Toxicogenomics Summary Report

Analysis of Triptonide and Triptolide Analogue in Rat Primary Hepatocyte

Prepared for Emory

September, 2007

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1. PROJECT DESCRIPTION

1.1. GOAL

The objective of this project is perform basic toxicogenomic analysis of rat primary hepatocyte gene expression data of two compounds (Triptonide and Triptolide Analogue), and deliver a brief written report including findings based on Drug Signature analysis, pathway impact and Gene Ontology analysis of significantly changed genes, and expression profile similarities.

1.2. STUDY DESIGN

Male Sprague Dawley rat primary hepatocytes were treated with two compounds (Triptonide and Triptolide Analogue) at single TC_{20} doses for 16 and 24h by Entelos scientists. Triptonide was determined to have a TC_{20} of 100μM and the TC_{20} for Triptolide Analogue was determined to be 32.5μM. Experiments were performed using 3 replicates for each *in vitro* dose/time-point combination. RNA samples were extracted from treated rat primary hepatocytes and were hybridized onto the Affymetrix Rat Genome 230 2.0 GeneChip by Entelos scientists. The analysis was performed in the context of rat primary hepatocyte experiments profiled on Affymetrix RG230-2 array in the DrugMatrix reference database. Data are represented as the average $\log_{10}$ ratios of treated to animal and treatment time matched controls.

2. EXECUTIVE SUMMARY

2.1. ANALYSIS SUMMARY IN RAT PRIMARY HEPATOCYTES

Treatment with Triptonide is predicted to cause hepatotoxicity in rats while the Triptolide Analogue is expected to have significantly fewer hepatotoxic liabilities. These predictions are based on strong Drug Signature matches, pathway impact, and Gene Ontology analyses. While primary rat hepatocytes were treated with equitoxic doses (TC_{20}) of each compound, Triptolide caused a much higher number of significant gene perturbations as compared to Triptolide Analogue. In addition, Triptonide has an overall greater impact than the Triptolide Analogue on Drug Signatures, pathways impacted by induced genes and repressed genes, and gene ontology (GO) terms.

The predicted rat hepatotoxicity for Triptonide treatment is supported by several lines of evidence. Triptonide treatments perturbed between 12,000 and 14,000 genes at a P value of <0.05 on the Affymetrix RG230-2 rat whole genome array at either time point, significantly higher than the average range of gene perturbations by most RPH experiments in DrugMatrix. In contrast, the Triptolide Analogue perturbed approximately 2700 genes at the 16 hour time point and 1331 genes at the 24 hour time point, both within the average range of gene changes induced by other RPH experiments in DrugMatrix. Correlation analysis at the level of global gene expression shows Triptonide treatment similar to ifosfamide, lomustine, cisplatin, and staurosporine treatments at both time points (all cc between 0.2 – 0.3). Hypergeometric enrichment analysis shows moderate similarity between Triptonide and DNA damaging free radical generators, PKC inhibitors, and H+/K+ ATPase inhibitor RPH treatments profiled on Affymetrix RG230-2 arrays in DrugMatrix. In contrast, the Triptolide Analogue had moderate similarity only to H+/K+ ATPase inhibitors at both time points. Triptonide had a number of strong matches to Drug Signatures indicating the compound may cause liver toxicity in rats. Triptonide showed strong matches to the “hepatotoxic to rat liver”, “cross-tissue phospholipidosis”, and “cholestasis” signatures. The Triptolide Analogue did not show a match to these signatures. The Triptonide and Triptolide Analogue both had weak-to-moderate matches to the “apoptosis” and “in vitro GSH depletion” signatures.

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Gene Ontology (GO) term analysis revealed that Triptonide showed statistically significant perturbation of genes classified in ribosomal protein synthesis and nucleic acid binding terms while Triptolide Analogue treatment led to statistically significant perturbation of genes classified in nucleic acid binding and metabolism as well as mitochondrial oxidoreductase activity. Pathway impact analysis indicated that treatment with Triptonide led to statistically significant induction of genes in mitochondrial oxidative phosphorylation, oxidative stress response mediated by Nrf2, acute phase response, and ubiquitin-proteosome and protein degradation pathways. Downregulation was seen with Triptonide treatment at both time points in TGF-β signaling, Bcr-Abl signaling, and MAP kinase signaling pathways. Treatment with Triptolide Analogue induced genes in oxidative stress response mediated by Nrf2 and xenobiotic metabolism pathways while repressing genes in complement activation, P450 family, and integrin signaling pathways.

In summary, Triptonide is predicted to cause moderate toxicity in rat liver at the tested dose while the Triptolide Analogue is predicted to be less toxic than Triptonide. Drug Signature analysis revealed a different pattern of toxicity for Triptonide (hepatotoxic to rat liver, cross-tissue phospholipidosis, and cholestasis) than for Triptolide Analogue. Triptolide Analogue had a weaker impact than Triptonide on overall gene expression changes, signature matches, pathways impacted by induced and repressed genes, and gene ontology terms.

1.2. RECOMMENDATIONS FOR FOLLOW-UP ANALYSIS OF GENE EXPRESSION DATA

If further follow-up work is desired, we recommend in-depth contextual analysis to characterize the toxicological and pharmacological properties of Triptonide and Triptolide Analogue:

1. Contextual analysis into the mechanism(s) of predicted hepatotoxicity, cross-tissue phospholipidosis, and cholestasis induced by Triptonide and possible apoptosis and GSH depletion by both Triptonide and Triptolide Analogue.

2. Further compare Triptonide and Triptolide Analogue to other hepatocyte treatments in DrugMatrix to determine the significance of the induction of noted pathways and Gene Ontology processes, particularly with respect to the observed similarities to compounds causing perturbations in oxidative stress related pathways.

3. Analysis of additional highly impacted biological pathways, similarity to other hepatocyte treatments and their relevance to predicted toxicities.

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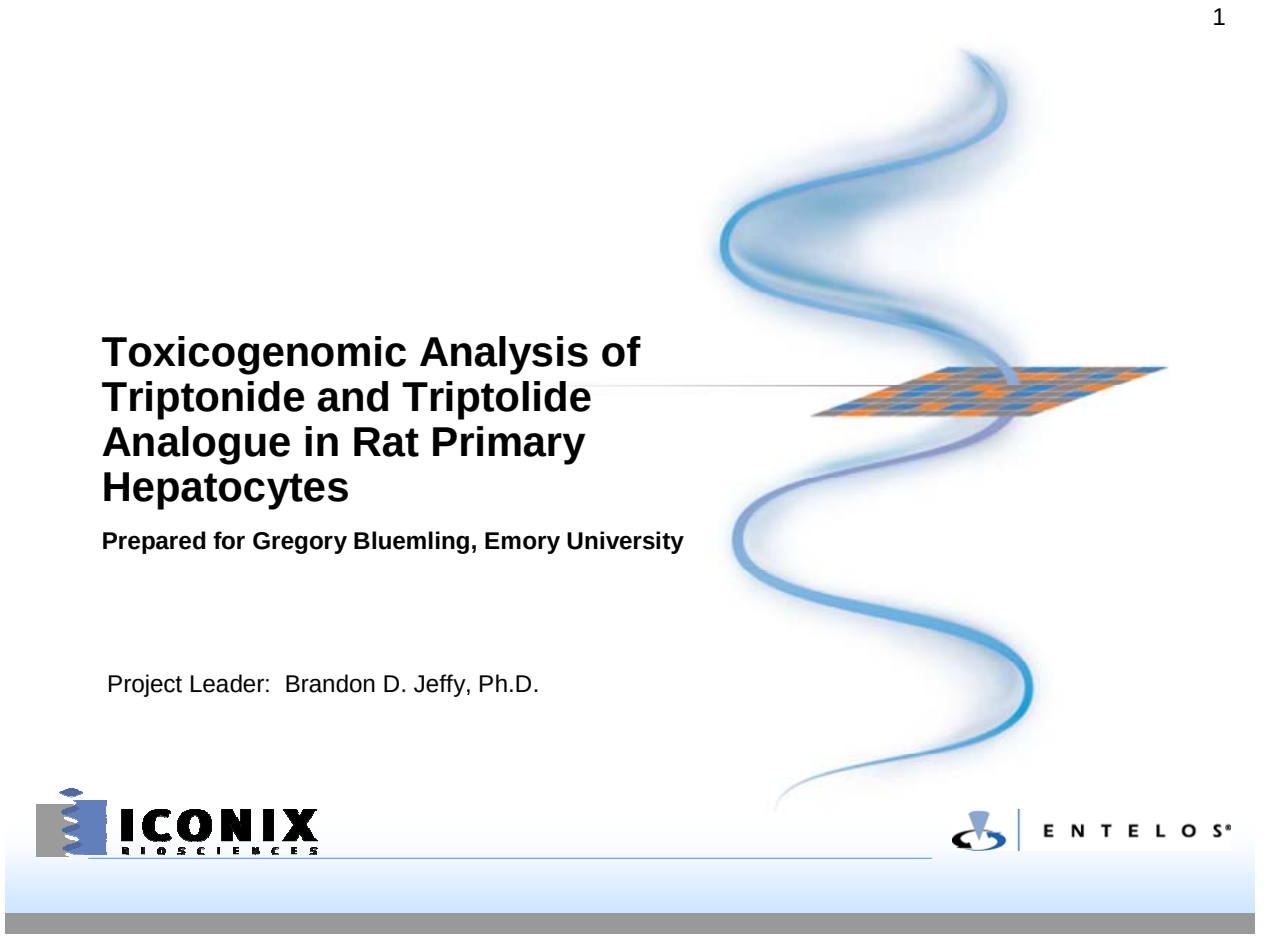
3. SLIDE PRESENTATION



Slide 1: Toxicogenomic analysis of triptonide and triptolide analogue in rat primary hepatocytes

A team of scientists at Entelos conducted this project. Dr. Brandon Jeffy was the project leader.

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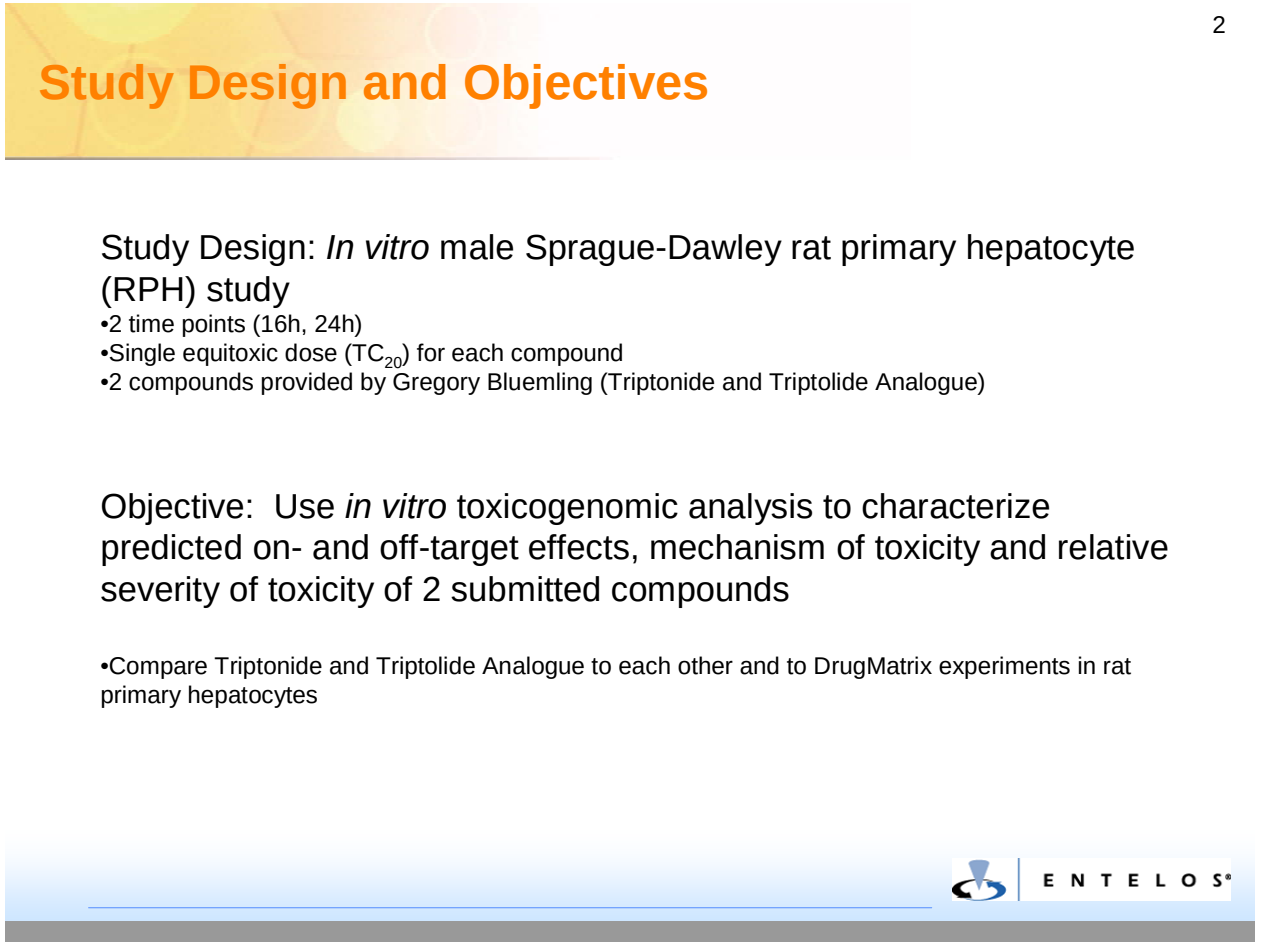

Slide 2: Study design and objectives

Refer to the executive summary for discussion of the study design and objectives.

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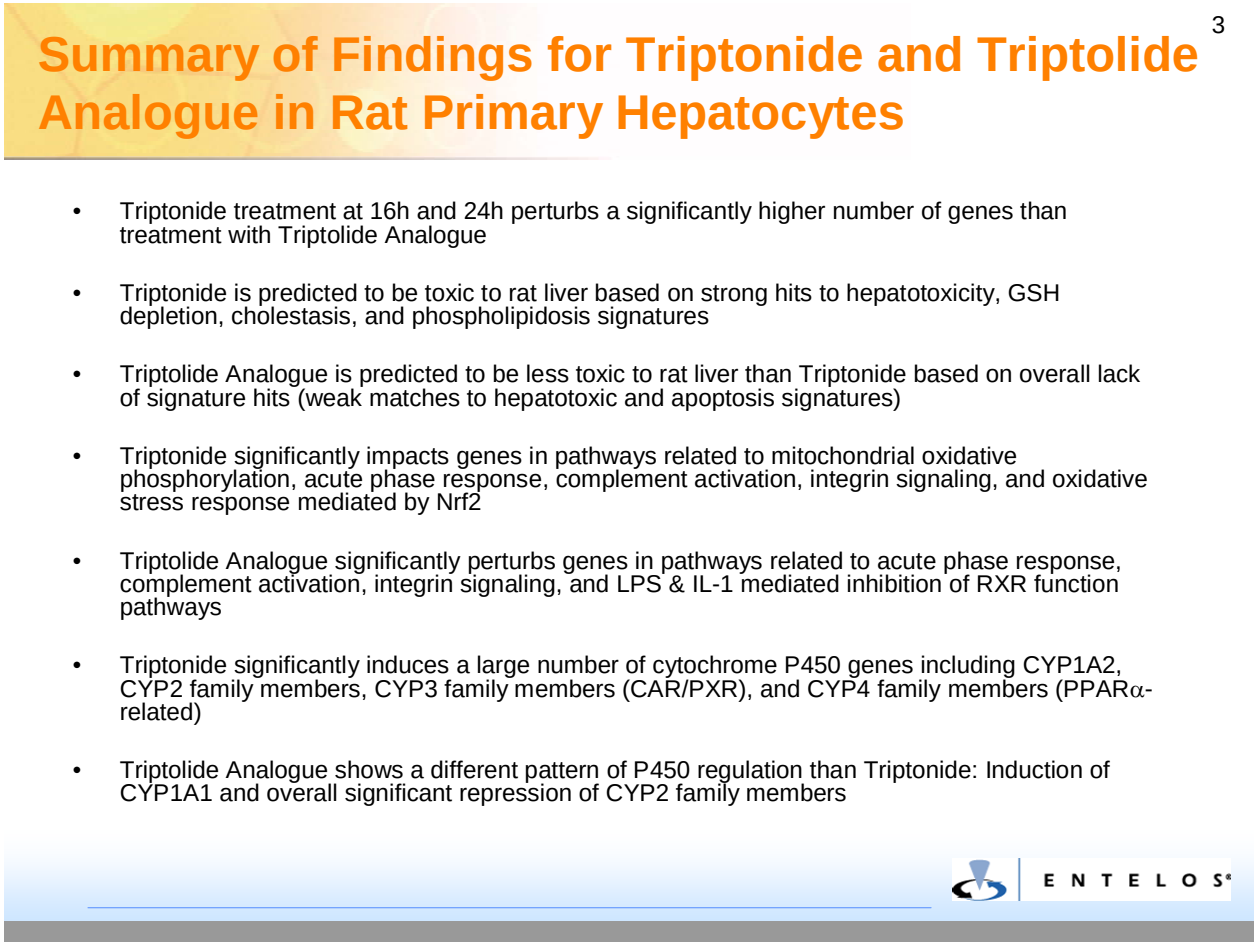

Slide 3: Summary of findings for triptonide and triptolide analogue in rat primary hepatocytes

Refer to the executive summary for discussion of the findings.

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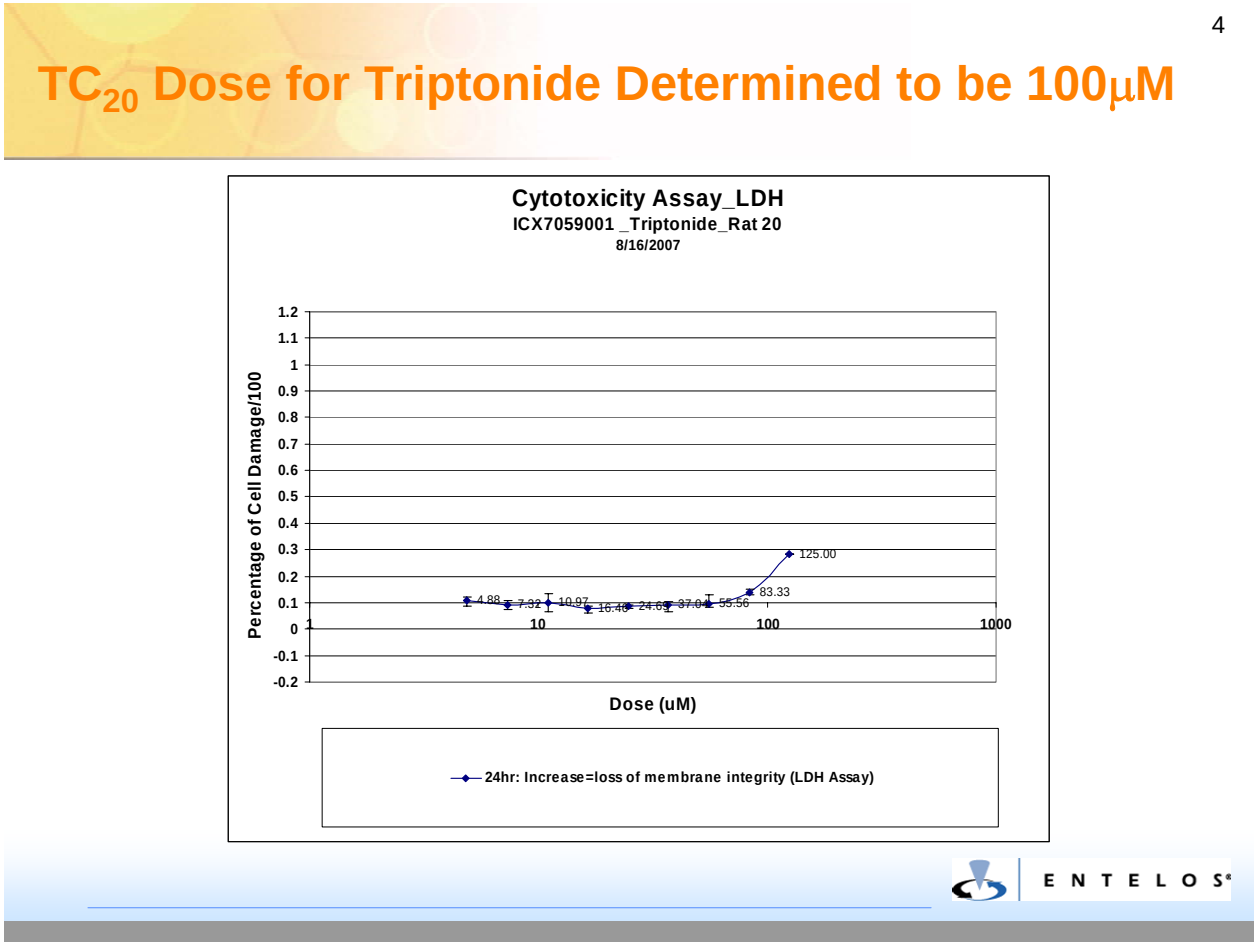

Slide 4: TC_{20} Dose for triptonide determined to be 100μM

Rat primary hepatocytes were treated with increasing concentrations of triptonide for 24 hrs. Cytotoxicity was estimated using an LDH assay (Promega). The concentration that killed 20% of the cells (TC20) was determined by interpolation of the dose response curve using Graphpad.

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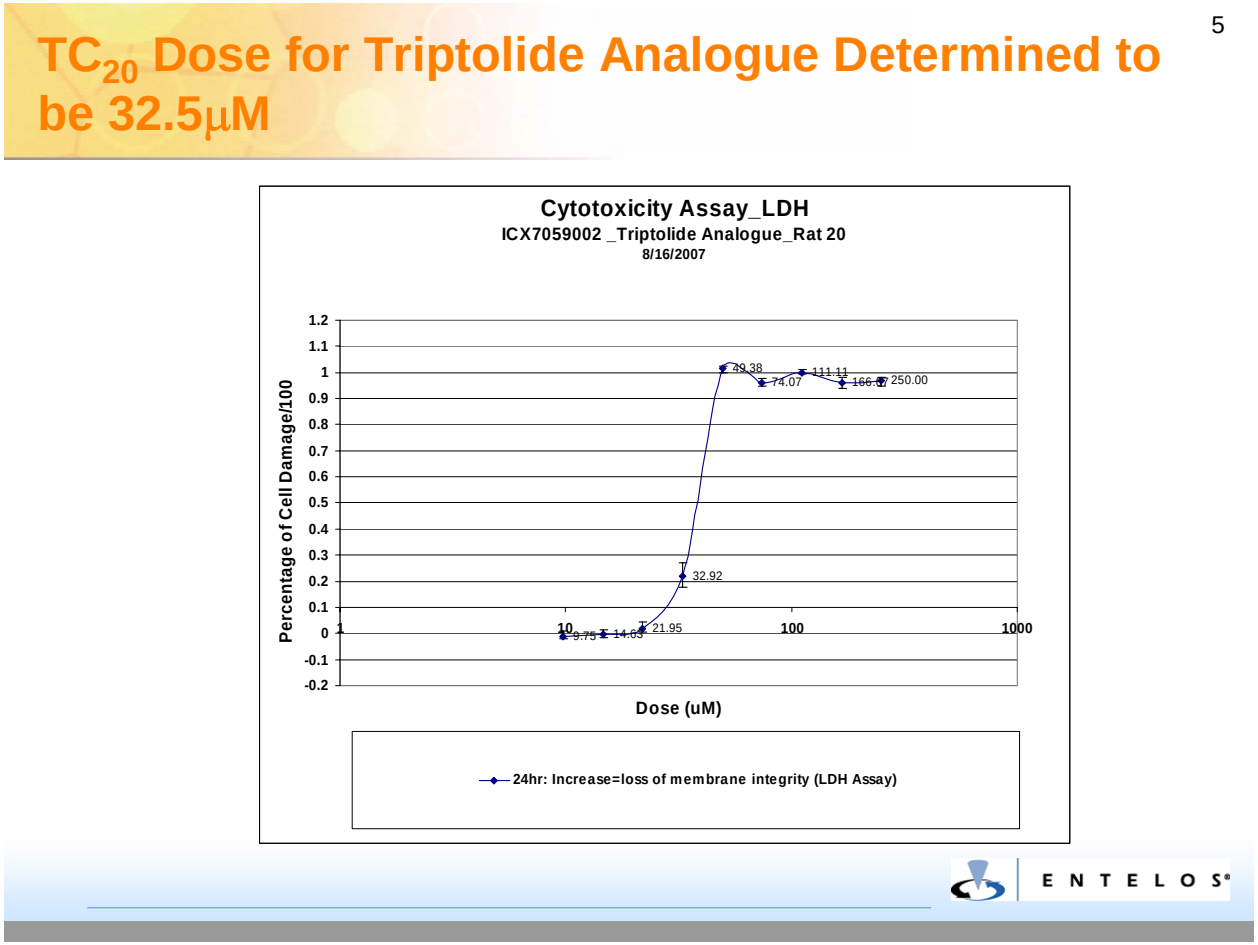

Slide 5: TC_{20} Dose for triptolide analogue determined to be 32.5μM

Rat primary hepatocytes were treated with increasing concentrations of triptolide analogue for 24 hrs. Cytotoxicity was estimated using an LDH assay (Promega). The concentration that killed 20% of the cells (TC20) was determined by interpolation of the dose response curve using Graphpad.

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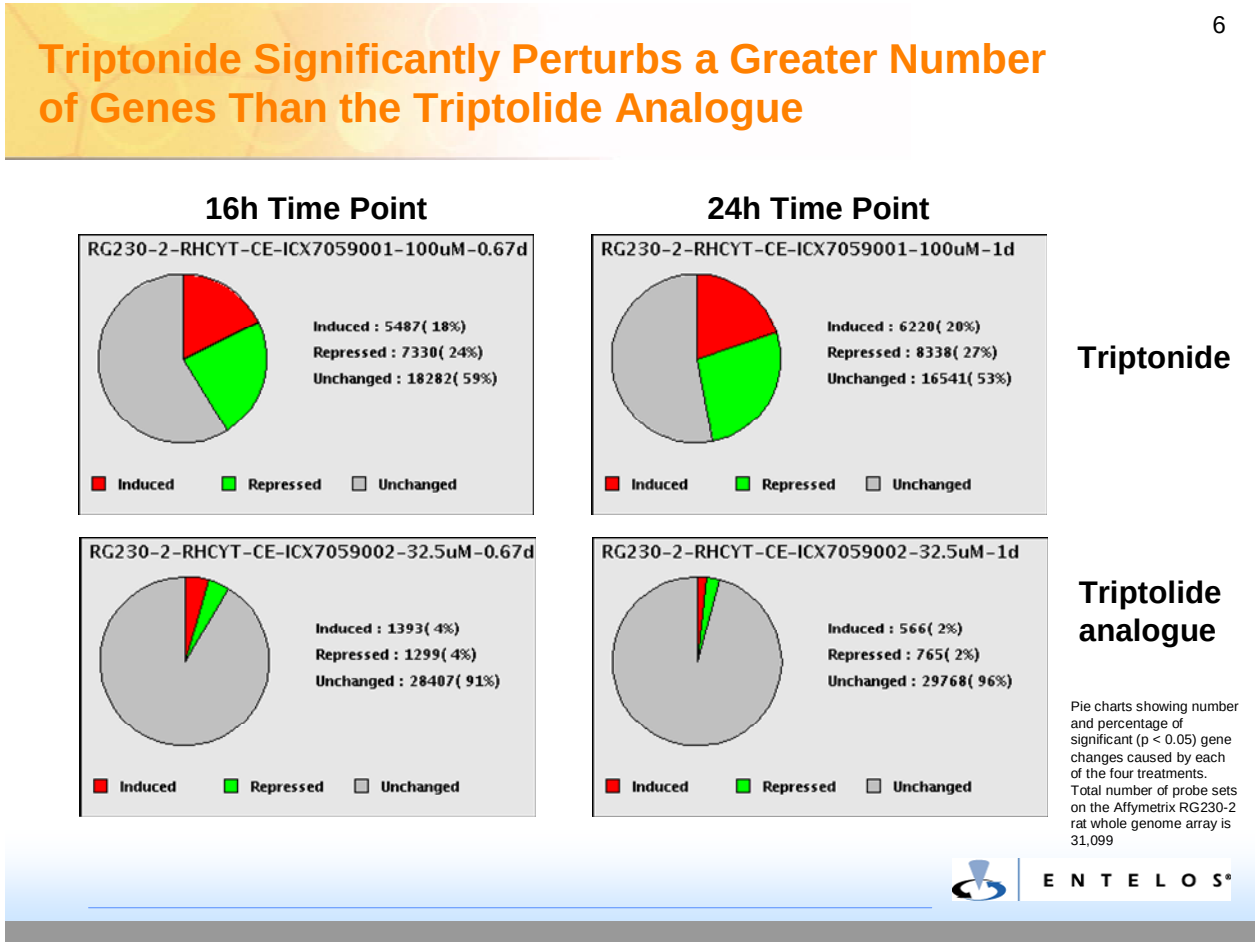

Slide 6: Triptonide significantly perturbs a greater number of genes than the triptolide analogue

The number and percentage of significantly induced or repressed genes at p<0.05 by each of the triptonide and triptolide analogue treatment are shown in the pie chart. Red indicates induced genes while green indicates repressed genes.

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Compounds Similar to Triptonide 24h Treatment Are Enriched in Free Radical Generators, PKC Inhibitors, and H+/K+ ATPase Inhibitors

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EXPERIMENT_NAME	SIMILARITY
TRIPTONIDE-.67d-100uM	1.000
TRIPTONIDE-1d-100uM	0.812
IFOSFAMIDE-1d-11000uM	0.327
IFOSFAMIDE-.67d-11000uM	0.252
LOMUSTINE-.67d-560uM	0.249
STAUROSPORIN-.67d-1.3uM	0.246
CISPLATIN-.67d-63uM	0.242
SPARTEINE-1d-2222uM	0.225
IFOSFAMIDE-1d-11000uM	0.210
CARMUSTINE-.67d-550uM	0.207
CISPLATIN-1d-63uM	0.206
CYTOCHALASIN-1d-167uM	0.196
CARMUSTINE-1d-550uM	0.188
SPORIDESMIN-1d-.15uM	0.188
KETOCONAZOLE-1d-90uM	0.184
4,4'-METHYLE-.67d-296uM	0.179
VINBLASTINE-1d-45uM	0.173
HALOPERIDOL-1d-77uM	0.172
DIETHYLSTILB-1d-120uM	0.171
TACROLIMUS-.67d-50uM	0.170

16h Triptonide

EXPERIMENT_NAME	SIMILARITY
TRIPTONIDE-1d-100uM	1.000
TRIPTONIDE-.67d-100uM	0.812
IFOSFAMIDE-1d-11000uM	0.369
LOMUSTINE-.67d-560uM	0.290
CISPLATIN-.67d-63uM	0.288
IFOSFAMIDE-.67d-11000uM	0.278
STAUROSPORIN-.67d-1.3uM	0.277
SPARTEINE-1d-2222uM	0.262
IFOSFAMIDE-1d-11000uM	0.241
CYTOCHALASIN-1d-167uM	0.240
CISPLATIN-1d-63uM	0.239
CARMUSTINE-1d-550uM	0.230
CARMUSTINE-.67d-550uM	0.230
KETOCONAZOLE-1d-90uM	0.229
SPORIDESMIN-1d-.15uM	0.220
TACROLIMUS-.67d-50uM	0.217
VINBLASTINE-1d-45uM	0.214
DIETHYLSTILB-1d-120uM	0.205
TRIPTOLIDE A-1d-32.5uM	0.204
HALOPERIDOL-1d-77uM	0.202

24h Triptonide

Most similar experiments to 24h Triptonide treatment are enriched in:

CATEGORY	TERM	ADJUSTED PVALUE
STRUCTURE_ACTIVITY	DNA damaging, free oxygen radical generator, nitrosourea	0.00505661
STRUCTURE_ACTIVITY	Protein-serine/threonine kinase (PKC) inhibitor	6.71E-05
STRUCTURE_ACTIVITY	H+/K+-ATPase inhibitor	2.89E-08
ACTIVITY_CLASS	H+/K+-ATPase inhibitor	2.92E-08

No enrichment in structure activity, activity class, or signature training set for experiments similar to 16h Triptonide treatment

Pearson correlation calculated across all RG230-2 probe sets. Top 20 most similar DrugMatrix hepatocyte experiments are shown. Hypergeometric feature enrichment calculation on all similar experiments with cc> 0.15. Bonferroni correction applied to p value.

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Slide 7: Compounds similar to triptonide 24h treatment are enriched in free radical generators, PKC inhibitors and H+/K+-ATPase inhibitors

The triptonide treatments at 16 and 24hr were compared to all experiments in DrugMatrix that were profiled in rat primary hepatocyte using Pearson's correlation across all genes on the RG230-2 array. The top 20 most similar experiments, ranked by the correlation coefficient, are shown. Hypergeometric feature enrichment was calculated on all similar experiments with correlation coefficient higher than 0.15. Bonferroni correction was applied to p value. Triptonide 24hr experiment is similar to free radical generators, PKC inhibitors and H+/K+-ATPase inhibitors.

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Compounds Similar to Triptolide Analogue 16h Treatment Are Enriched in H+/K+ ATPase Inhibitors, Histamine Receptor Antagonists, and Monoamine Re-uptake Inhibitors

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EXPERIMENT_NAME	SIMILARITY
TRIPTOLIDE A-.67d-32.5uM	1.000
STAVUDINE-1d-1120uM	0.379
METHAPYRILEN-1d-300uM	0.373
LANSOPRAZOLE-1d-240uM	0.360
ETHINYLESTRA-1d-190uM	0.347
ETHINYLESTRA-.67d-190uM	0.345
ISOTRETINOIN-1d-500uM	0.344
BETA-ESTRADI-.67d-250uM	0.342
METHAPYRILEN-.67d-300uM	0.342
PANTOPRAZOLE-1d-650uM	0.331
OLANZAPINE-1d-250uM	0.331
1-NAPHTHYL I-.67d-166.67uM	0.327
ITRACONAZOLE-1d-10uM	0.326
4,4'-METHYLE-1d-296uM	0.323
1-NAPHTHYL I-1d-166.67uM	0.321
STAVUDINE-.67d-1120uM	0.320
LABETALOL-1d-250uM	0.317
4,4'-DIETHYL-1d-20uM	0.317
LANSOPRAZOLE-.67d-240uM	0.317
CHLORPROMAZI-1d-43.9uM	0.316

16h Triptolide Analogue

Most similar DrugMatrix primary rat hepatocyte experiments to 16h Triptolide analogue treatment are enriched in:

CATEGORY	TERM	ADJUSTED PVALUE
STRUCTURE_ACTIVITY	H+/K+-ATPase inhibitor	5.81E-10
STRUCTURE_ACTIVITY	Histamine receptor (H1) antagonist, tricyclic	0.0147
ACTIVITY_CLASS	Monoamine re-uptake/oxidase inhibitor	0.0353
ACTIVITY_CLASS	Histamine antagonist	0.0025
ACTIVITY_CLASS	H+/K+-ATPase inhibitor	5.92E-10

Pearson correlation calculated across all RG230-2 probe sets. Top 20 most similar DrugMatrix hepatocyte experiments are shown. Hypergeometric feature enrichment calculation on all similar experiments with cc> 0.15. Bonferroni correction applied to p value.

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Slide 8: Compounds similar to triptolide analogue 16h treatment are enriched in H+/K+-ATPase inhibitors, histamine receptor antagonists, and monoamine reuptake inhibitors

The triptolide analogue treatment at 16hr was compared to all experiments in DrugMatrix that were profiled in rat primary hepatocyte using Pearson's correlation across all genes on the RG230-2 array. The top 20 most similar experiments, ranked by the correlation coefficient, are shown. Hypergeometric feature enrichment was calculated on all similar experiments with correlation coefficient higher than 0.15. Bonferroni correction was applied to p value. Triptolide analogue 16hr experiment is similar to H+/K+-ATPase inhibitors, histamine receptor antagonists and monoamine reuptake inhibitors.

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Compounds Similar to Triptolide Analogue 24h Treatment Enriched in H+/K+ ATPase Inhibitors

EXPERIMENT_NAME	SIMILARITY
TRIPTOLIDE A-1d-32.5uM	1.000
METHAPYRILEN-1d-300uM	0.270
ETHINYLESTRA-1d-190uM	0.268
BETA-ESTRADI-1d-250uM	0.266
BETA-ESTRADI-.67d-250uM	0.255
1-NAPHTHYL I-1d-166.67uM	0.252
ITRACONAZOLE-1d-10uM	0.250
ETHINYLESTRA-.67d-190uM	0.249
1-NAPHTHYL I-.67d-166.67uM	0.248
LANSOPRAZOLE-1d-240uM	0.245
4,4'-DIETHYL-1d-20uM	0.242
STAVUDINE-1d-1120uM	0.241
ISOTRETINOIN-1d-500uM	0.238
ITRACONAZOLE-.67d-10uM	0.235
DIETHYLSTILB-.67d-120uM	0.233
CHLORPROMAZI-1d-43.9uM	0.233
COLCHICINE-1d-1000uM	0.231
LABETALOL-1d-250uM	0.228
PANTOPRAZOLE-1d-650uM	0.225
4,4'-METHYLE-1d-296uM	0.225

24h Triptolide Analogue

Most similar DrugMatrix primary rat hepatocyte experiments to 24h Triptolide analogue treatment are enriched in:

CATEGORY	TERM	ADJUSTED PVALUE
STRUCTURE_ACTIVITY	H+/K+-ATPase inhibitor	0.0051
ACTIVITY_CLASS	H+/K+-ATPase inhibitor	0.0051

Pearson correlation calculated across all RG230-2 probe sets. Top 20 most similar DrugMatrix hepatocyte experiments are shown. Hypergeometric feature enrichment calculation on all similar experiments with cc> 0.15. Bonferroni correction applied to p value.

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Slide 9: Compounds similar to triptolide analogue 24h treatment enriched in H+/K+-ATPase inhibitors

The triptolide analogue treatment at 24hr was compared to all experiments in DrugMatrix that were profiled in rat primary hepatocyte using Pearson's correlation across all genes on the RG230-2 array. The top 20 most similar experiments, ranked by the correlation coefficient, are shown. Hypergeometric feature enrichment was calculated on all similar experiments with correlation coefficient higher than 0.15. Bonferroni correction was applied to p value. Triptolide analogue 24hr experiment is similar to H+/K+-ATPase inhibitors.

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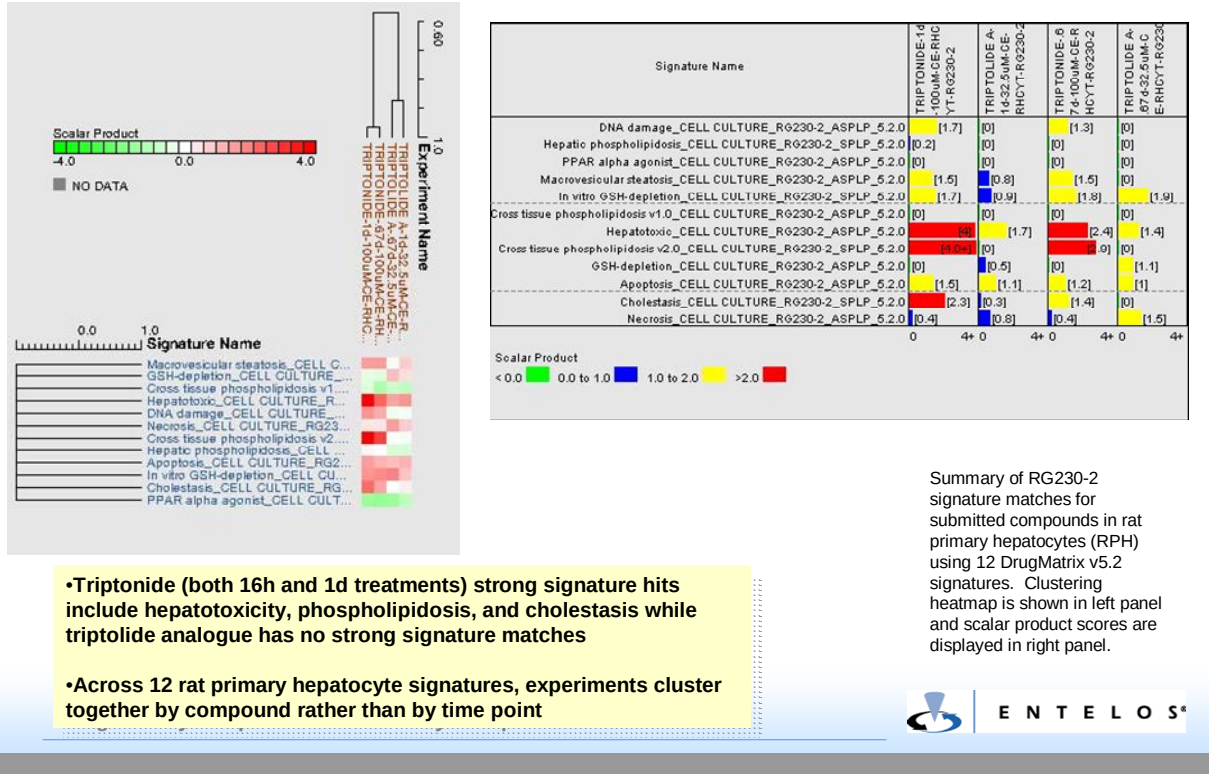
DrugMatrix Rat Primary Hepatocyte Signatures Predict Triptonide to be More Hepatotoxic Than Triptolide Analogue



Summary of RG230-2 signature matches for submitted compounds in rat primary hepatocytes (RPH) using 12 DrugMatrix v5.2 signatures. Clustering heatmap is shown in left panel and scalar product scores are displayed in right panel.

- **Triptonide (both 16h and 1d treatments) strong signature hits include hepatotoxicity, phospholipidosis, and cholestasis while triptolide analogue has no strong signature matches**
- **Across 12 rat primary hepatocyte signatures, experiments cluster together by compound rather than by time point**

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Slide 10: DrugMatrix rat primary hepatocyte signatures predict triptonide to be more hepatotoxic than triptolide analogue

Triptonide and triptolide analogue were evaluated using Drug Signatures in rat primary hepatocyte. Positive scalar product scores indicate the compound is classified positive for the endpoint predicted by the signature. Scalar product score higher than 1 indicates strong match to the signature. Triptonide strongly matched hepatotoxicity, phospholipidosis, and cholestasis signatures while triptolide analogue showed relatively weak match.

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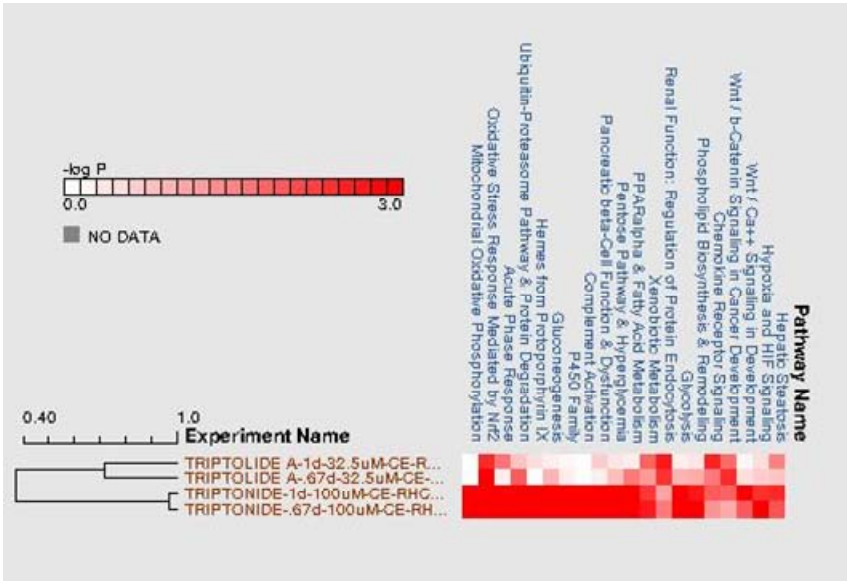
Pathway Impact Analysis with Induced Genes: Greater Impact from Treatment with Triptonide as Compared to Triptolide Analogue



- **Genes induced by Triptonide (both 16h and 1d treatments) have overall greater impact on pathways than Triptolide analogue**
- **Significantly induced pathways with Triptolide analogue include xenobiotic metabolism, chemokine receptor signaling, and Wnt/b-catenin signaling in cancer development**
- **Genes in oxidative stress response mediated by Nrf2 are induced by both compounds**

Pathway impact of treatment-induced genes (p<0.05) on 135 Iconix-curated pathways. Top 20 impacted pathways are displayed. Pathways are sorted left to right by average impact per pathway across all experiments in the set.

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Slide 11: Pathway impact analysis with induced genes: greater impact from treatment with triptonide as compared to triptolide analogue

Each experiment was evaluated for its impact on the 135 pathways using significantly induced genes (p <0.05). Pathways are sorted from left to right based on the average impact score [-log(p-value)] per pathway across experiments. Experiments are clustered based on the pattern of impact on pathways. Triptonide has overall greater impact on pathways than triptolide analogue. Triptonide treatments at both 16 and 24hr induced genes that strongly impact a number of pathways, including mitochondrial oxidative phosphorylation, oxidative stress, acute phase response, ubiquitin-mediated protein degradation, heme synthesis, and gluconeogenesis. Genes in oxidative stress response pathway were induced by both compounds.

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Pathway Impact Analysis with Repressed Genes: Triptonide and Triptolide Analogue Show Different Patterns of Repression Across DrugMatrix Pathways

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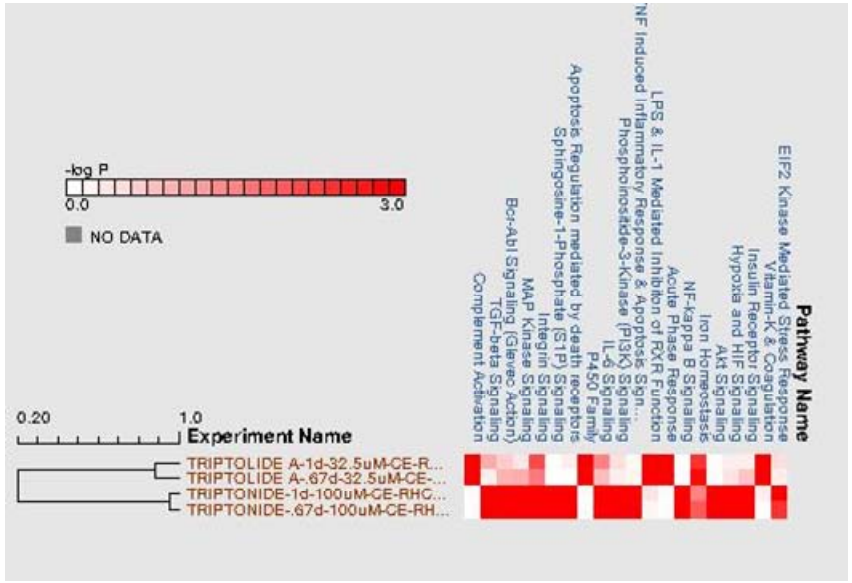

- **Triptonide (both 16h and 1d treatments) has overall greater impact on pathways than Triptolide analogue**
- **Pathways repressed to a greater level by Triptolide analogue include complement activation, P450 family, LPS & IL-1 mediated inhibition of RXR function, acute phase response, iron homeostasis, and vitamin K and coagulation**

Pathway impact of treatment-repressed genes (p<0.05) on 135 Iconix-curated pathways. Top 20 impacted pathways are displayed. Pathways are sorted left to right by average impact per pathway across all experiments in the set.

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Slide 12: Pathway impact analysis with repressed genes: triptonide and triptolide analogue show different patterns of repression across DrugMatrix pathways

Each experiment was evaluated for its impact on the 135 pathways using significantly repressed genes (p <0.05). Pathways are sorted from left to right based on the average impact score [-log(p-value)] per pathway across experiments. Experiments are clustered based on the pattern of impact on pathways. Triptonide and triptolide analogue showed different patterns of repression across those pathways. Triptonide treatments at both 16 and 24hr repressed genes that strongly impact pathways including TGF-beta, Bcr-Abl, MAP kinase and integrin signaling. Triptolide analogue repressed genes involved in complement activation, P450 family, acute phase response and Vitamin K and coagulation pathways.

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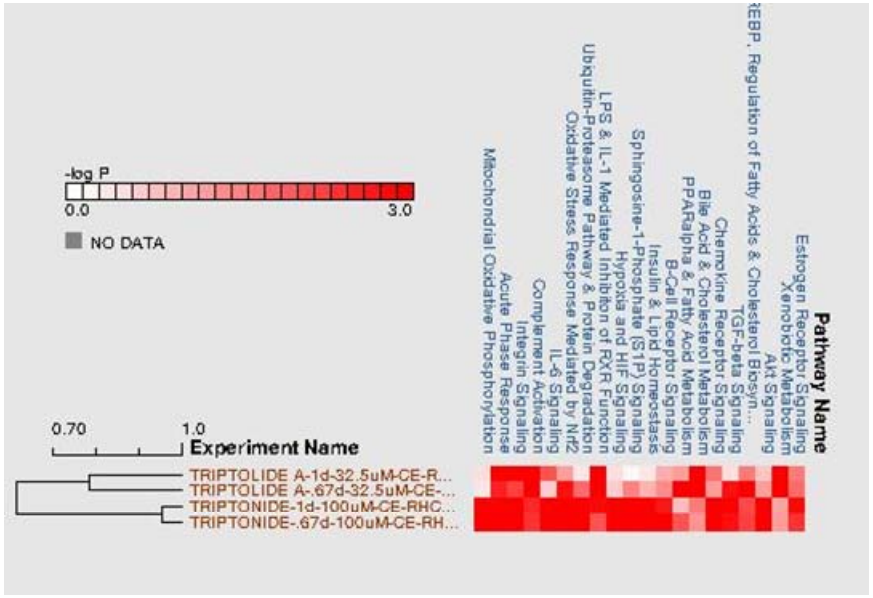
Pathway Impact with Perturbed Genes: Both Compounds Perturb Genes Involved in Oxidative Stress and Immune Response



Pathways perturbed by Triptonide and Triptolide analogue include mitochondrial oxidative phosphorylation, acute phase response, integrin signaling, complement activation, IL-6 signaling, and oxidative stress response mediated by Nrf2

Pathway impact of treatment-perturbed genes ($p<0.05$) on 135 Iconix-curated pathways. Top 20 impacted pathways are displayed. Pathways are sorted left to right by average impact per pathway across all experiments in the set.

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Slide 13: Pathway impact with perturbed genes: both compounds perturb genes involved in oxidative stress and immune response

Each experiment was evaluated for its impact on the 135 pathways using significantly perturbed genes ($p < 0.05$). Pathways are sorted from left to right based on the average impact score [-log(p-value)] per pathway across experiments. Experiments are clustered based on the pattern of impact on pathways. Triptonide and triptolide analogue both perturbed pathways including acute phase response, integrin signaling, and complement activation.

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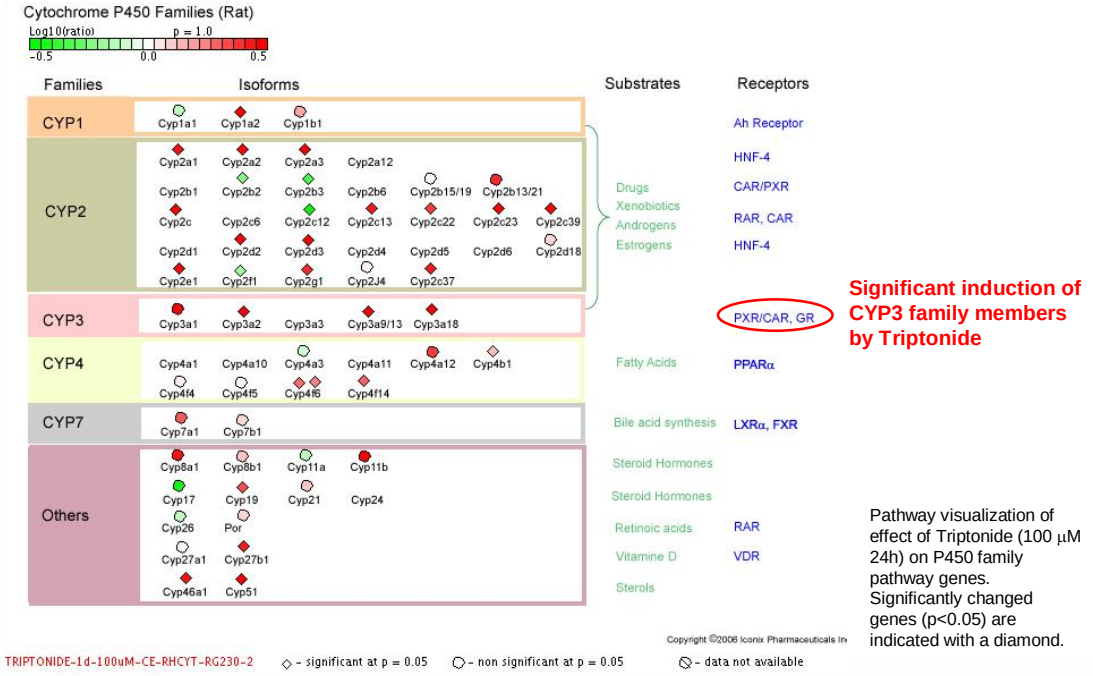
Triptonide Shows Significant Induction of a Large Number of Phase I Biotransformation Enzymes



Significant induction of CYP3 family members by Triptonide

Pathway visualization of effect of Triptonide (100 μM 24h) on P450 family pathway genes. Significantly changed genes ($p<0.05$) are indicated with a diamond.

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Slide 14: Triptonide shows significant induction of a large number of phase I biotransformation enzymes

Shown is the visualization of the effects of triptonide at 24hr on P450 pathway. The response from triptonide treatment on this pathway is indicated by circles and diamonds. Significantly induced or repressed genes ($p<0.05$) are indicated with a diamond. The coloring is based on the direction of the gene changes observed, where green represents downregulation and red represents upregulation. Triptonide significantly induced a large number of cytochrome P450 genes, especially Cyp3 family members.

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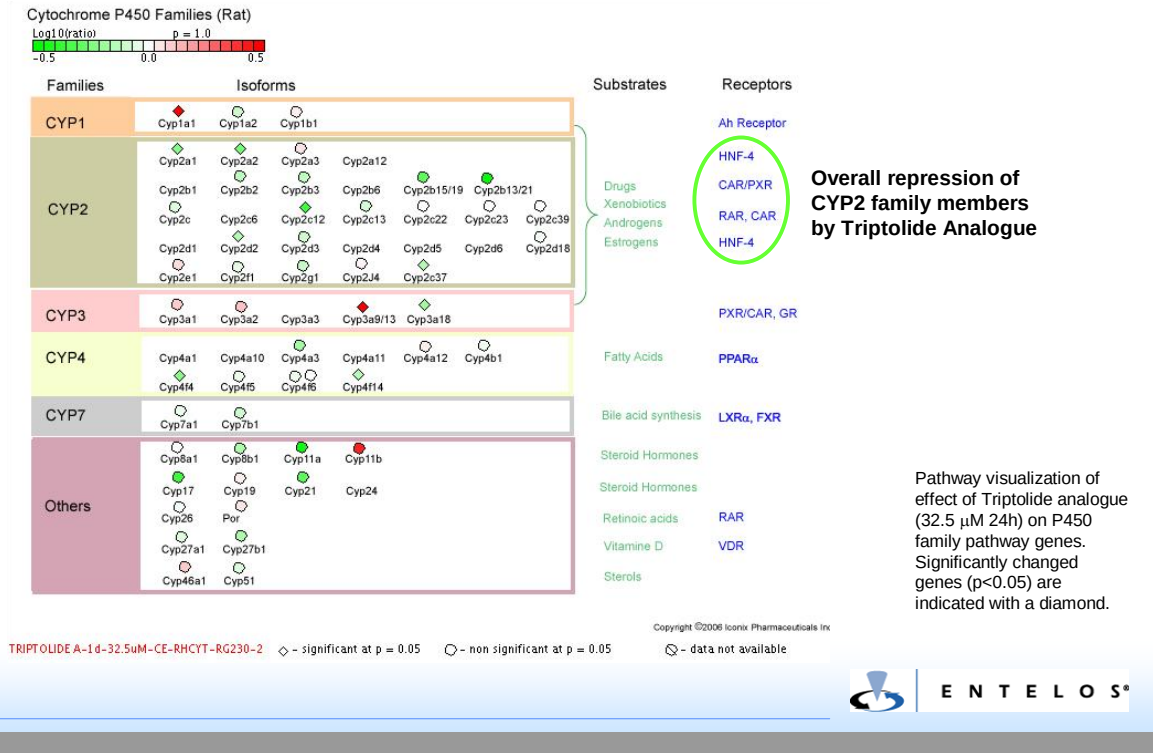
Triptolide Analogue Shows Significant Repression of a Large Number of Phase I Biotransformation Enzymes in the Cyp2 Family



Overall repression of CYP2 family members by Triptolide Analogue

Pathway visualization of effect of Triptolide analogue (32.5 μM 24h) on P450 family pathway genes. Significantly changed genes (p<0.05) are indicated with a diamond.

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Slide 15: Triptolide analogue shows significant repression of a large number of phase I biotransformation enzymes in the Cyp2 family

Shown is the visualization of the effects of triptolide analogue at 24hr on P450 pathway. The response from triptolide analogue treatment on this pathway is indicated by circles and diamonds. Significantly induced or repressed genes (p<0.05) are indicated with a diamond. The coloring is based on the direction of the gene changes observed, where green represents downregulation and red represents upregulation. Triptolide analogue significantly induced Cyp1a1, but repressed a number of cytochrome P450 genes, especially the Cyp2 family members.

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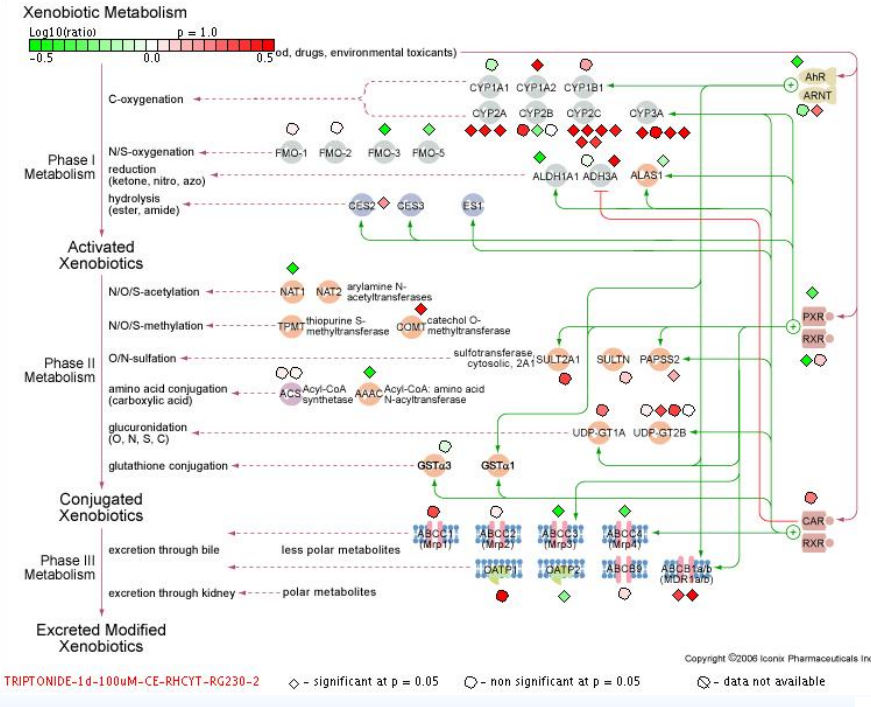
Triptonide Shows Significant Perturbation of Phase I and Phase II Biotransformation Enzymes



- **Significant repression of AhR, PXR, RXR, NAT1, and ABCC3 and 4**
- **Significant induction of CYP1A2, CYP2A, CYP2C, CYP3A, COMT, and MDR1**

Pathway visualization of effect of Triptonide (100 μM 24h) on xenobiotic metabolism pathway genes. Significantly changed genes ($p<0.05$) are indicated with a diamond.

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Slide 16 Triptonide shows significant perturbation of phase I and phase II biotransformation enzymes

Shown is the visualization of the effects of triptonide at 24hr on xenobiotic metabolism pathway. The response from triptonide treatment on this pathway is indicated by circles and diamonds. Significantly induced or repressed genes ($p<0.05$) are indicated with a diamond. The coloring is based on the direction of the gene changes observed, where green represents downregulation and red represents upregulation. Triptonide significantly perturbed a large number of genes involved in phase I, II and III drug metabolism.

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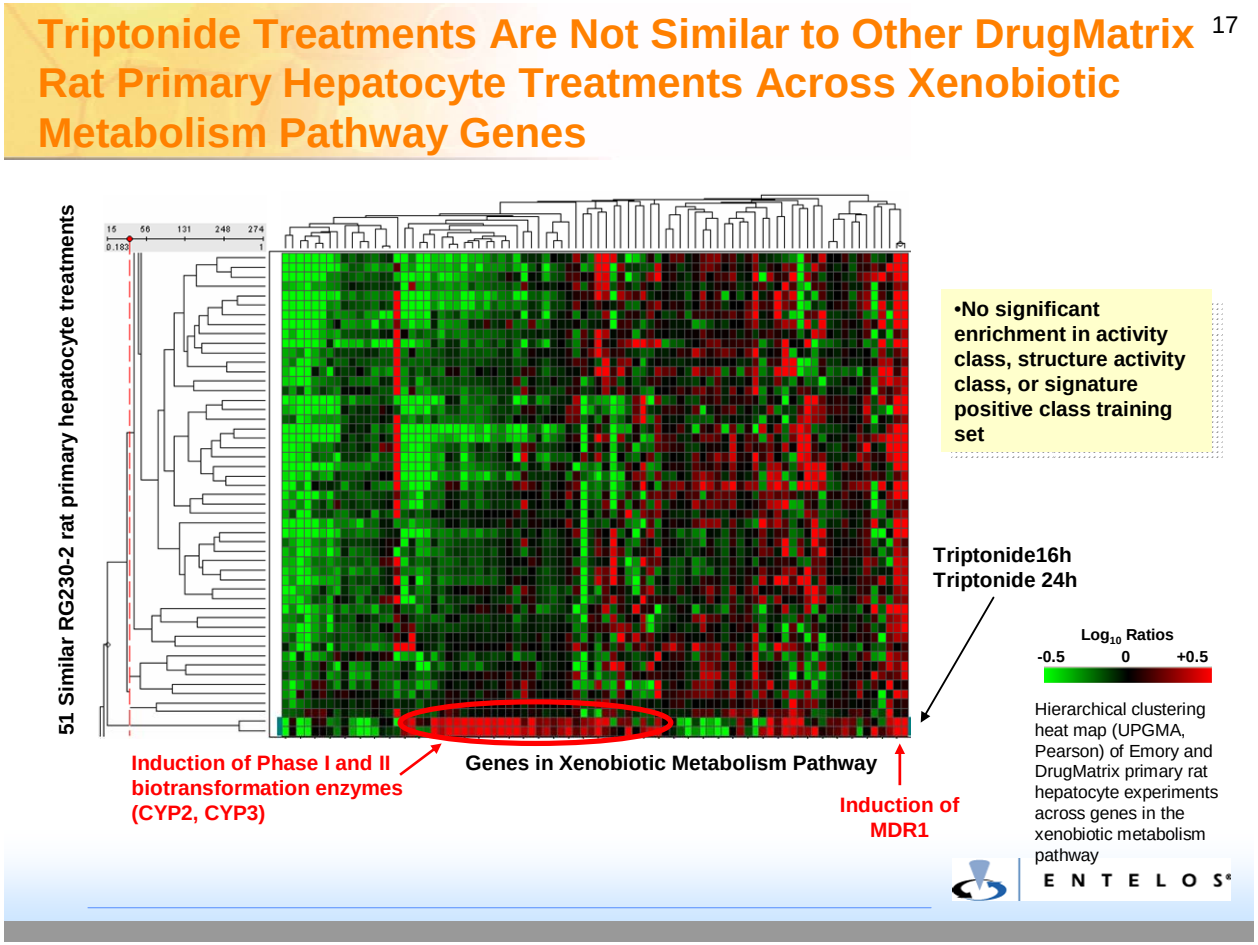

Slide 17: Triptonide treatments are not similar to other DrugMatrix rat primary hepatocyte treatments across xenobiotic metabolism pathway genes

Shown is the 2-D hierarchical clustering (UPGMA) of triptonide and triptolide analogue experiments and DrugMatrix rat primary hepatocyte experiments across the xenobiotic metabolism pathway genes. The result indicates that triptonide perturbed xenobiotic metabolism genes in a pattern distinct from other DrugMatrix experiments.

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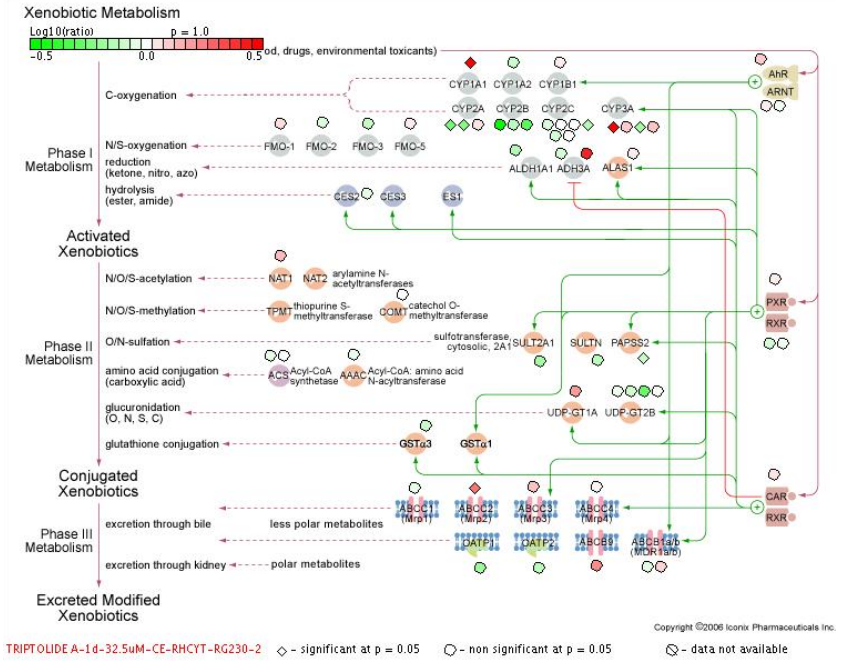
Triptolide Analogue Shows a Weaker Overall Impact on Xenobiotic Metabolism Genes Than Triptonide



- **Induction of CYP1A1 by Triptolide Analogue and CYP1A2 by Triptonide**
- **Induction of CYP2A and CYP2C by Triptonide opposite of repression by Triptolide Analogue**
- **Triptolide Analogue has minimal impact on receptor gene expression (AhR, PXR, RXR, CAR)**

Pathway visualization of effect of Triptolide analogue (32.5 μM 24h) on xenobiotic metabolism pathway genes. Significantly changed genes (p<0.05) are indicated with a diamond.

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Slide 18: Triptolide analogue shows a weaker overall impact on xenobiotic metabolism genes than triptonide

Shown is the visualization of the effects of triptolide analogue at 24hr on xenobiotic metabolism pathway. The response from triptolide analogue treatment on this pathway is indicated by circles and diamonds. Significantly induced or repressed genes (p<0.05) are indicated with a diamond. The coloring is based on the direction of the gene changes observed, where green represents downregulation and red represents upregulation. Triptolide analogue showed a weaker overall impact on the xenobiotic metabolism pathway than triptonide.

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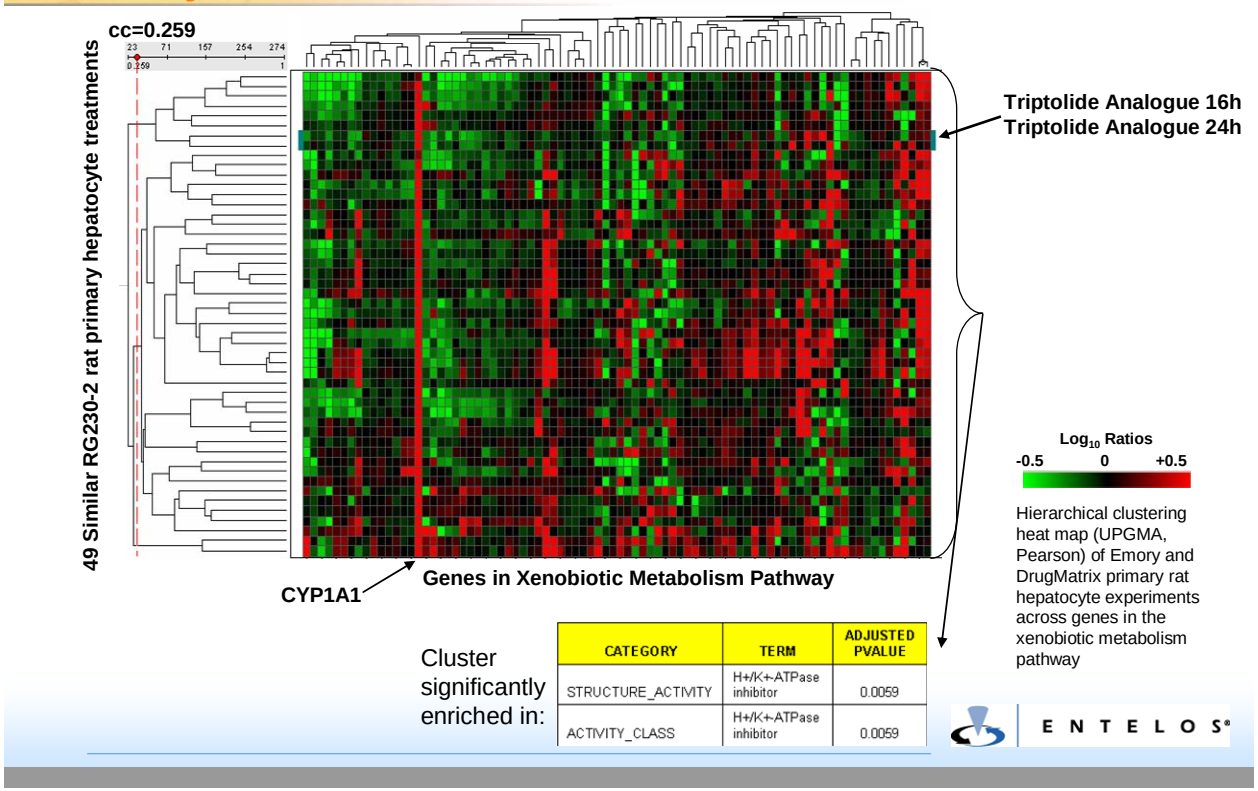
Triptolide Analogue is Similar to H+/K+ ATPase Inhibitors in Rat Primary Hepatocytes Across Xenobiotic Metabolism Pathway Genes



Slide 19: Triptolide analogue is similar to H+/K+ ATPase inhibitors in rat primary hepatocytes across xenobiotic metabolism pathway genes

Shown is the 2-D hierarchical clustering (UPGMA) of triptonide and triptolide analogue experiments and DrugMatrix rat primary hepatocyte experiments across the xenobiotic metabolism pathway genes. The result indicates that triptolide analogue perturbed xenobiotic metabolism genes in a pattern similar to H+/K+-ATPase inhibitors.

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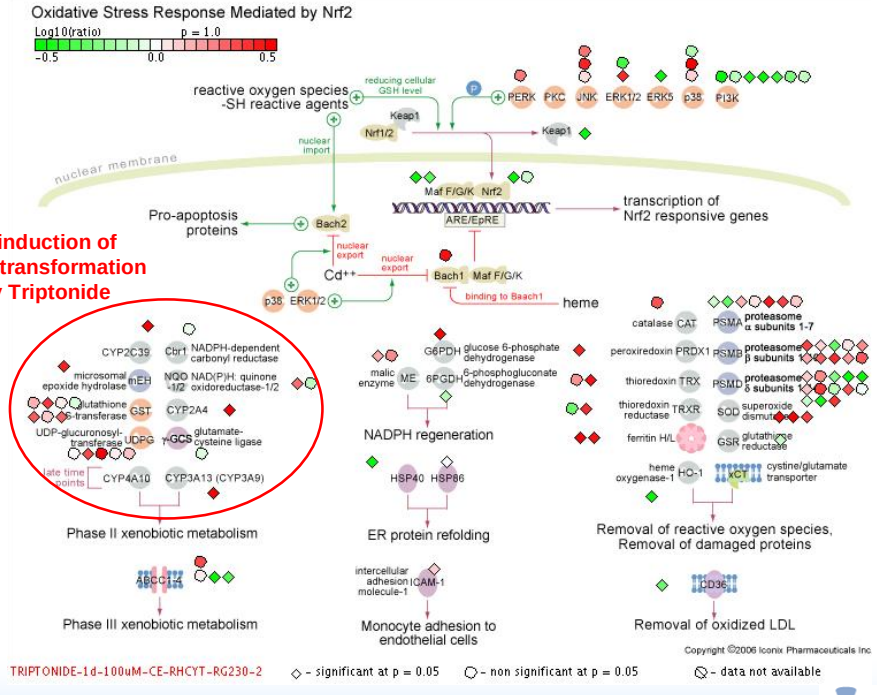
Triptonide Treatment Results in Significant Perturbation of Genes In Oxidative Stress Mediated by Nrf2 Pathway



Significant induction of phase II biotransformation enzymes by Triptonide

•Induction of genes related to antioxidant defense response (SOD, TRX, PRDX1, catalase)

Pathway visualization of effect of Triptonide (100 μM 24h) on oxidative stress response mediated by Nrf2 pathway genes. Significantly changed genes (p<0.05) are indicated with a diamond.

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Slide 20: Triptonide treatment results in significant perturbation of genes in oxidative stress mediated by Nrf2 pathways

Shown is the visualization of the effects of triptonide at 24hr on oxidative stress mediated by Nrf2 pathway. The response from triptonide treatment on this pathway is indicated by circles and diamonds. Significantly induced or repressed genes (p<0.05) are indicated with a diamond. The coloring is based on the direction of the gene changes observed, where green represents downregulation and red represents upregulation. Triptonide significantly induced genes involved in antioxidant defense response.

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Triptolide Analogue Treatment Results in Overall Weak Impact on Oxidative Stress Response Mediated by Nrf2 Pathway Genes

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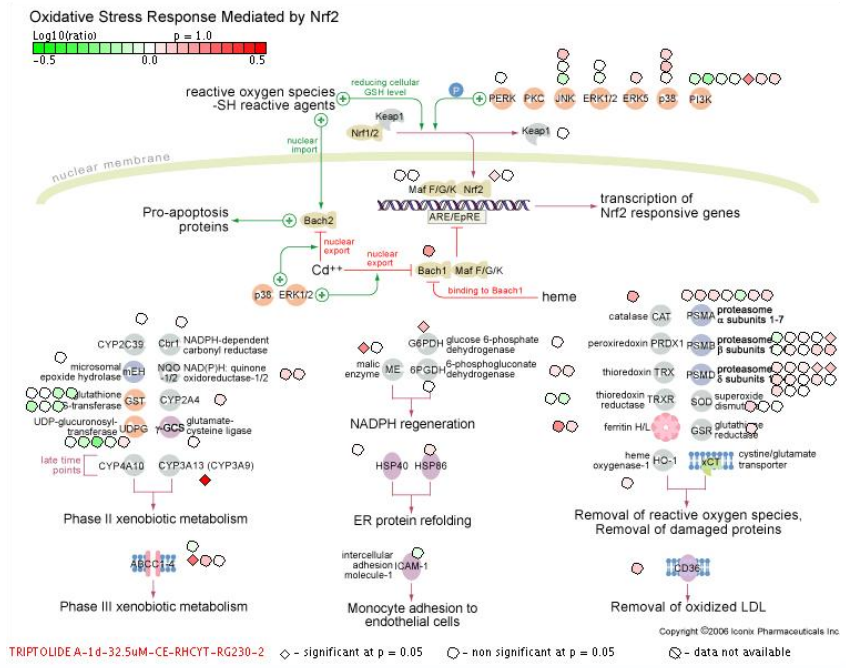

Pathway visualization of effect of Triptolide analogue (32.5 μM 24h) on oxidative stress response mediated by Nrf2 pathway genes. Significantly changed genes (p<0.05) are indicated with a diamond.

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Slide 21: Triptolide analogue treatment results in overall weak impact on oxidative stress response mediated by Nrf2 pathway genes

Shown is the visualization of the effects of triptolide analogue at 24hr on the oxidative stress response mediated by Nrf2 pathway. The response from triptolide analogue treatment on this pathway is indicated by circles and diamonds. Significantly induced or repressed genes (p<0.05) are indicated with a diamond. The coloring is based on the direction of the gene changes observed, where green represents downregulation and red represents upregulation. Triptolide analogue showed a weaker overall impact on the oxidative stress response mediated by Nrf2 pathway than triptonide.

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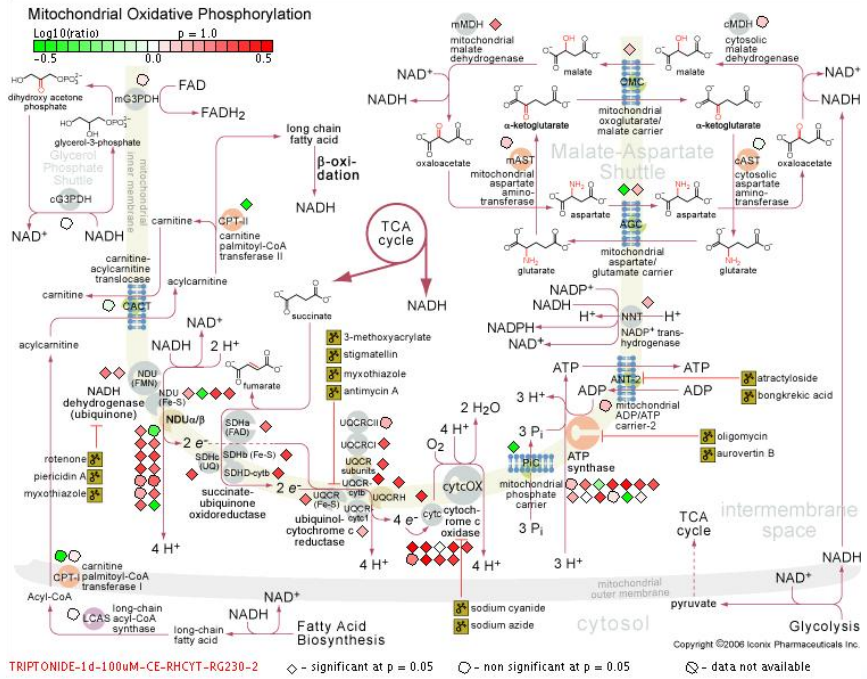
Triptonide Treatment Results in Significant Induction of Genes in Mitochondrial Oxidative Phosphorylation Pathway



Pathway visualization of effect of Triptonide (100 μM 24h) on mitochondrial oxidative phosphorylation pathway genes. Significantly changed genes (p<0.05) are indicated with a diamond.

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Slide 22: Triptonide treatment results in significant induction of genes in mitochondrial oxidative phosphorylation pathway

Shown is the visualization of the effects of triptonide at 24hr on mitochondrial oxidative phosphorylation pathway. The response from triptonide treatment on this pathway is indicated by circles and diamonds. Significantly induced or repressed genes (p<0.05) are indicated with a diamond. The coloring is based on the direction of the gene changes observed, where green represents downregulation and red represents upregulation. Triptonide significantly induced a large number of genes involved in mitochondrial oxidative phosphorylation.

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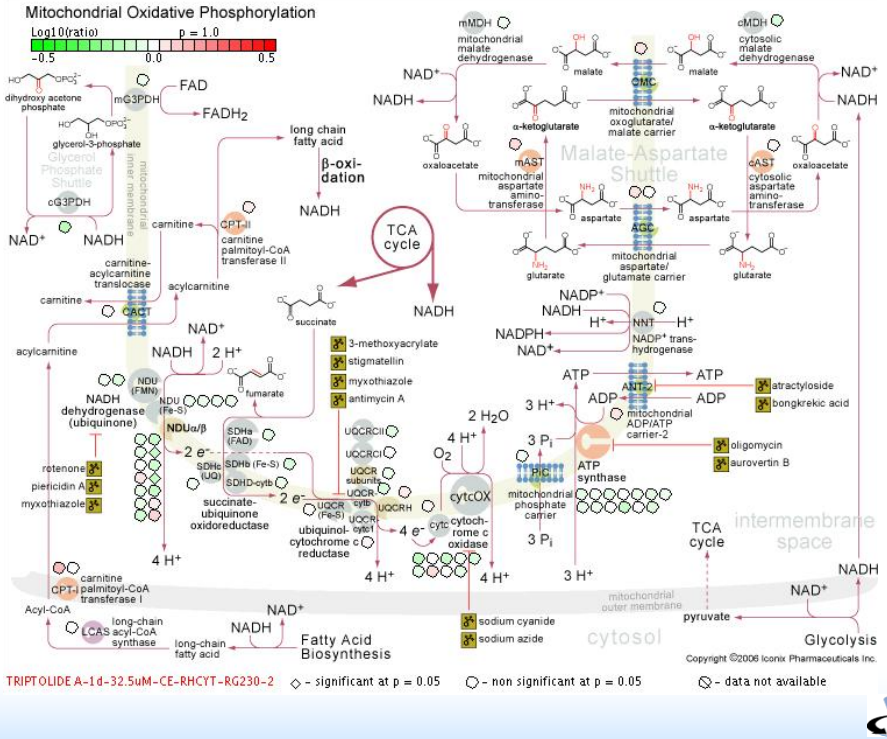
Triptolide Analogue Treatment Results in Minimal Perturbation of Genes in Mitochondrial Oxidative Phosphorylation Pathway



- **Overall weak response compared to Triptonide treatments**
- **Similar effect seen with 16h treatment**

Pathway visualization of effect of Triptolide analogue (32.5 μM 24h) on mitochondrial oxidative phosphorylation pathway genes. Significantly changed genes (p<0.05) are indicated with a diamond.

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Slide 23: Triptolide analogue treatment results in minimal perturbation of genes in mitochondrial oxidative phosphorylation pathway

Shown is the visualization of the effects of triptolide analogue at 24hr on the mitochondrial oxidative phosphorylation pathway. The response from triptolide analogue treatment on this pathway is indicated by circles and diamonds. Significantly induced or repressed genes (p<0.05) are indicated with a diamond. The coloring is based on the direction of the gene changes observed, where green represents downregulation and red represents upregulation. Triptolide analogue treatment resulted in minimal perturbation of genes in the mitochondrial oxidative phosphorylation pathway.

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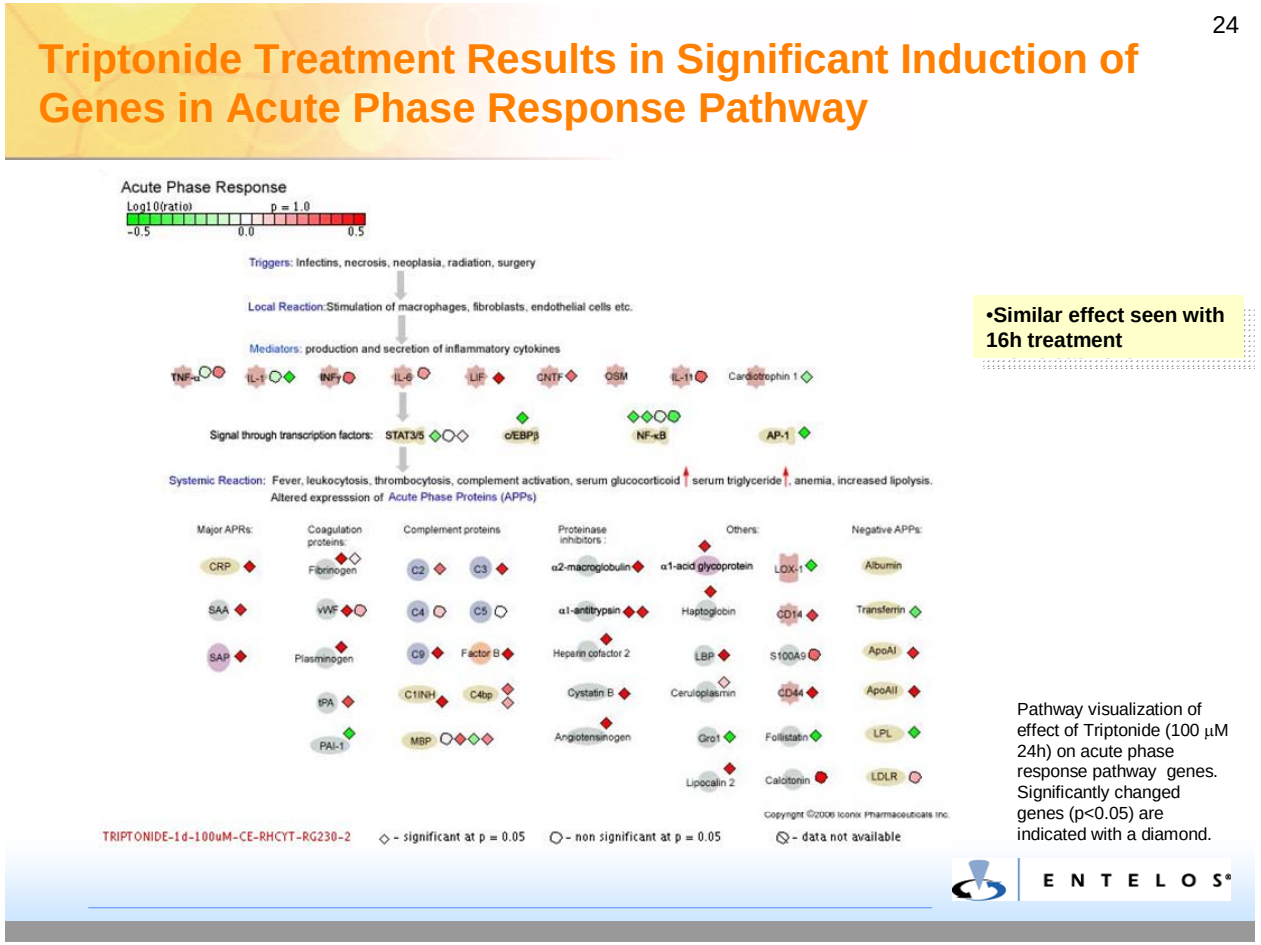

Slide 24: Triptonide treatment results in significant induction of genes in acute phase response pathway

Shown is the visualization of the effects of triptonide at 24hr on acute phase response pathway. The response from triptonide treatment on this pathway is indicated by circles and diamonds. Significantly induced or repressed genes ($p<0.05$) are indicated with a diamond. The coloring is based on the direction of the gene changes observed, where green represents downregulation and red represents upregulation. Triptonide significantly induced genes involved in acute phase response.

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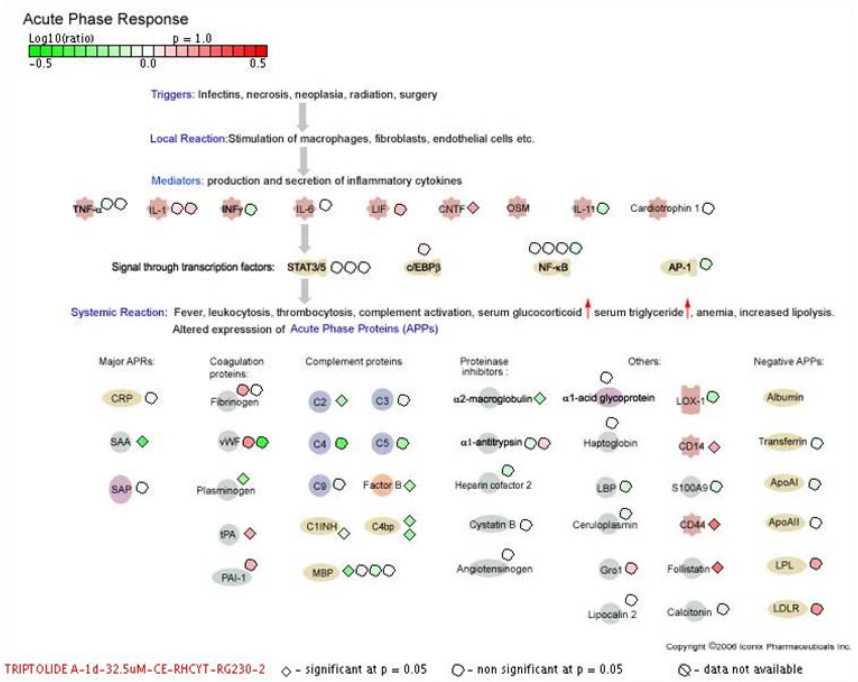
Triptolide Analogue Treatment Results in Minimal Perturbation of Genes in Acute Phase Response Pathway



- **Overall weak response compared to Triptonide treatments**
- **Similar effect seen with 16h treatment**

Pathway visualization of effect of Triptolide analogue (32.5 μM 24h) on acute phase response pathway genes. Significantly changed genes ($p<0.05$) are indicated with a diamond.

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Slide 25: Triptolide analogue treatment results in minimal perturbation of genes in acute phase response pathway

Shown is the visualization of the effects of triptolide analogue at 24hr on acute phase response pathway. The response from triptolide analogue treatment on this pathway is indicated by circles and diamonds. Significantly induced or repressed genes ($p<0.05$) are indicated with a diamond. The coloring is based on the direction of the gene changes observed, where green represents downregulation and red represents upregulation. Triptolide analogue treatment resulted in minimal perturbation of genes in the acute phase response pathway.

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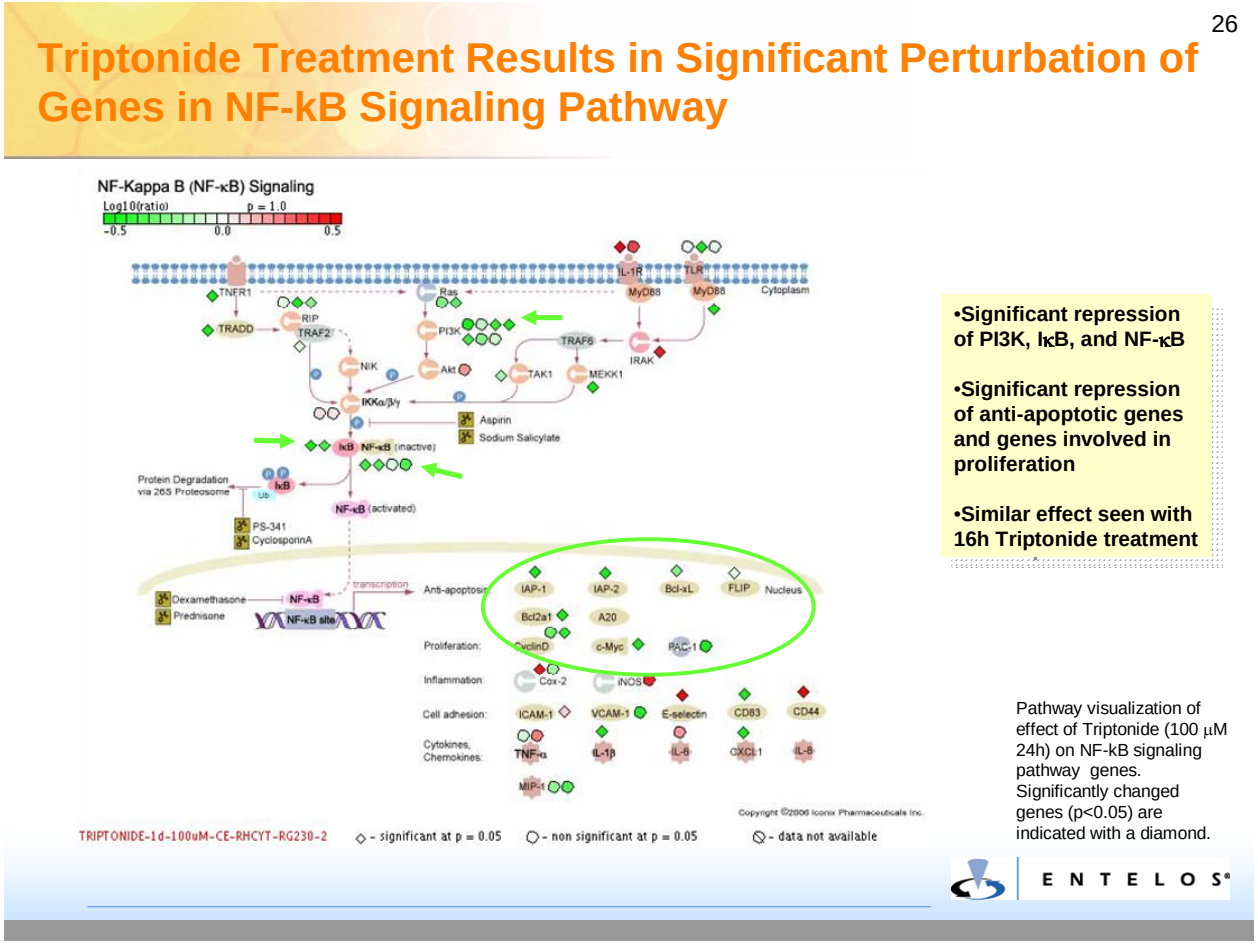

Slide 26: Triptonide treatment results in significant perturbation of genes in NF-kB signaling pathway

Shown is the visualization of the effects of triptonide at 24hr on NF-kB signaling pathway. The response from triptonide treatment on this pathway is indicated by circles and diamonds. Significantly induced or repressed genes ($p<0.05$) are indicated with a diamond. The coloring is based on the direction of the gene changes observed, where green represents downregulation and red represents upregulation. Triptonide significantly induced IL-1R and IRAK, but repressed down-stream factor PI3K, IkB and NF-kB. Significant repression of anti-apoptotic genes and genes involved in proliferation was observed following triptonide treatment, but Cox-2 and iNOS were induced.

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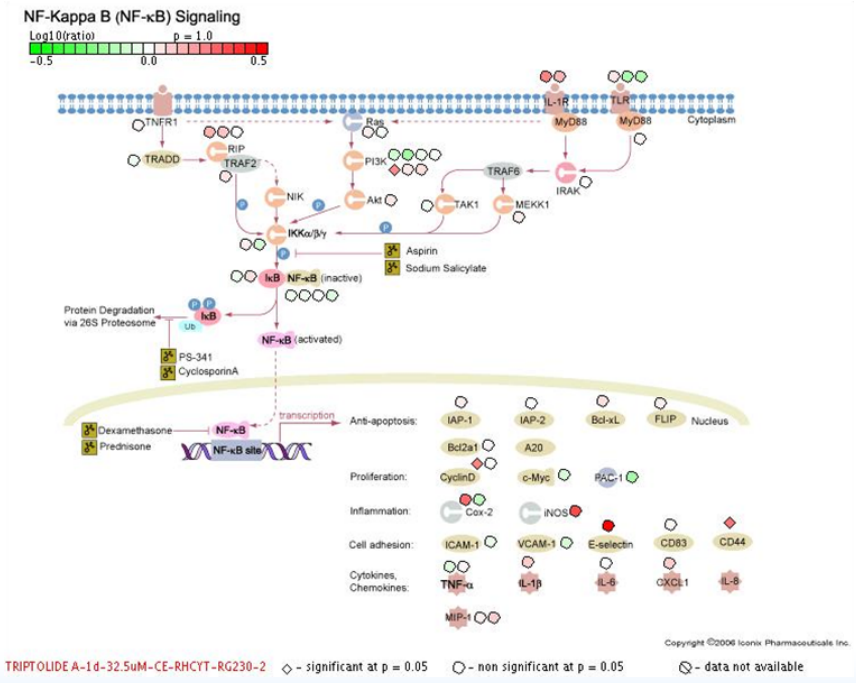
Triptolide Analogue Treatment Results in Minimal Perturbation of Genes in NF-kB Signaling Pathway



- **Overall weak response compared to Triptonide treatments**
- **No significant repression of IκB, NF-κB, or PI3K**
- **Similar effect seen with 16h treatment**

Pathway visualization of effect of Triptolide analogue (32.5 μM 24h) on NF-kB signaling pathway genes. Significantly changed genes (p<0.05) are indicated with a diamond.

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Slide 27: Triptolide analogue treatment results in minimal perturbation of genes in NF-kB signaling pathway

Shown is the visualization of the effects of triptolide analogue at 24hr on the NF-kB signaling pathway. The response from triptolide analogue treatment on this pathway is indicated by circles and diamonds. Significantly induced or repressed genes (p<0.05) are indicated with a diamond. The coloring is based on the direction of the gene changes observed, where green represents downregulation and red represents upregulation. Triptolide analogue treatment resulted in minimal perturbation of genes in NF-kB signaling pathway.

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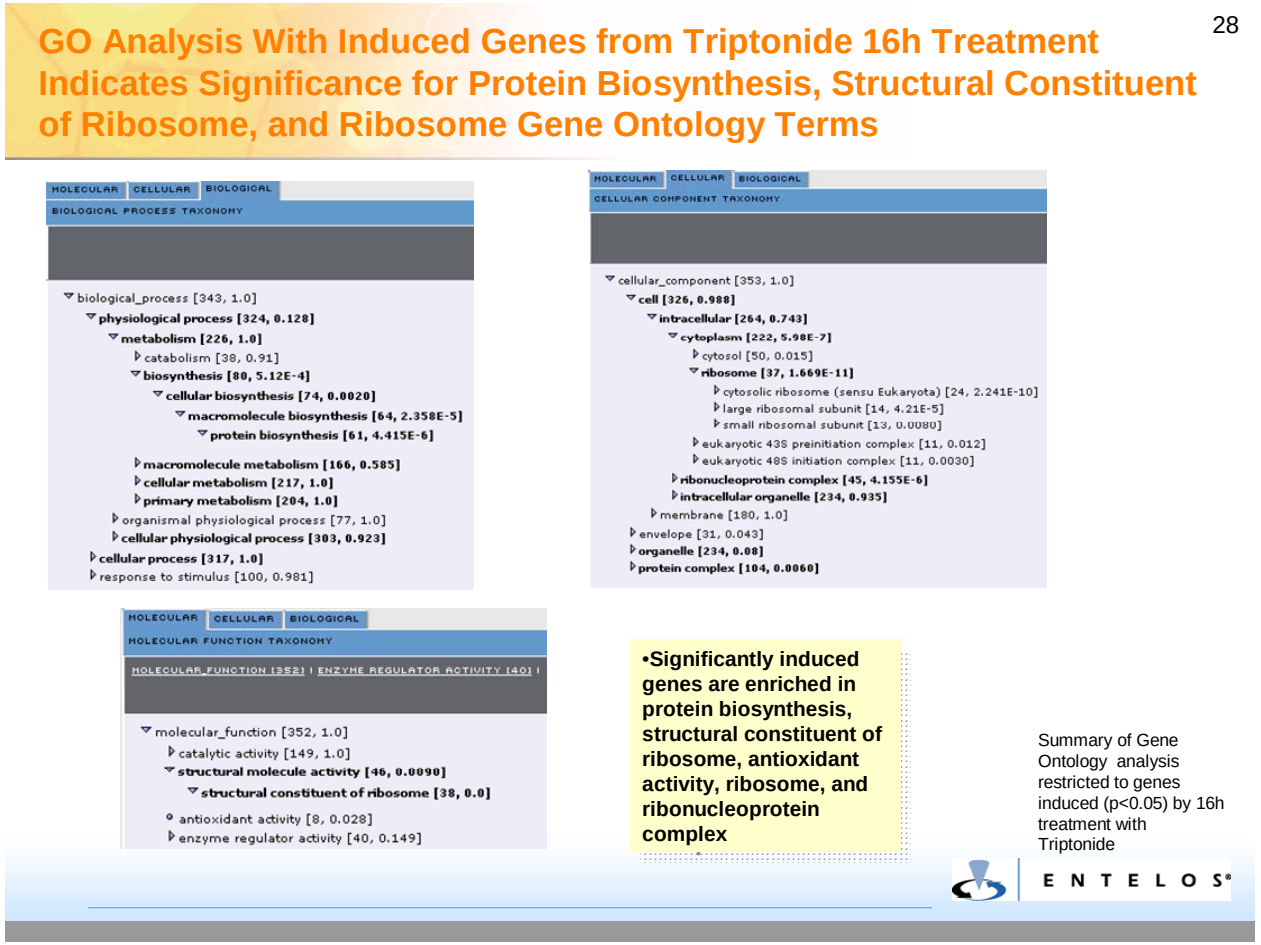

Slide 28: GO analysis with induced genes from triptonide 16h treatment indicates significance for protein biosynthesis, structural constituent of ribosome, and ribosome Gene Ontology terms

The Gene Ontology consortium has curated information for a large fraction of the genes that are used on the Affymetrix arrays. The Gene Ontology analysis determines whether a subset of selected genes is enriched in any Gene Ontology (GO) terms. The probability of the coincidence of the genes within the selected subset and the genes in each GO category is determined by the hypergeometric distribution and represented by a p value. Genes significantly ($p<0.05$) induced by triptonide treatment at 16hr were submitted to the Gene Ontology analysis. Protein biosynthesis and structural constituent of ribosome are significantly impacted Gene Ontology terms by triptonide

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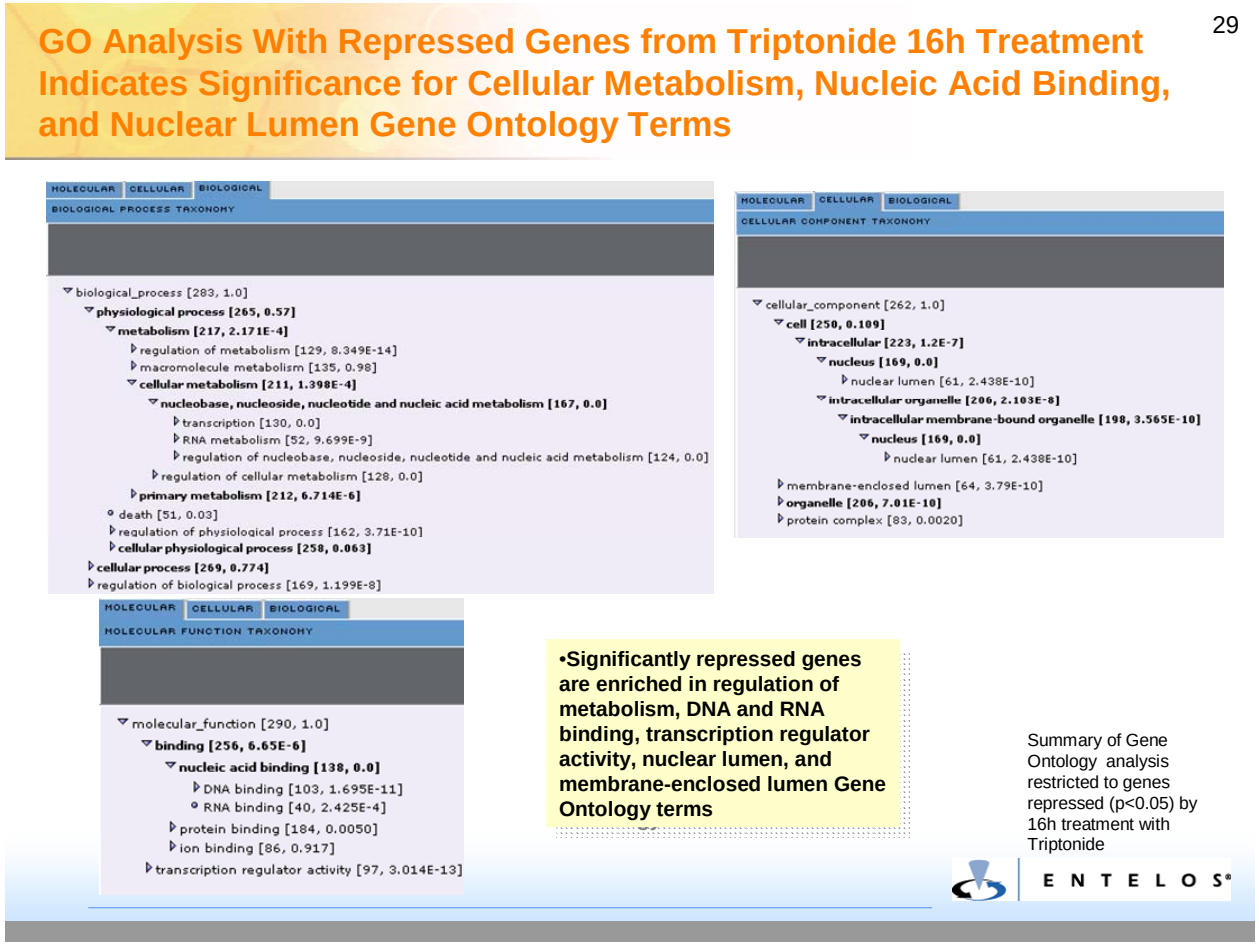

Slide 29: GO analysis with repressed genes from triptonide 16h treatment indicates significance for cellular metabolism, nucleic acid binding, and nuclear lumen Gene Ontology terms

Shown is the Gene Ontology analysis using genes significantly ($p<0.05$) repressed by triptonide treatment at 16hr. Cellular metabolism, nucleic acid binding are significantly impacted Gene Ontology terms by triptonide.

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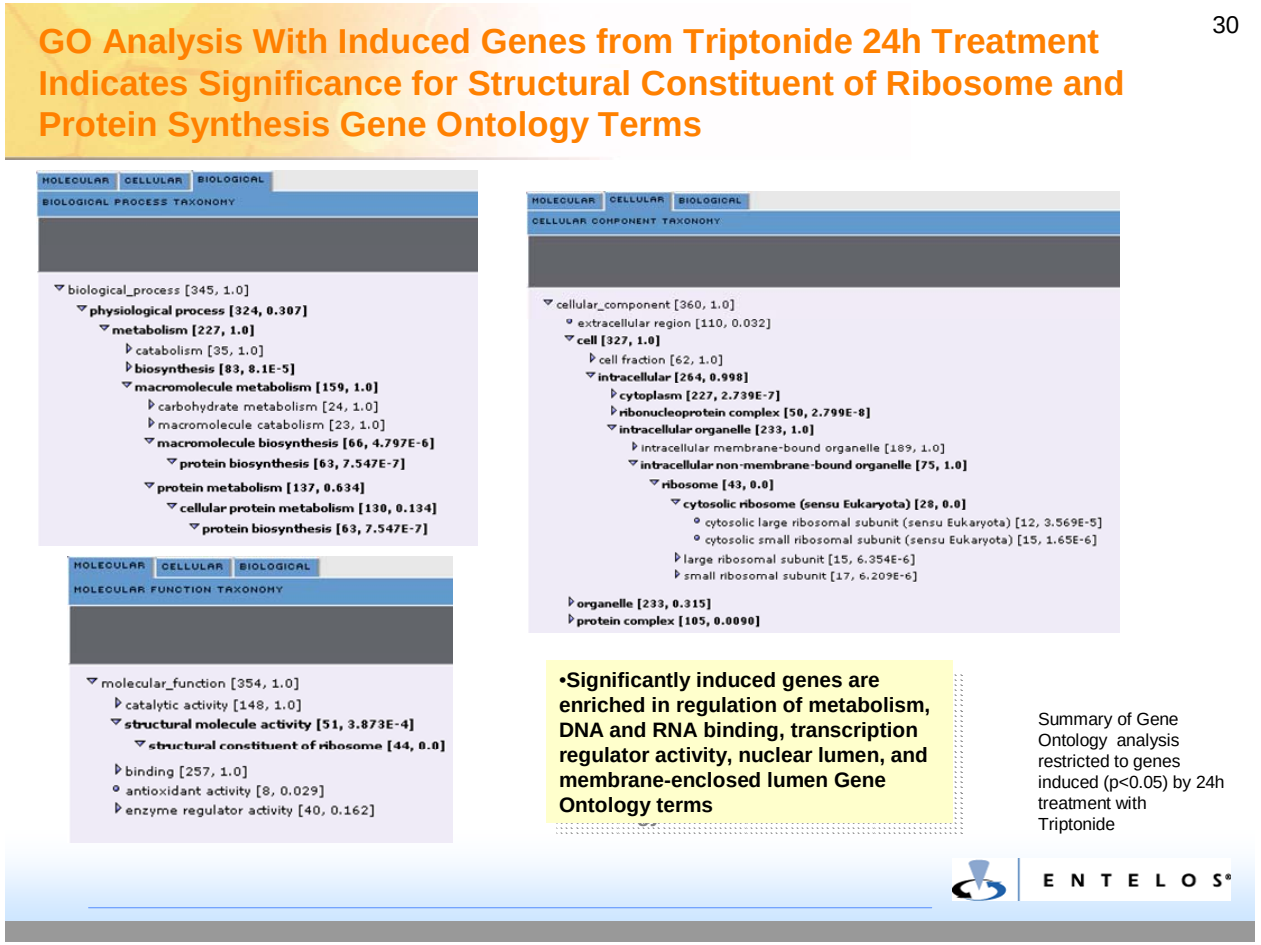

Slide 30: GO analysis with induced genes from triptonide 24h treatment indicates significance for structural constituent of ribosome and protein synthesis Gene Ontology terms

Shown is the Gene Ontology analysis using genes significantly ($p<0.05$) induced by triptonide treatment at 24hr. Structural constituent of ribosome and protein biosynthesis are significantly impacted Gene Ontology terms by triptonide.

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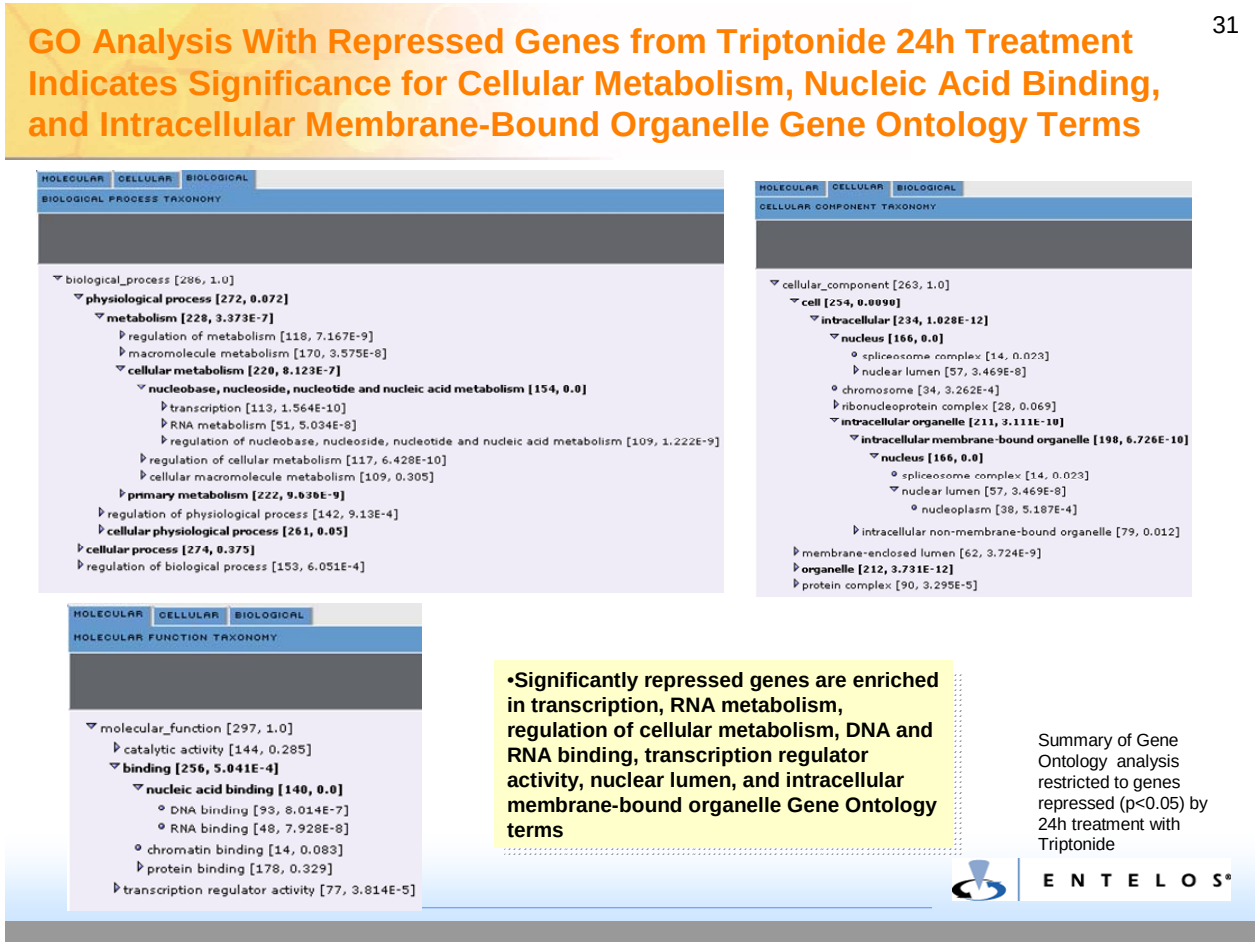

Slide 31: GO analysis with repressed genes from triptonide 24h treatment indicates significance for cellular metabolism, nucleic acid binding, and intracellular membrane-bound organelle Gene Ontology terms

Shown is the Gene Ontology analysis using genes significantly ($p<0.05$) repressed by triptonide treatment at 24hr. Cellular metabolism, nucleic acid binding, and intracellular membrane-bound organelle are significantly impacted Gene Ontology terms by triptonide.

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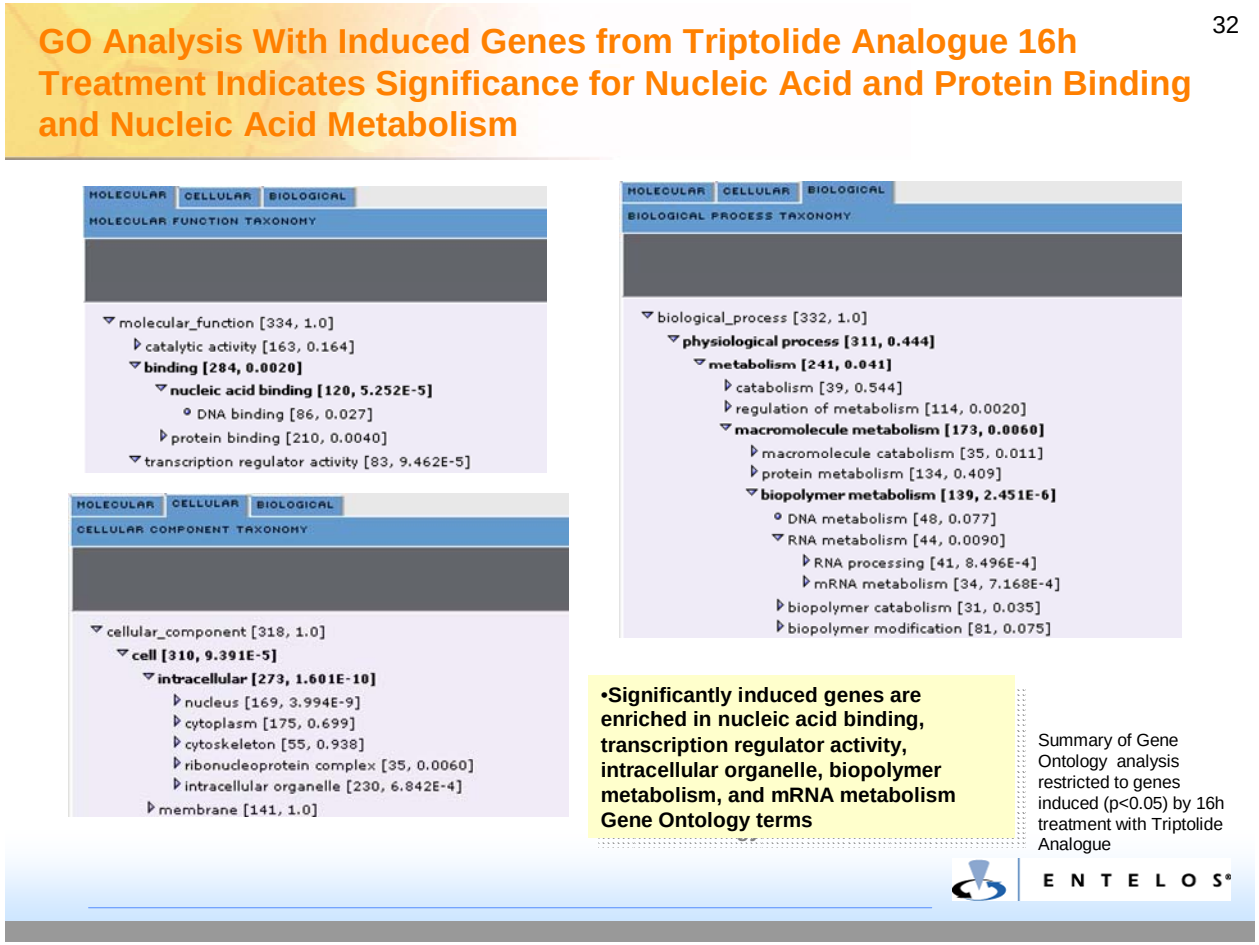

Slide 32: GO analysis with induced genes from triptolide analogue 16h treatment indicates significance for nucleic acid and protein binding and nucleic acid metabolism

Shown is the Gene Ontology analysis using genes significantly ($p<0.05$) induced by triptolide analogue treatment at 16hr. Nucleic acid and protein binding and nucleic acid metabolism are significantly impacted Gene Ontology terms by triptolide analogue.

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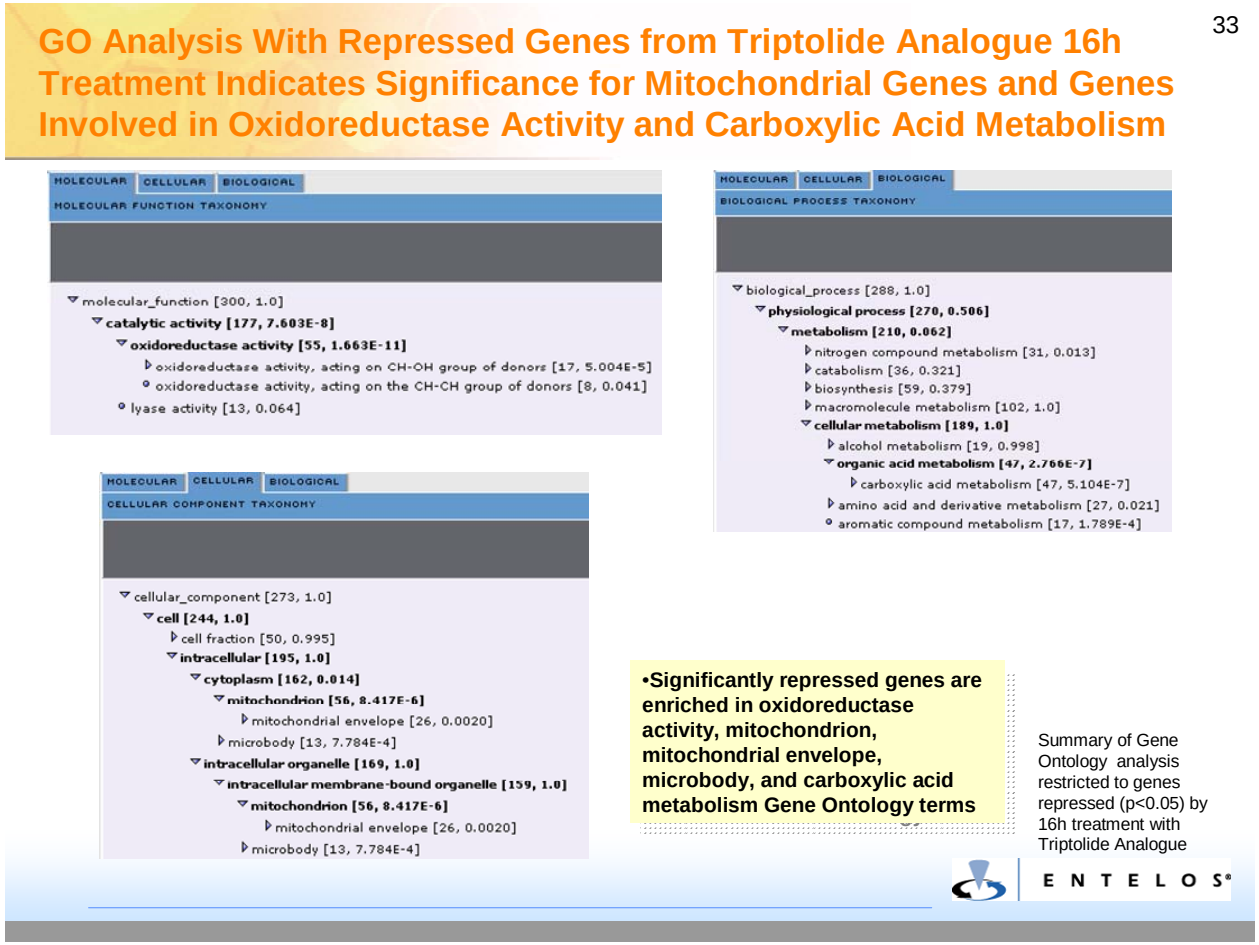

Slide 33: GO analysis with repressed genes from triptolide analogue 16h treatment indicates significance for mitochondrial genes and genes involved in oxidoreductase activity and carboxylic acid metabolism

Shown is the Gene Ontology analysis using genes significantly ($p<0.05$) repressed by triptolide analogue treatment at 16hr. Mitochondrion, oxidoreductase activity and carboxylic acid metabolism are significantly impacted Gene Ontology terms by triptolide analogue.

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GO Analysis With Induced Genes from Triptolide Analogue 24h Treatment Indicates Significance for Genes in Protein Binding

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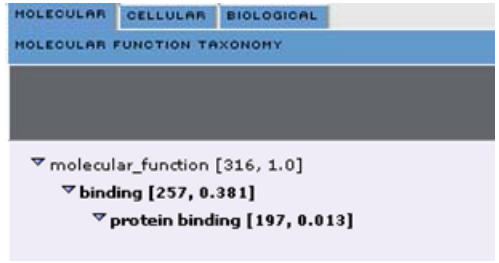

•No enrichment in Cellular Component or Biological Process Gene Ontology terms from genes induced by 24h treatment with Triptolide Analogue

Summary of Gene Ontology analysis restricted to genes induced ($p<0.05$) by 24h treatment with Triptolide Analogue

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Slide 34: GO analysis with induced genes from triptolide analogue 24h treatment indicates significance for genes in protein binding

Shown is the Gene Ontology analysis using genes significantly ($p<0.05$) induced by triptolide analogue treatment at 24hr. Protein binding is significantly impacted Gene Ontology term by triptolide analogue.

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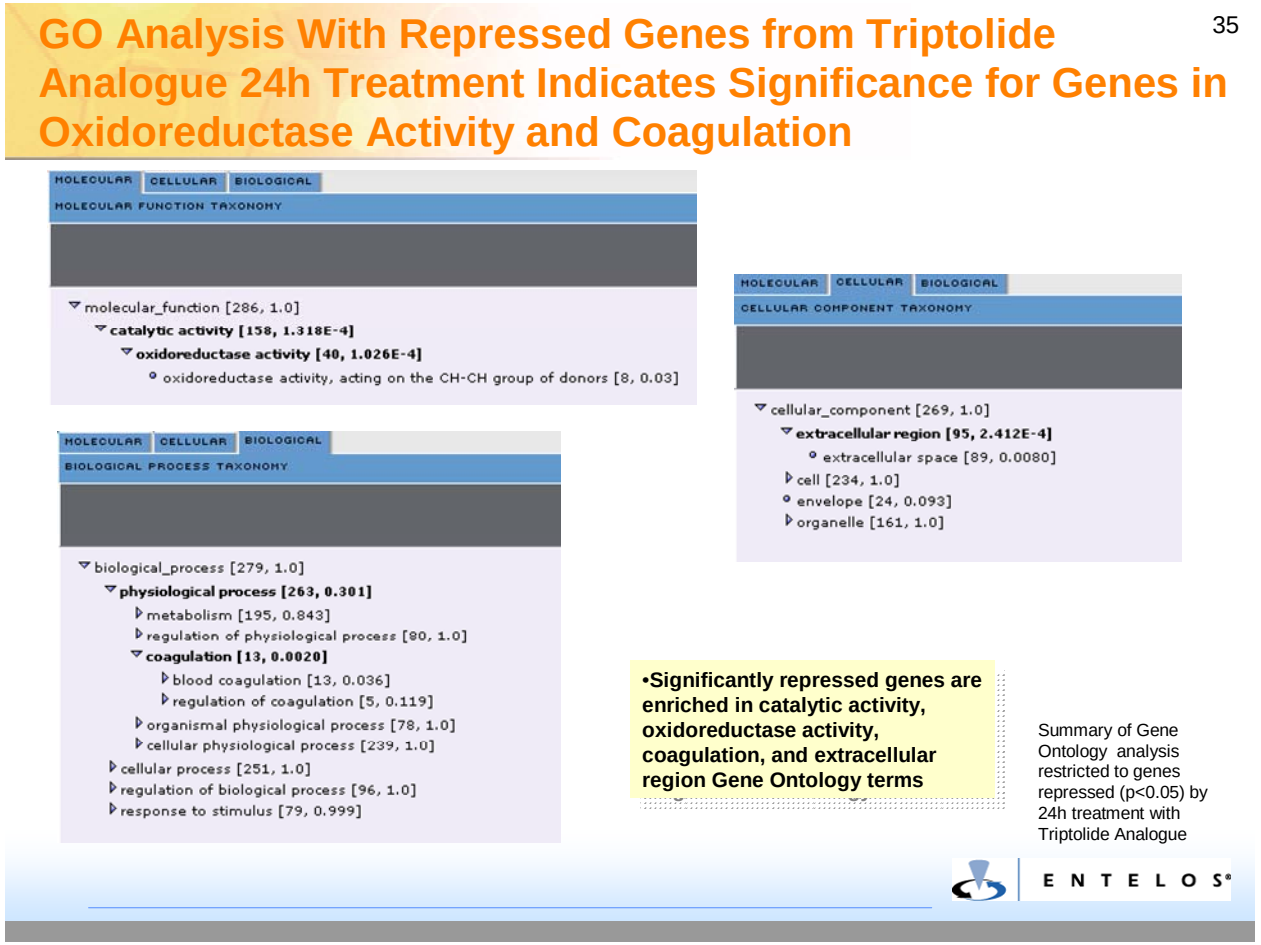

Slide 35: GO analysis with repressed genes from triptolide analogue 24h treatment indicates significance for genes in oxidoreductase activity and coagulation

Shown is the Gene Ontology analysis using genes significantly ($p<0.05$) repressed by triptolide analogue treatment at 24hr. Oxidoreductase activity and coagulation are significantly impacted Gene Ontology terms by triptolide analogue.

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Summary of Gene Ontology Analysis Results

	Induced	Repressed
Triptonide	Ribosomal protein synthesis (16hr, 24hr)	Nucleic acid binding and metabolism (16hr, 24hr)
Triptolide Analogue	Protein binding (24hr)	Oxidoreductase activity, coagulation (24hr)
	Nucleic acid, protein binding and DNA, RNA metabolism (16hr)	Mitochondrial oxidoreductase activity, carboxylic acid metabolism (16hr)

Slide 36: Summary of gene ontology analysis results

Triptonide treatments at 16 and 24hr show similar impact on Gene Ontology terms, inducing ribosomal protein synthesis and repressing nucleic acid binding and metabolism. Triptolide analogue induces protein binding and represses oxidoreductase activity at both time-points. Triptolide analogue also induces nucleic acid binding and metabolism and represses carboxylic acid metabolism at 16hr.

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Summary of Gene Expression Findings

	Triptonide	Triptolide Analogue
Gene Perturbation	40-50%	<10%
Compound Similarity	Similar to free radical generator	Similar to H+/K+-ATPase inhibitor
Drug Signature Match	Strong match to hepatotoxicity, phospholipidosis, cholestasis and GSH depletion signatures	Weak to moderate match to hepatotoxicity, apoptosis signatures
Pathway Perturbation	Strongly impact pathways including mitochondrial oxidative phosphorylation, acute phase response, oxidative stress and xenobiotics metabolism	Minimal impact on pathways
Drug Metabolism	Strong induction of phase I and phase II drug metabolism genes, potential PXR/CAR activator	Significant induction of Cyp1a1, overall repression of Cyp2 family members
Oxidative Stress	Strong induction of antioxidant defense response and glutathione metabolism genes, indicative of oxidative stress	Minimal perturbation of genes involved in oxidative stress response
Gene Ontology	Induction of ribosomal protein biosynthesis and repression of nucleic acid binding and metabolism	Induction of nucleic acid and protein binding, repression of oxidoreductase activity

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Slide 37: Summary of gene expression findings

Refer to the executive summary for discussions on gene expression findings.

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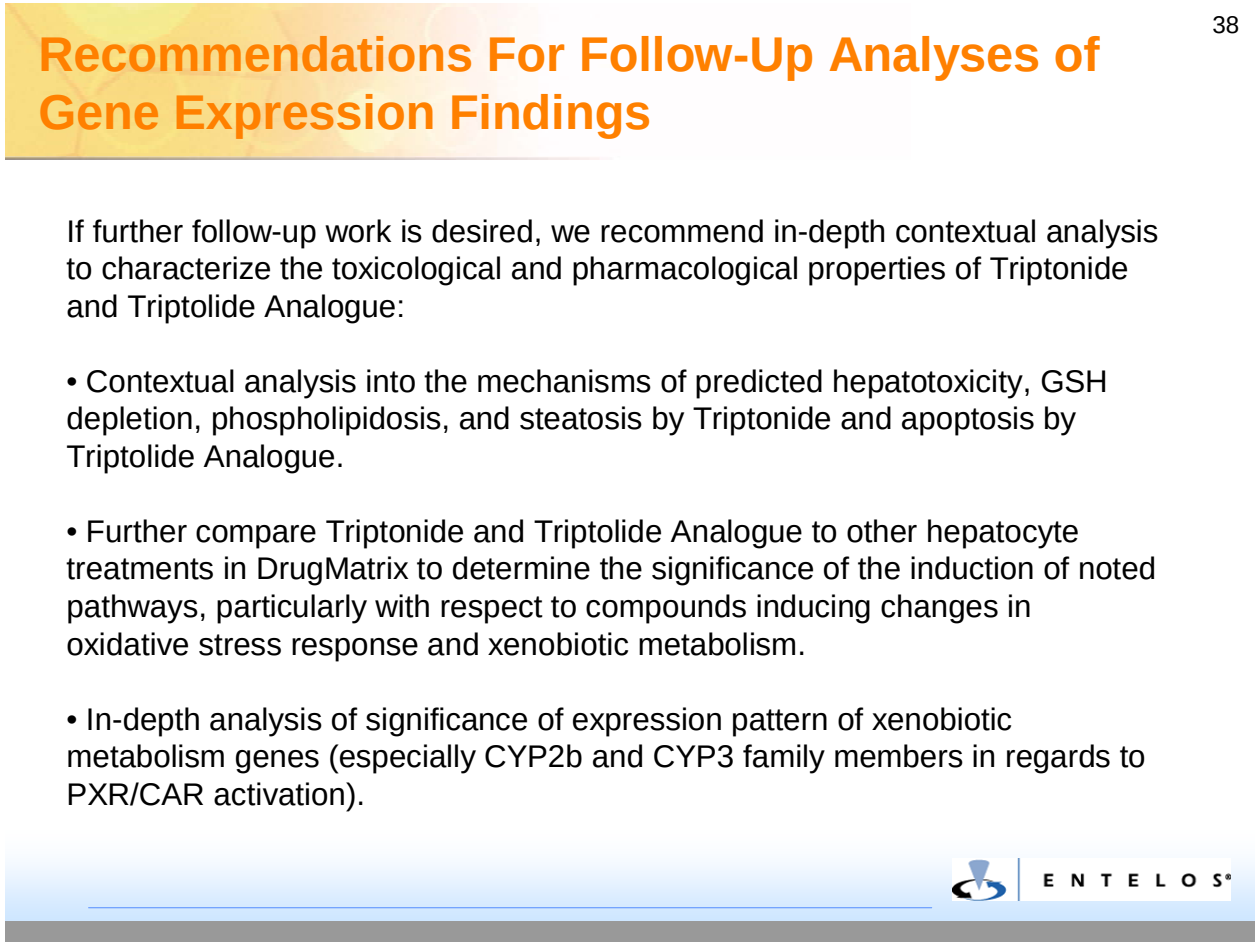

Slide 38: Recommendations for follow-up analyses of gene expression findings

Refer to the executive summary for discussions on recommendations.

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