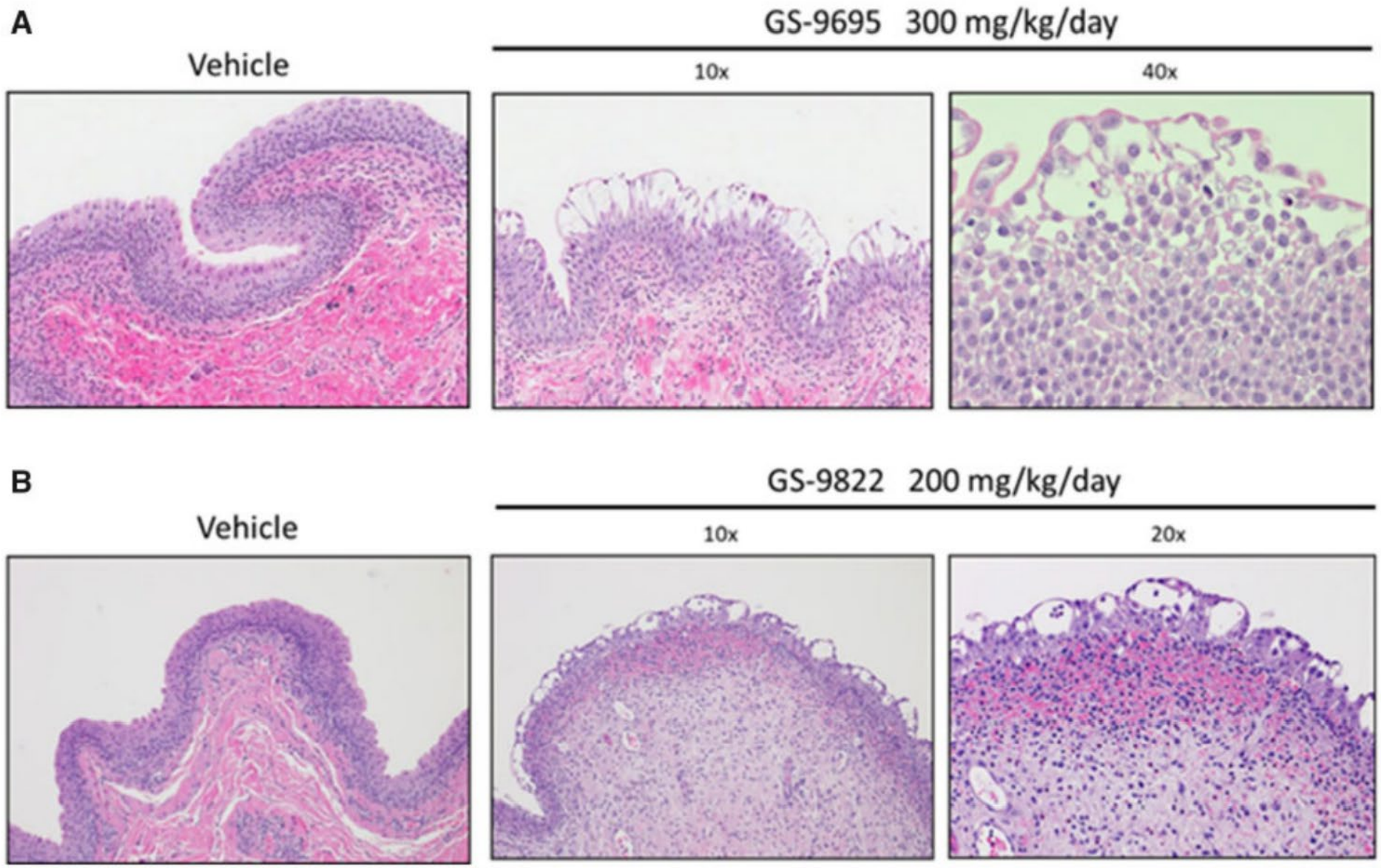
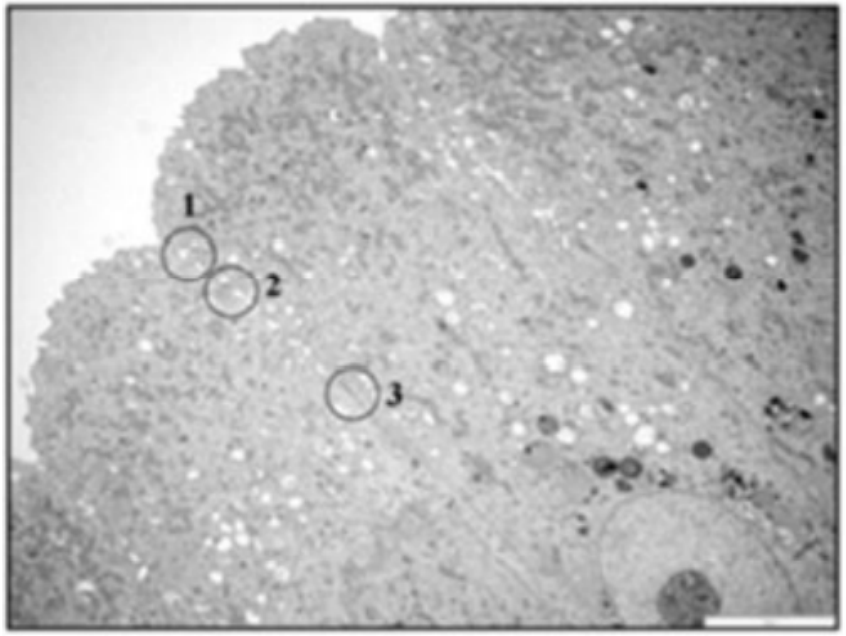


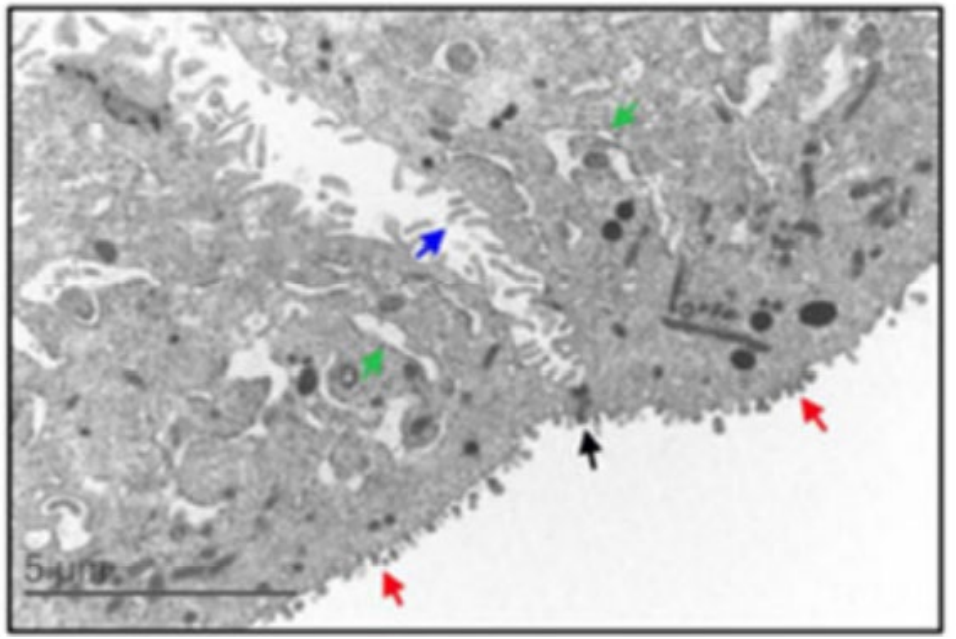
Figure 2. Representative images of cynomolgus monkey bladder urothelium stained with hematoxylin and eosin at 7 days oral gavage treatment with either (A) GS-9695 at 0 (vehicle; 10× magnification) or 300 mg/kg/day at 10× magnification (middle) or 40× magnification (right) or (B) GS-9822 at 0 (vehicle; 10× magnification) or 200 mg/kg/day at 10× magnification (middle) or 20× magnification (right). Transitional cell vacuolation, inflammation and hemorrhage are evident in the treated sections compared with the vehicle only controls.

Figure 3. Representative TEM images of cynomolgus monkey urothelium.



Control

Junctional complexes are intact:
1 tight junction or zona occludens;
2 intermediate junction or zona adherens;
3 desmosome or macula adherens



GS-9822

Green arrows - 2 moderately injured superficial cells with increased cytoplasmic vacuolation.
Blue arrows - marked separation of the cells, with the presence of prominent microvilli
Red arrows - projections from the luminal surface are shorter and plumper than in controls

So now what?

- Immunological activation* – some changes but highly variable and inconsistent with the observed species specificity
- Urinary stability* - see next slide
- Mitochondrial toxicity?
- Interference with adhesion?

*: mentioned earlier in passing as unlikely

- **Urinary concentration and stability GS-9695**
- Concentration - although group means varied between rats and cyno, data points overlapped between species
- Solubility in urine was similar between rat and cynomolgus monkey
- No unexplained solid material, crystalline or otherwise, was detected in any urine samples

Mitochondrial toxicity

- Mitochondrial toxicity was evident with both compounds
- Difficult to reach any conclusion on relevance for the observed species differences in the bladder effect since in PC3 human cells
- Unlikely as a MOA since histopathology indicated inflammation and disruption of the urothelial morphology rather than cellular necrosis or loss of cellular viability
- Also if mitochondrial dysfunction was the primary MOA, we would have expected to see more systemic and widespread toxicity, especially in tissues such as the heart that are highly susceptible to mitochondrial toxicants

So now what?

- Immunological activation* – some changes but highly variable and inconsistent with the observed species specificity
- Urinary stability* - see next slide
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Immunohistochemistry for E-Cadherin and Pan-cytokeratin in Cyno Tissues

Table 4. Expression patterns of E-Cadherin and Pan-Cytokeratin Staining in Cynomolgus Monkeys Treated With GS-9695

		Control	Treated
E-cadherin	Superficial and upper intermediate urothelium	+++	+
	Lower intermediate and basal urothelium	+++	+++
Pan-cytokeratin	Superficial and upper intermediate urothelium	++	+
	Lower intermediate and basal urothelium	++	++

Staining intensity: 0, negative; +, mild; ++, moderate; +++, marked.

Reduction in E-cadherin (implicated in cell-cell interactions)
Reduction in pan-cytokeratin (implicated in intra-cellular stability)
But:

- No data for the rat bladder
- No plausible reason for the observed species differences in the bladder lesion
- Interpretation of these findings in the context of a possible MOA is challenging.

So now what?

- Brain storming session (another one!)
- Lesion is on the urinary side of the urethra (not just bladder)
- The lesion looks like sloughing of entire sheets of viable cells
- Urine is the only unbuffered solution in the body…..

Idea!

- Observed lesions are irritant-like?
- Physicochemical changes?
- Urine differs markedly from plasma in osmolality and acidity and is not buffered?
- Might GS-9822 be demonstrating detergent-like properties in monkey urine?

Dr Richard A. Campbell

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The University of Manchester

'I work at the boundary between physical chemistry and physical pharmacy. Surface-sensitive experimental techniques including optical and neutron reflectometry are used in my research to probe mixed systems at fluid interfaces. The underlying aim is to solve complex interactions mechanisms that are often dominated by non-equilibrium effects.'

Approach

- Evaluation of the surface activity of GS-9822 with respect to pH in solutions with salts added to mimic ionic strength of urine
- Range of pH values reflected urinary pH which is wider in nonhuman primates (normal range 5.5– 7.4) and humans (normal range 4.8–7.8) compared with rodents (7.3–8.5)
- Two parameters
  - Surface pressure - ability of the drug to lower the surface tension of the air/water interface (ie, potential to act as a surfactant)
  - Ellipsometric phase shift, d(delta) - a measure of the amount of drug adsorbed to the air/water interface.

Results

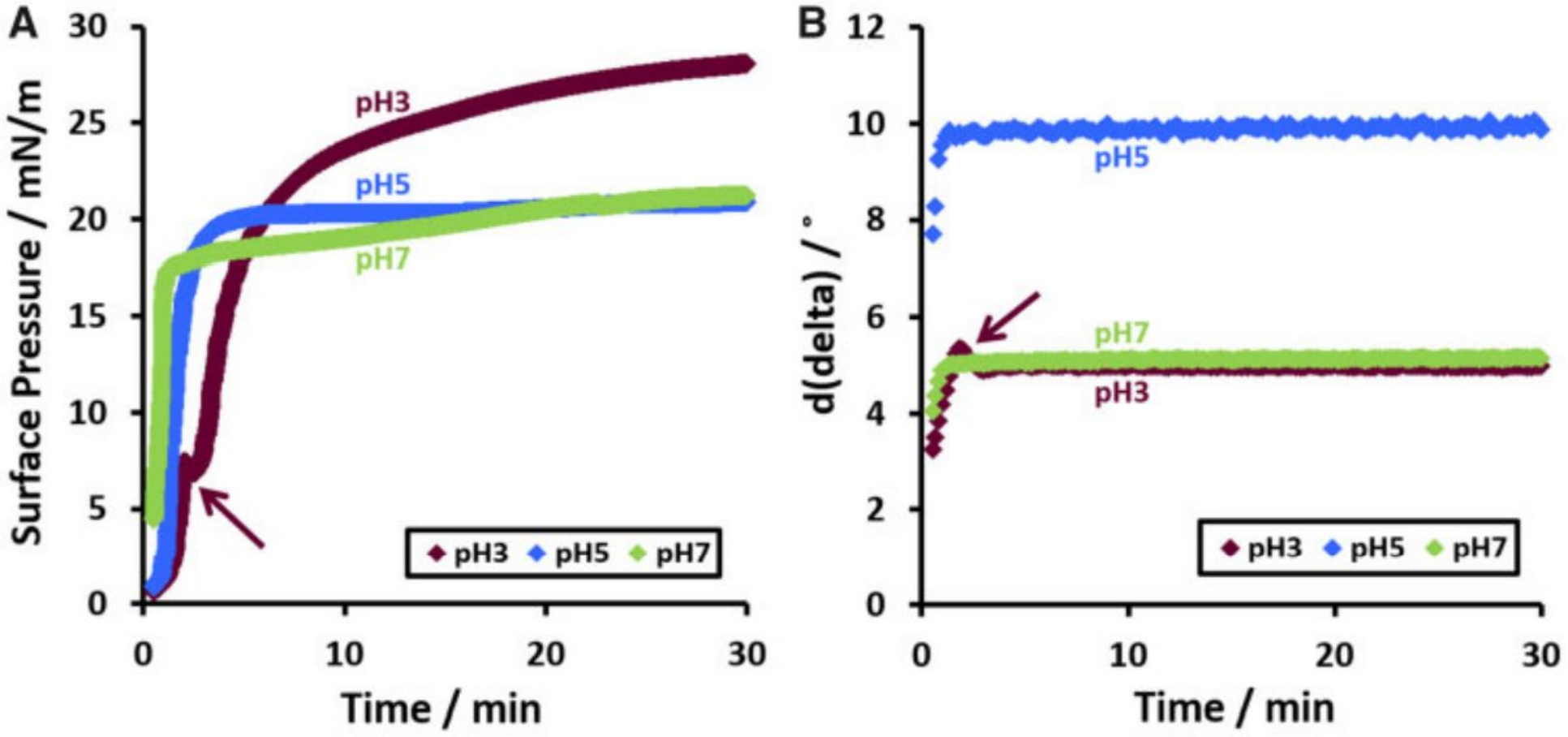
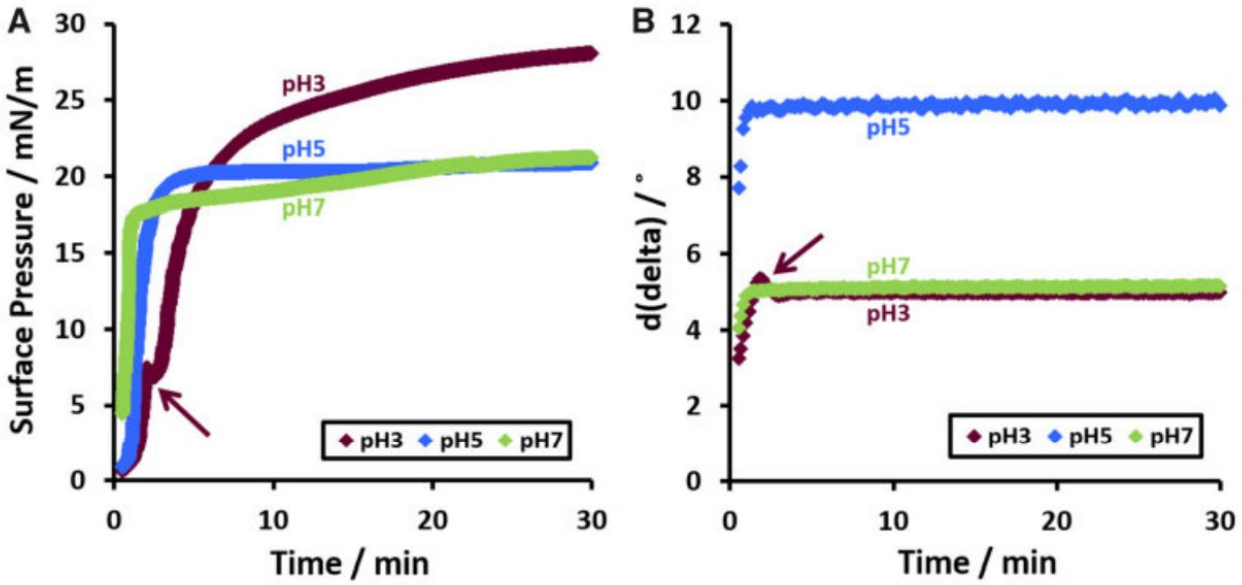


Figure 5. (A) the surface pressure and (B) ellipsometric phase shift at the air/water interface for solutions of 20 μM GS-9822 The former (A) is related to the ability of the drug to lower the surface tension of the air/water interface and the latter (B) is a measure of the amount of adsorbed drug.

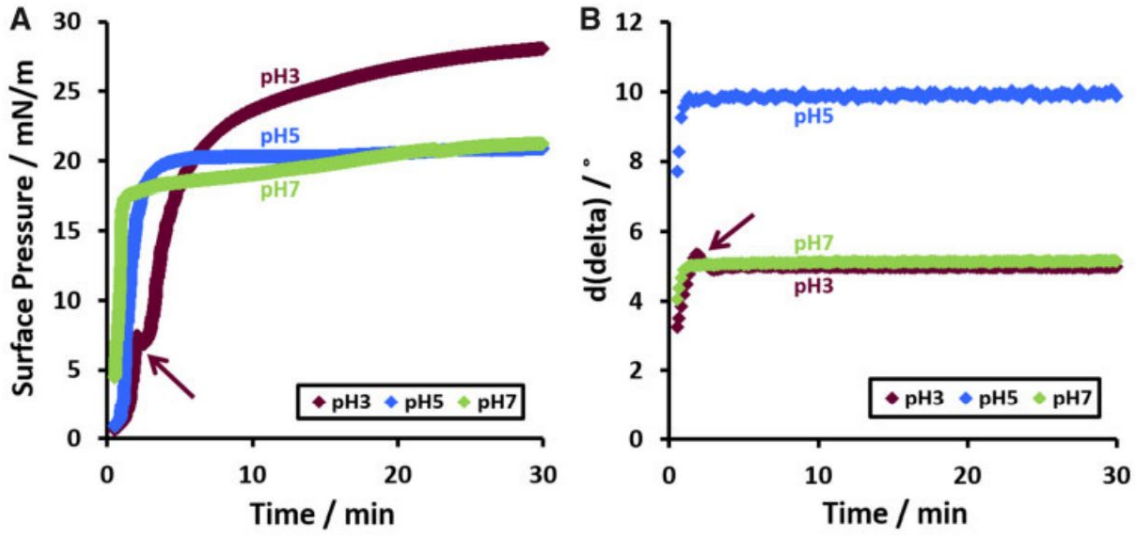
Results

- The molecule showed an unusual transition from a monolayer to a bilayer at the air/water interface at pH 5



- Attributed to the strong association between drug molecules in adjacent bilayer leaflets resulting from a zwitterionic characteristic that exists over only a narrow range of pH values (pH 4–7)

Implications: molecular transitions

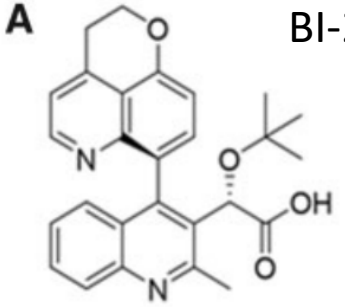


- This surface transition would not be expected to occur in rat urine due to a different pH range
- Plausible that it may occur in humans
- A transition of an adsorbed surfactant from monolayer to a bilayer at the air/water interface is a newly discovered phenomenon, first reported as an unexpected finding recently (Honnigfort et al., 2020).

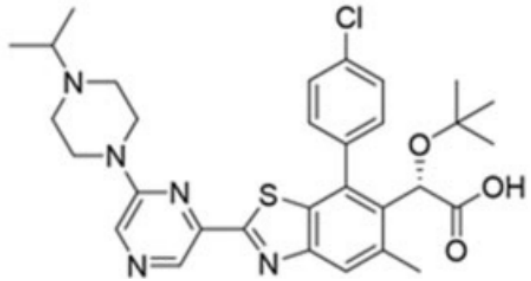
Physicochemical Analysis

- GS-9822 and GS-9695 are rigid due to aromatic groups which restrict conformational change
- Plus, the zwitterionic nature of the molecule means the groups at either end have different potentials for ionization depending on the pH
- Importantly, BI-224436 is markedly different - there is neither a piperidine nor a piperazine group in BI-224436 and so its carboxylic group will transition from neutral to cationic (never zwitterionic) with decreasing pH

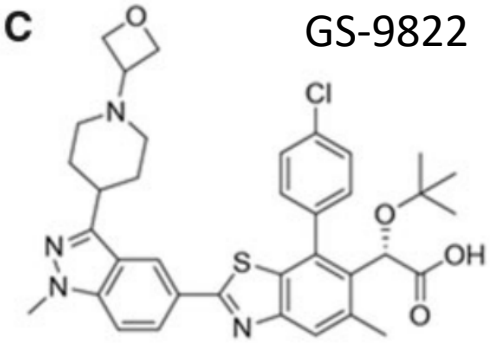
A BI-224436



B GS-9695



C GS-9822



Hence it follows that the exotic surface behavior observed with GS-9822 would be absent in BI-224436

Studies that would have been ‘useful’…

- Re-run the cyno studies with the new hypothesis in mind!
  - Collect urines for accurate PK
  - Measure urinary pH
  - Include BI-224436 as a comparator
- Rodent study to generate treated tissues for immunohistochemistry
- Structural characterization of GS-9822 using neutron reflectometry – resolves structural transitions of drugs and their interaction with biological membranes

….but cannot be conducted due to ethical and practical reasons

Key Conclusions

- GS-9695 and GS-9822 are next-generation NCINIs
- A bladder lesion in the monkey halted development
- Potential MOAs inconclusive
  - secondary pharmacology
  - mitochondrial respiration
  - immune activation
  - interference with the expression of function of cellular adhesion molecules
- Studies of surface pressure pointed to a surface rearrangement during the course of drug adsorption where the drug molecules switch conformation, a transformation expected to be highly disruptive to the integrity of the cell membrane
- The structure-toxicity relationship inferred from the data on GS-9822/9695 compared with BI-224436 suggest that novel NCINIs can be developed providing modelling is employed to ensure the rigid zwitterionic characteristics described here can be avoided

Acknowledgments – the authoring team!

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Locations of Contributing Organisations

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G2, Kibron, Finland

EP4, Accurion, Germany

Covance, Madison, WI

EUROFINS, Panlab, Taiwan

WIL Ohio

Gilead, Foster City, CA

EPL, Virginia

Virginia Tech, Blacksburg

EPL Durham, NC

Mitologix, Paris France

Charles River Laboratories,
St Germain-Nuelles

In Summary

- Many thanks to DDTSS for choosing our paper!
- A complex toxicological story with many twists and turns
- Multiple organisations contributing
- A tribute to team work, imagination and collaboration!

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