



Prediction of non-genotoxic carcinogenesis in rats using changes in gene expression following acute dosing.

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Outline

- Background
 - Genotoxic versus non-genotoxic carcinogenicity testing
 - Possible alternative strategies for detection of non-genotoxic carcinogens
- Data
 - testing of gene signatures for detection of non-genotoxic carcinogens
- Conclusions

Carcinogenicity Testing

- Genotoxic Carcinogen Screening
 - Highly predictive short term *in vitro/in vivo* tests are available
 - Ames test
 - bone marrow/*in vitro* micronucleus
 - unscheduled DNA synthesis
 - transcriptional signatures
- Non-genotoxic Carcinogen Screening
 - Gold standard remains the 2-year rodent bioassay
 - time and cost intensive
 - positive results may require additional investigative studies to determine human risk.

2-Year Rodent Bioassay

- Alternative short-term tests
 - Transgenic mice e.g. Tg/AC, $p53^{+/-}$
 - Results in 6-months.
 - Approach could replace standard mouse bioassay but 2 year study in rats still required.
 - Further validation required.
 - Transcriptomics
 - Treat rodents with test article for short durations and determine gene expression changes in particular organ systems.
 - Use data to predict the outcome of the 2-year bioassay.
 - Would allow pro-active investigational studies to commence in parallel with bioassay.

Gene Expression Signatures for Non-Genotoxic Carcinogenesis

- Several published reports of gene expression changes that appear to be predictive of a positive outcome in the bioassay.
 - Nie *et al* (2006). *Mol. Carcinog.* (45), 914-933 (J&J)
 - Rat, Liver
 - RNA isolated 24h after a single dose of compound
 - Derived a 6-gene signature.
 - Fielden *et al* (2007). *Tox. Sci.* (99), 90-100 (Iconix)
 - Rat, Liver
 - RNA isolated after 3, 5 or 7 days of once daily dosing
 - Derived 37-gene signature
 - Subsequent work by Fielden and Gollub reduced this to 11 genes.
 - Ellinger-Ziegelbauer *et al* (2005). *Mutat. Res.* (575), 61-84 (Bayer)
 - Rat, Liver
 - RNA isolated after 1, 3, 7 and 14 days of once daily dosing
 - Identified pathways that were altered by NGTCs but did not establish a predictive panel of genes

Aims of Schering-Plough Study

- Independent confirmation using qPCR
 - All studies used microarray so important to confirm gene changes by qPCR
- No overlap in lists of genes from separate studies
 - how predictive and specific are these signatures when different experimental paradigms are used?
- Could we identify a novel panel of genes from the Ellinger-Ziegelbauer et al (2005) study that could be useful in detecting NGTCs?

Experimental Design

- Selected 2 non-carcinogens (NCs) and 3 non-genotoxic carcinogens (NGTCs).
- Treated rats for 1 or 5 days.
- Harvested liver and performed qPCR analysis.

Compound	Dose (mg/kg)	Classification	Treated Previously? (POS/NEG)	Reference
Fluoxetine	100	NC	[4] (NEG) [5]*	http://potency.berkeley.edu http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CCRIS
Ranitidine	1000	NC	[4] (NEG)	http://us.gsk.com/products/assets/us_zantac.pdf
Methapyrilene	100	NGTC	[4] (POS) [5]* [8] (NA)	http://potency.berkeley.edu http://ntp-server.niehs.nih.gov
Acetaminophen	950	NGTC	[5] (POS)	http://potency.berkeley.edu http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CCRIS
Phenobarbital	50	NGTC	[4] (POS) [5]*	http://potency.berkeley.edu http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CCRIS

Details of qPCR Analysis

- The following gene signatures were tested:
 - Nie et al., 2006, 6 gene signature.
 - Fielden, 2007, 11 gene signature.
- Additionally we analyzed changes in the expressions of genes identified as being responsive to NGTCs in Ellinger-Ziegelbauer et al., 2005.
 - Selected 10 genes.
 - induced by NGTCs and not genotoxic carcinogens.
 - induced >2-fold by at least $\frac{3}{4}$ NGTCs.
 - linked to cell division or carcinogenesis.

What is a “gene signature?”

- A gene activity pattern characteristic for a specific disease.
- Mathematically derived “weight” and “bias” terms add sensitivity and specificity.
- Example – J&J signature using 24h fluoxetine data.

Gene Symbol	Gene Acc	Weight (W)	Log(2)Ratio 2-??Ct	Impact (W x LR)
<i>Mat1a</i>	NM_012860	-0.02	0.49	-0.0093
<i>Mt1a</i>	NM_138826	-0.20	0.14	-0.0270
<i>NTF2</i>	BC061569	0.27	0.58	0.1550
<i>Pgrmc1</i>	NM_021766	0.20	0.68	0.1364
<i>Sel1h</i>	NM_177933	-0.92	0.38	-0.3520
<i>Udpgr2</i>	NM_173295	0.02	3.17	0.0490
Bias				0.0804
Scalar Product				-0.1284
Prediction:		if Scalar Product		
NGTC	> 0			
NC	≤ 0			

Results of J&J Gene Signature

Day 1

COMPOUND	GENE ANALYZED (FOLD CHANGE)						S
	Induced Genes			Repressed Genes			
	<i>Pgrmc1</i>	<i>Ntf2</i>	<i>Udpgr2</i>	<i>Sel1h</i>	<i>Mat1a</i>	<i>MT1a</i>	
Fluoxetine (NC)	1.63	1.48	8.96	1.30	1.42	1.08	-0.13
Ranitidine (NC)	1.04	1.22	1.57	-1.05	-1.27	1.06	-0.02
Methapyrilene (NGTC)	1.44	1.51	2.45	-1.39	1.14	-1.54	0.77
Acetaminophen (NGTC)	3.02	2.51	2.95	1.13	1.12	-1.11	0.51
Phenobarbital (NGTC)	2.06	1.71	16.83	1.04	-1.59	-1.63	0.56

Day 5

COMPOUND	GENE ANALYZED (FOLD CHANGE)						S
	Induced Genes			Repressed Genes			
	<i>Pgrmc1</i>	<i>Ntf2</i>	<i>Udpgr2</i>	<i>Sel1h</i>	<i>Mat1a</i>	<i>MT1a</i>	
Fluoxetine (NC)	1.09	1.02	7.71	1.18	1.48	1.35	-0.36
Ranitidine (NC)	1.24	1.22	1.18	1.20	1.43	1.42	-0.30
Methapyrilene (NGTC)	1.27	2.25	1.79	1.01	1.04	-3.04	0.67
Acetaminophen (NGTC)	3.15	1.92	3.35	1.23	1.42	1.90	0.10
Phenobarbital (NGTC)	2.18	1.75	15.71	1.33	-1.03	-1.04	0.09

100% sensitivity/specificity at 1 and 5 days
For NGTCs, day 1 data is more convincing

Results of Iconix Gene Signature

Day 1

COMPOUND	GENE ANALYZED (FOLD CHANGE)											S
	Induced Genes					Repressed Genes						
	<i>Cited4</i>	<i>Ica1</i>	<i>Litaf</i>	<i>Psmb9</i>	<i>AW143969</i>	<i>Usp2</i>	<i>Cdkn1a</i>	<i>AI232085</i>	<i>AW915076</i>	<i>AW533663</i>	<i>Pspla1</i>	
Fluoxetine (NC)	2.23	1.29	1.43	1.31	1.56	2.48	1.26	1.251	1.19	2.14	1.28	-1.54
Ranitidine (NC)	1.07	-1.13	-1.04	-1.08	1.12	1.40	-1.78	-1.122	-1.16	-1.10	1.07	-1.50
Methapyrilene (NGTC)	1.24	2.50	1.12	1.10	1.23	1.41	5.29	1.012	-1.47	-1.73	1.07	-0.21
Acetaminophen (NGTC)	-2.34	3.95	1.56	6.24	1.77	1.97	4.60	1.363	-1.34	-1.64	2.80	-1.07
Phenobarbital (NGTC)	-1.64	1.19	1.14	-1.00	1.35	1.28	-2.58	1.102	-1.03	-1.34	1.39	-1.66

Day 5

COMPOUND	GENE ANALYZED (FOLD CHANGE)											S
	Induced Genes					Repressed Genes						
	<i>Cited4</i>	<i>Ica1</i>	<i>Litaf</i>	<i>Psmb9</i>	<i>AW143969</i>	<i>Usp2</i>	<i>Cdkn1a</i>	<i>AI232085</i>	<i>AW915076</i>	<i>AW533663</i>	<i>Pspla1</i>	
Fluoxetine (NC)	3.54	1.42	1.16	-1.56	1.03	1.68	1.36	-1.079	-1.08	1.41	1.32	-1.11
Ranitidine (NC)	1.96	1.33	1.01	1.02	1.50	1.56	1.30	-6.724	1.19	1.44	-1.01	-0.48
Methapyrilene (NGTC)	2.59	8.68	1.89	3.00	2.39	-1.17	18.17	1.725	-1.48	-2.76	2.25	1.65
Acetaminophen (NGTC)	-1.30	3.70	1.27	2.30	1.37	1.70	1.28	1.165	-1.17	-1.60	-1.08	0.04
Phenobarbital (NGTC)	1.66	1.44	1.24	-1.15	1.20	1.25	1.27	1.223	-1.20	-1.65	-1.13	-0.36

Day 1: 2/2 NCs and 0/3 NGTCs predicted

Day 5: 2/2 NCs and 2/3 NGTCs predicted

Additional Genes Selected for Analysis

- 10 genes selected from Ellinger-Ziegelbauer study
 - induced by NGTCs and not genotoxic carcinogens.
 - induced >2-fold by at least ¾ NGTCs.
 - linked to cell division or carcinogenesis.

Gene Name	Symbol	Function of gene product
Apurinic/aprimidinic endonuclease 1	Apex1	Major AP endonuclease that functions in the repair of premutagenic DNA lesions
v-myc myelocytomatosis viral oncogene homolog (avian)	Myc	Nuclear phosphoprotein that functions as a transcription factor. Overexpression associated with numerous forms of cancer.
Histidine decarboxylase	Hdc	Synthesizes histamine from histidine. High levels have been found in tumor specimens.
Cell division cycle 2	Cdc2	Kinase involved in driving cells through their cycle
High mobility group box 2	Hmgb2	Causes DNA bends to facilitate transcription and may also be involved in DNA damage repair. May modulate p53 expression.
Minichromosome maintenance complex component 6	Mcm6	Key component of pre-replication fork complex. Central role in initiation of genome replication.
Cell division cycle 20	Cdc20	Kinase involved in driving cells through their cycle
Tubulin beta-5	Tubb5	Polymerizes to form microtubules.
Pituitary tumor transforming 1 (Securin)	Pttg1	Prevents separins promoting sister chromatid separation. APC substrate. Transforming activity <i>in vitro</i> and <i>in vivo</i> . Highly expressed in various tumors.
Stathmin 1/Oncoprotein 18	Stmn1	May promote microtubule depolymerization. May function as a relay protein for second messengers involved in cell growth and proliferation.

Expression Changes in Genes Linked to Carcinogenesis/Cell Proliferation.

Day 1											
COMPOUND	GENE ANALYZED (FOLD CHANGE)										
	<i>Apex1</i>	<i>Myc</i>	<i>Hdc</i>	<i>Cdc2</i>	<i>Hmgb2</i>	<i>Mcm6</i>	<i>Cdc20</i>	<i>Tubb5</i>	<i>Pttg1</i>	<i>Stmn1</i>	NGTCis
Fluoxetine (NC)	1.47	1.09	-1.01	1.03	1.31	1.18	1.59	1.71	1.35	1.37	1.55
Ranitidine (NC)	1.01	-1.42	-1.06	-1.13	-1.18	-1.18	-1.23	1.04	1.03	-1.02	1.28
Methapyrilene (NGTC)	2.25	3.04	2.23	2.04	1.17	1.39	1.28	1.70	1.43	1.27	1.95
Acetaminophen (NGTC)	2.92	5.60	2.27	4.78	2.00	13.12	2.36	5.98	1.38	2.36	5.65*
Phenobarbital (NGTC)	1.29	-1.22	1.42	4.56	1.22	1.63	1.38	1.58	4.69	2.27	2.35*

Day 5											
COMPOUND	GENE ANALYZED (FOLD CHANGE)										
	<i>Apex1</i>	<i>Myc</i>	<i>Hdc</i>	<i>Cdc2</i>	<i>Hmgb2</i>	<i>Mcm6</i>	<i>Cdc20</i>	<i>Tubb5</i>	<i>Pttg1</i>	<i>Stmn1</i>	NGTCis
Fluoxetine (NC)	1.27	1.66	-1.30	-6.00	-1.09	1.07	-1.68	-1.01	-7.07	-1.69	1.24
Ranitidine (NC)	-1.03	1.58	1.78	-1.01	1.16	1.17	-1.26	1.03	-2.62	1.13	1.43
Methapyrilene (NGTC)	3.52	15.55	7.62	5.28	2.45	3.94	2.00	3.70	2.38	4.73	5.72*
Acetaminophen (NGTC)	2.00	5.29	1.14	1.09	1.08	2.50	1.30	1.88	-3.60	-1.01	2.04*
Phenobarbital (NGTC)	1.35	1.79	3.36	1.37	1.12	1.29	1.23	1.16	-4.35	2.29	1.81

NGTCis – Average fold induction in gene expression

*significantly different from values obtained for NCs (p=0.05)

Summary/Conclusions

- Gene signatures demonstrated mixed results.
 - J&J signature most robust in our experiments
 - Iconix signature functioned best with 5 day samples.
- Gene expression changes are temporally regulated.
 - Changing dosing duration significantly alters gene expression patterns affected by certain compounds.
 - Predictive power of signatures likely reliant on specific dosing durations.
- Additional set of genes involved in carcinogenesis/cellular proliferation may have potential as biomarkers.
 - Significantly modulated by NGTCs on day 1 and 5.
 - Further work needed to determine sensitivity/specificity.

Future Work

- Are acute changes in gene expression truly predictive of cellular transformation?
 - Signatures require rigorous testing to fully define sensitivity and specificity
 - e.g. use of liver toxicants which are non-carcinogenic
 - Initiating events likely to be heterogeneous for different compounds so perhaps a larger gene set will be required.
 - Mechanistic understanding of changes in gene expression is required
 - will allow rationalization of role of specific genes in cell transformation
- Ultimately transcriptomics should be used as one component of a multifaceted weight of evidence approach for identification of non-genotoxic carcinogens.

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