An Investigation into Retigabine (Ezogabine) Associated Dyspigmentation in Rat Eye by MALDI Imaging Mass Spectrometry (IMS)

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Introduction

Retigabine (RTG) (other names: Potiga, ezogabine)[1]

- Potassium channel opener - KV7.2-7.5 (KCNQ2/3) in brain neurons
- adjunct treatment of refractory partial-onset seizures in adults

FDA Safety Communication (April 2013)

- RTG could cause changes in retinal pigmentation and discoloration of skin resulting in a blue appearance (**Figure 1**)
- Associated with long RTG treatment intervals of over four years on average
- 38 of 605 patients had developed skin discoloration (6.3%)
- 11 of 36 patients given eye exams were found to have retinal pigment abnormalities – unknown if these changes could result in vision loss

Figure 1 Examples of blue skin discoloration associated with RTG (FDA Drug Safety Communication)

FDA Issues Black Box Warning (Oct. 2013)

WARNING: RETINAL ABNORMALITIES AND POTENTIAL VISION LOSS

GSK initiated numerous studies to gain mechanistic understanding of the origins of retinal pigment changes. One of these efforts was a long term ocular toxicity risk assessment in rats. MALDI IMS was used to determine the presence and distribution of RTG and its metabolites in the rat eye following 13 and 39 weeks of dosing.

Methods

All animal procedures were conducted in an American Association for the Accreditation of Laboratory Animal Care (AALAC)-accredited facility at GlaxoSmithKline (GSK) in accordance with GSK policies on the care, welfare and treatment of laboratory animals and they were reviewed and approved by GSK's Institutional Animal Care and Use Committee (IACUC) as appropriate. RTG was administered to pigmented male Long Evans (HsdBlu:LE) rats for 91 days (Group 2) or 271 days (Group 3) by oral diet administration at a dose of 0 (vehicle; Group 1) or nominal dose of 100 mg/kg/day (Groups 2&3). Rats were necropsied on days 91 or 272 and eye tissues were collected on days 91 or 272 for evaluation by MALDI IMS.

Eye tissues were embedded in pHPMA (150 mg/mL pHPMA in H_2O). Thin sections (6 μm) of the embedded eye tissues were collected in a cryostat and mounted onto ITO coated glass microscope slides. An optical image of each tissue section was generated using an Aperio ScanScope (Leica, Buffalo Grove, IL) digital slide scanner (20x magnification) prior to matrix application. Serial sections (6 μm) were collected for H&E staining.

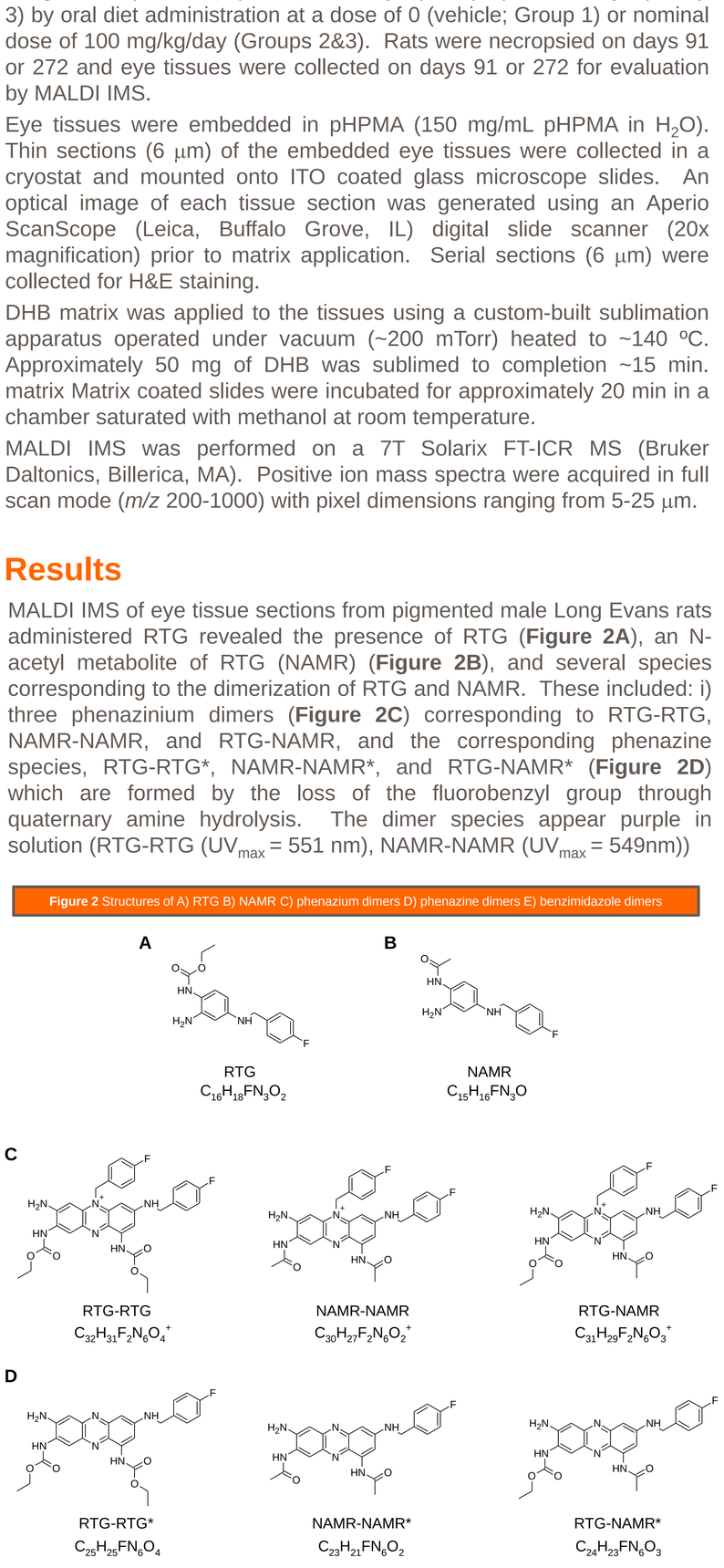
DHB matrix was applied to the tissues using a custom-built sublimation apparatus operated under vacuum (~200 mTorr) heated to ~140 ºC. Approximately 50 mg of DHB was sublimed to completion ~15 min. matrix Matrix coated slides were incubated for approximately 20 min in a chamber saturated with methanol at room temperature.

MALDI IMS was performed on a 7T Solarix FT-ICR MS (Bruker Daltonics, Billerica, MA). Positive ion mass spectra were acquired in full scan mode (*m/z* 200-1000) with pixel dimensions ranging from 5-25 μm.

Results

MALDI IMS of eye tissue sections from pigmented male Long Evans rats administered RTG revealed the presence of RTG (**Figure 2A**), an N-acetyl metabolite of RTG (NAMR) (**Figure 2B**), and several species corresponding to the dimerization of RTG and NAMR. These included: i) three phenazinium dimers (**Figure 2C**) corresponding to RTG-RTG, NAMR-NAMR, and RTG-NAMR, and the corresponding phenazine species, RTG-RTG*, NAMR-NAMR*, and RTG-NAMR* (**Figure 2D**) which are formed by the loss of the fluorobenzyl group through quaternary amine hydrolysis. The dimer species appear purple in solution (RTG-RTG (UV_{max} = 551 nm), NAMR-NAMR (UV_{max} = 549nm))

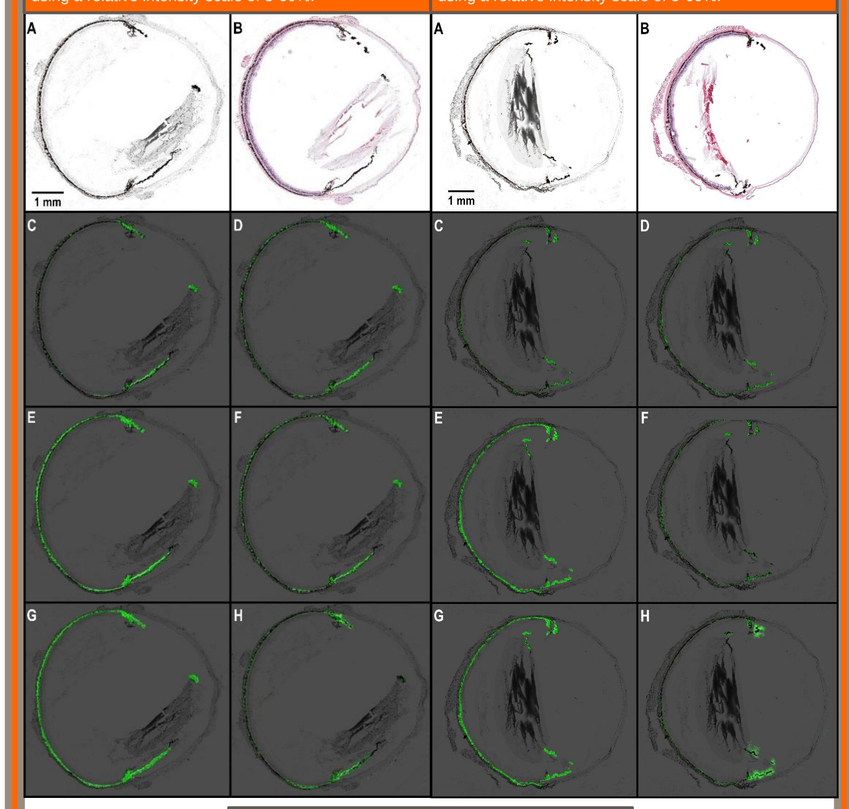
Figure 2 Structures of A) RTG B) NAMR C) phenazium dimers D) phenazine dimers E) benzimidazole dimers



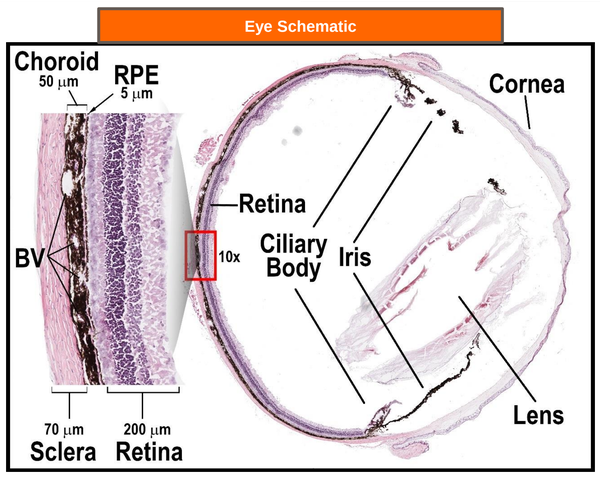
Distribution of several of RTG-related species detected by MALDI IMS (25 μm pixel dimensions) from two separate rats are displayed in **Figures 3** and **4**. The same spatial distribution was observed for these species and they appeared to be localized to the melanin containing layers of the uveal tract including the choroid, ciliary body and iris (see eye schematic).

Figure 3 Group 2 **Rat-1** eye A) Pre-matrix optical B) Serial H&E C) NAMR [M+K]+ (m/z 312.0909) D) RTG [M+K]+ (m/z 342.1015) E) NAMR-NAMR* [M+K]+ (m/z 471.1342) F) RTG-RTG* [M+K]+ (m/z 531.1553) G) RTG-NAMR* [M+K]+ (m/z 501.14473) H) RTG-NAMR [M]+ (m/z 571.22637). Ion images are all displayed using a relative intensity scale of 5-60%.

Figure 4 Group 2 **Rat-2** eye A) Pre-matrix optical B) Serial H&E C) NAMR [M+K]+ (m/z 312.0909) D) RTG [M+K]+ (m/z 342.1015) E) NAMR-NAMR* [M+K]+ (m/z 471.1342) F) RTG-RTG* [M+K]+ (m/z 531.1553) G) RTG-NAMR* [M+K]+ (m/z 501.14473) H) RTG-NAMR [M]+ (m/z 571.22637). Ion images are all displayed using a relative intensity scale of 5-60%.

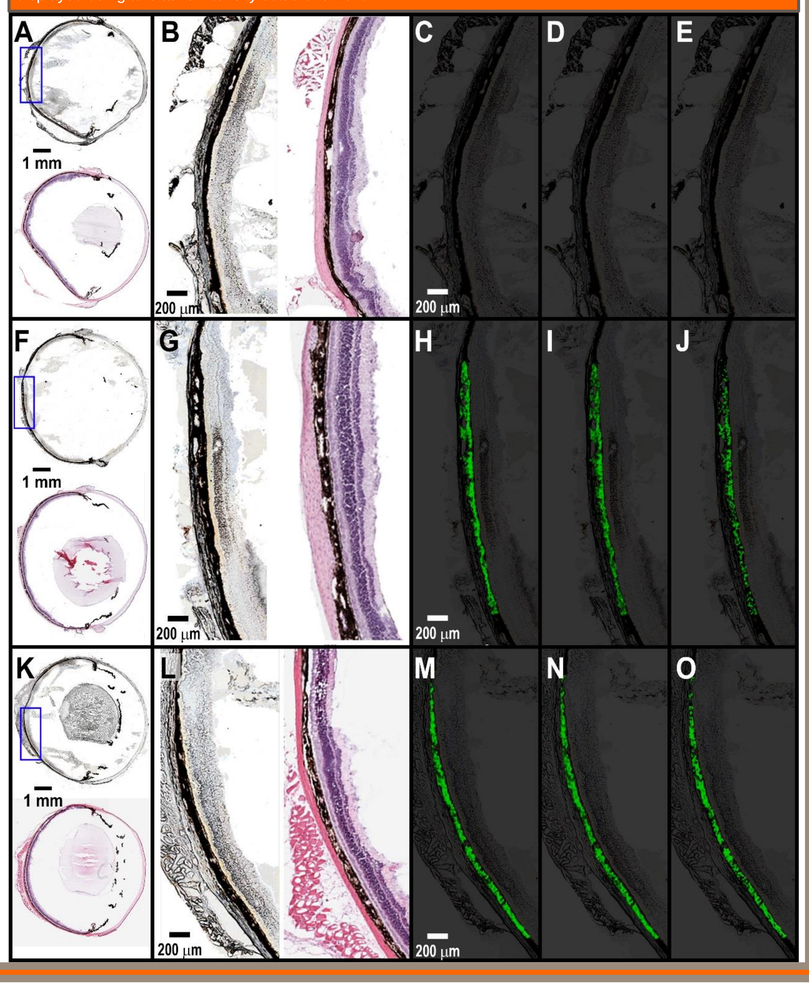


Eye Schematic



Several high spatial resolution imaging experiments conducted to further investigate the localized distribution of RTG-related material. **Figure 6** displays the 10 μm pixel images for the three major dimer species, detected as the phenazine species [M+K]+, in ocular tissue from an animal from each group: Group 1 (vehicle; 272 days), Group 2 (100 mg/kg/day; 91 days), and Group 3 (100 mg/kg/day; 272 days). These images further show the highly-localized distribution of the dimer species in the eyes of animals administered RTG. No differences were observed for RTG-related material between animals from Group 2 (91 days) and Group 3 (272 days).

Figure 6 Ion images (10 μm pixels) from eye tissues from 3 separate rats: Group 1 (control), Group 2 (91 days - 100 mg/kg/day) and Group 3 (272 days - 100 mg/kg/day) A, F, K) Pre-matrix optical and serial H&E stained sections B, G, L) 5x magnification of analysis region C, H, M) RTG–RTG* [M+K]+ (m/z 531.1553) D, I N) NAMR-NAMR*[M+K]+ (m/z 471.1342) E, J, O) RTG-NAMR* [M+K]+ (m/z 501.14473). Ion images are displayed using a relative intensity scale of 5-25%.



An important question for the risk assessment of the dimer species was whether they were localized only to the choroid or also to the RPE. We conducted a 5 μm pixel imaging experiment on eye tissue from a Group 2 rat to investigate this (**Figure 7**). In the ion image for the phenazine RTG-NAMR* [M+K]+, the localization appears to be primarily to the choroid layer with little or no ion intensity detected in the RPE. The large blood vessels within the choroid do not appear to contain any RTG-related material. This is consistent with the results of a plasma LC-MS analysis, where no dimers were detected in plasma.

Figure 7 MALDI IMS analysis of rat eye tissue section at 5 μm pixel dimensions from animal 41 (91 days) A) Pre-matrix optical scan B) Ion image for RTG-NAMR* [M+K]+ (m/z 501.14473) overlaid with the pre-matrix optical scan C) Ion image for RTG-NAMR* [M+K]+ (m/z 501.14473). Ion image is displayed using a relative intensity scale of 5-25%

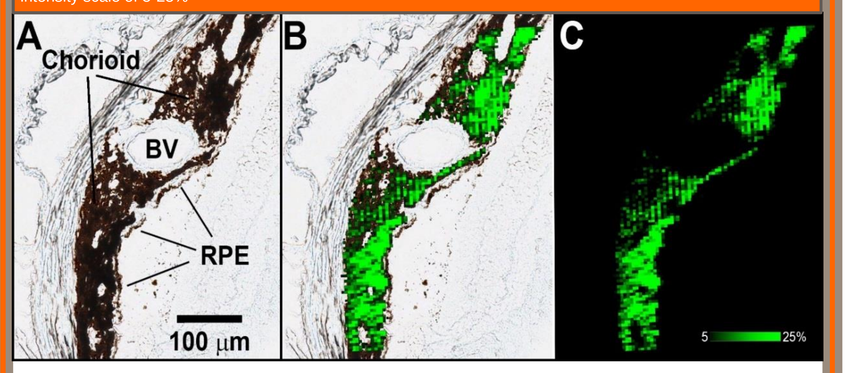
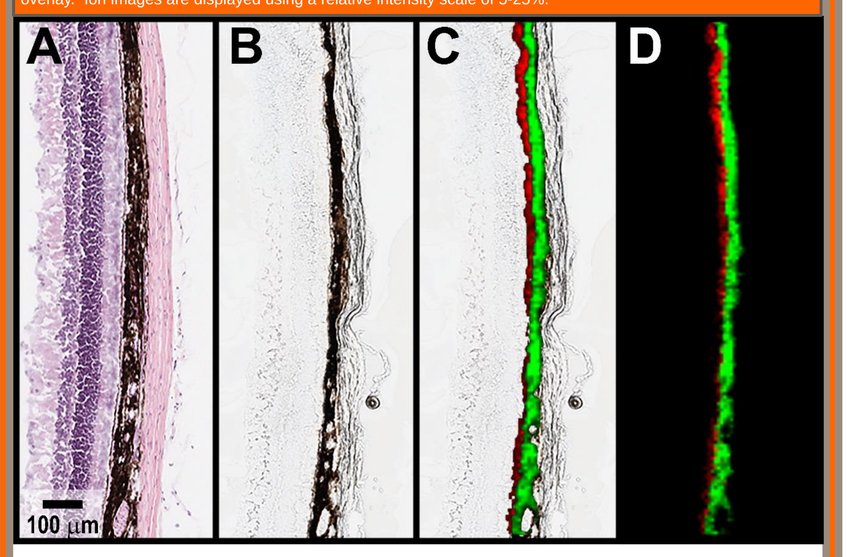
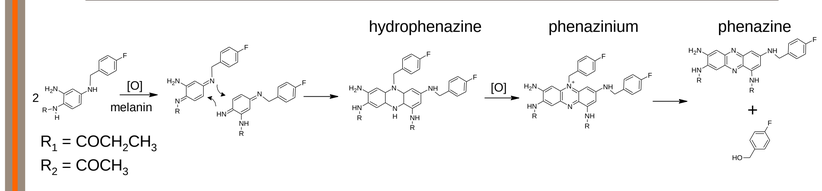


Figure 8 displays the ion image for the phenazine RTG-NAMR* [M+K]+ (green) overlaid with the ion image for bis-retinoid N-retinyl-N-retinylidene ethanolamine (A2E) (red). A2E is a by-product of the visual cycle and is known to accumulate in the RPE and Bruch's membrane.[3] The distinct distributions observed for these species is further evidence that the RTG-related material is localized specifically to the choroid layer and excludes the RPE.

Figure 8 Group-2 rat eye imaged with 10 μm pixels A) serial H&E B) Pre-matrix optical. Ion images for RTG-NAMR* [M+K]+ (m/z 501.14473) in green and A2E [M]+ (m/z 592.45129) in red C) overlaid on optical D) no overlay. Ion images are displayed using a relative intensity scale of 5-25%.



Proposed mechanism for the formation of phenazinium dimers



Conclusions

The detection of the three phenazinium dimers (RTG-RTG, NAMR-NAMR; RTG-NAMR) in the melanin containing ocular tissue, their absence in plasma or other non-melanin containing ocular tissues, and the high melanin association for both RTG and NAMR tissues suggests that the formation of these dimers occurs from melanin bound RTG and NAMR. In this hypothesis, the melanin binding of RTG and NAMR effectively concentrates the two compounds to enable mixed condensation reactions to occur when the binding provides the proper geometry in the oxidative environment of the uveal tract. Based upon their purple appearance and UV spectra, we propose that these dimers could also be responsible for the dyspigmentation (purple-grey appearance) in melanin containing skin tissues of patients with long term long RTG treatment.

Melanin binding within the retina, the region of the eye containing the photoreceptors, could have a greater potential risk for adverse events. Thus, the high resolution IMS experiments (**Figure 7 and 8**) demonstrate that no detectable levels of RTG related material including the dimers were able to penetrate the blood-retinal barrier associated with the RPE. This is consistent with the results of the histopathological analysis of rat eyes in this study, from which no ocular toxicity findings were reported. Though convincing in the rat, it should be noted that it is not clear how our findings in the rat model relate to the FDA warning of changes in retinal pigmentation associated with long term use of RTG in patients.

References

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