NIST

Assessment and Examination of Mitochondrial DNA Sequence Changes in Primary Human Hepatocytes Following *In Vitro* Exposure to Four Different Conazoles

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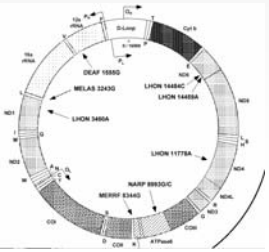
ABSTRACT

Conazoles are azole antifungal agents used both as pesticides and drugs. Some are hepatotoxic in mice, rats, and humans; and they have been shown to be hepatocarcinogenic in mice. Ketoconazole is reported to be a potent mitochondrial inhibitor, with complex I of the respiratory chain, a major target in rat hepatocytes. Mitochondrial DNA represents a target for oxidative damage. After sustaining an initial damage event, mitochondrial DNA can amplify oxidative stress by decreasing the expression of proteins involved in electron transport, leading to more reactive oxygen species generation. In this study, we have exposed primary human hepatocytes in duplicate to Ketoconazole (30uM), Propiconazole (300uM), Triadimefon (300uM), or Myclobutanil (300uM), for 24 hrs. Cellular DNA was isolated and the entire mitochondrial genome was amplified with 3 sets of forward-reverse mitochondrial primers. The resulting PCR amplicons were pooled and enzymatically fragmented, followed by labeling and hybridization to 12 human Affymetrix Mitochondrial Resequencing Chips v2, containing the entire mitochondrial genome (16,568 bp tiled in duplicate) including the D-loop sequence and the coding sequences for 37 mitochondrial genes. The resulting data were analyzed using Affymetrix GSEQ software. Analysis showed 26 sequences different from the reference sequence, which categorized cells into mtDNA specific haplo-groups. In addition, we observed one mutation at sequence mt188 after Ketoconazole and Propiconazole treatment but not Triadimefon or Myclobutanil. These results will be used to optimize further experiments establishing cell culture conditions including the time of exposure and chemical concentration. (This abstract does not reflect EPA policy.)

OBJECTIVE

Examine effect(s) of conazole exposure on human mitochondrial DNA



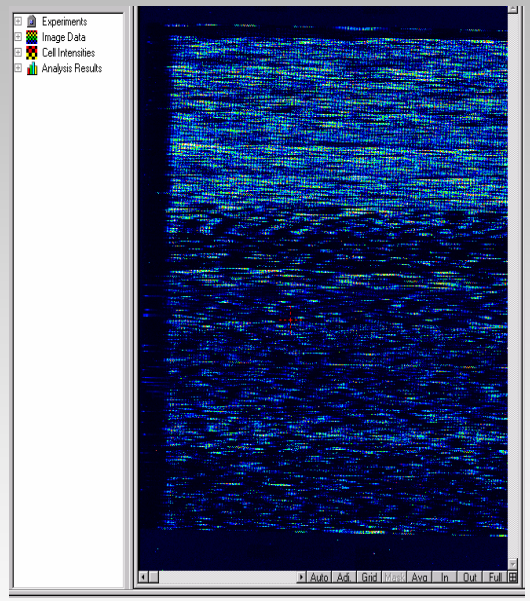
Mito genome

- 16.5 kb encodes 37 genes;
- 13 respiratory chain
- 1000 copies per cell
- All genes required for proper function of the organelle
- ROS exposure- genetic instability

METHODS

- 6 well plates (matrigel) primary human hepatocytes exposed to conazoles 300uM (Triadimefon, Propiconazole, Myclobutanil) and 30 uM Ketoconazole for 24h at 37^{0}C in 5% CO_2
- Isolated 100ug DNA (kit) Qiagen DNaEasy
- Amplify DNA with TaKaRA LA *Taq*-3 primer sets
- As a control for PCR amplification and subsequent hybridization, a 7.5-kb plasmid DNA (Tag IQ-EX template) was amplified with the test samples, using forward and reverse primers included in the CustomSeq Control kit (Affymetrix)
- Used QIAQuick PCR Clean up kit (QIAGEN), and the purified PCR products were resuspended in 30 µL volume of EB buffer (Affymetrix).
- The PCR reaction yield (ng/µL)was determined spectrophotometrically.
- PCR Pooling, DNA Fragmentation, Labeling, and Chip Hybridization- pooled equimolar amounts from the three amplified fragments to ensure that an equal number of targets existed for each probe.
- DNA fragmentation was performed with a 15-µL master mix containing Affymetrix fragmentation reagent (calculated as 0.2 U DNAse I/µg DNA), 5 µL OnePhorAll buffer (Amersham Life Sciences), and EB Buffer.
- Fragmented DNA was labeled by adding 1.5 µL Biotin-N6-ddATP (Perkin Elmer Life Sciences) and 1 µL of 20 U/µL rTdT enzyme
- Prehybridization, hybridization, washing, and scanning of the MitoChip were performed as described in the Affymetrix CustomSeq Resequencing protocol.
- Following hybridization, the chips were washed using preprogrammed CustomSeq Resequencing wash protocol [Affymetrix Microarray Suite (MAS) version 5.2].
- The raw pixel data (.DAT file) generated is thus digitized into a .CEL file for subsequent batch analysis.

Image of scan-Mito Chip .DAT



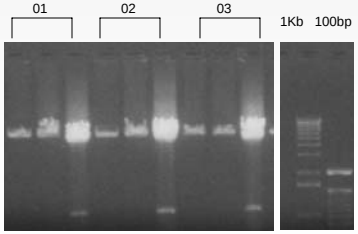
Sequence Batch Analysis:
26 sequences differ from reference

Fragment	Frag position	Ref	Keto	Myclo	neg	Propi	Tria	Heterozygous
human_mt	72	a	g	g	g	g	n	0
human_mt	188	a	g	n	a	g	n	0.44
human_mt	262	a	g	g	g	g	g	0
human_mt	708	g	a	a	a	a	a	0
human_mt	749	a	g	g	g	g	g	0
human_mt	1437	a	g	g	g	g	g	0
human_mt	2705	a	g	g	g	g	g	0
human_mt	3937	c	t	t	t	t	t	0
human_mt	4091	a	g	g	g	g	g	0
human_mt	4767	a	g	g	g	g	g	0
human_mt	5044	g	a	a	a	a	a	0
human_mt	7026	c	t	t	t	t	t	0
human_mt	8249	g	a	a	a	a	a	0
human_mt	8858	a	g	g	g	g	g	0
human_mt	8992	g	a	a	a	a	a	0
human_mt	11945	a	g	g	g	g	g	0
human_mt	12412	t	c	c	c	n	n	0
human_mt	12703	c	t	t	t	t	t	0
human_mt	13720	a	g	g	g	g	g	0
human_mt	14764	c	t	t	t	t	t	0
human_mt	15324	a	g	g	g	g	g	0
human_mt	15779	c	t	t	t	t	t	0
human_mt	15882	g	c	c	c	c	c	0
human_mt	16221	c	t	t	t	t	t	0
human_mt	16323	t	c	c	c	c	c	0
human_mt	16517	t	c	c	c	c	c	0

n=no call

RESULTS

example of 3-primer amplification: mito genome



CONCLUSIONS

- Deoxyguanosine is a common site for oxidative damage to DNA(8-oxo dG formation).
- Our observed results of a mutation at guanine in Ketoconazole is consistent with a finding by Rodriquez & Acosta (1996) indicating that Ketoconazole was a potent mitochondrial inhibitor and targets complex I of the respiratory chain.
- Furthermore, Propiconazole also induced a mutation at guanine suggesting a possible ROS mode of action for this chemical.
- This is a novel approach to examine chemically-induced oxidative damage to the mitochondria. A longer exposure may not be long enough to fix a mitochondrial mutation. Therefore, we will extend the chemical exposure to 5 days and repeat the experiment.