

**Ingenuity Applications Spectrum**

Target Identification	Target Validation	Lead Identification	Lead Optimization	Pre-Clinical/Clinical	Post-Market
<ul style="list-style-type: none"> <li>Novel Targets</li> <li>Biological Pathways</li> <li>Protein Families</li> <li>Cellular Functions</li> <li>Disease States</li> </ul>	<ul style="list-style-type: none"> <li>Drugability</li> <li>Target-to-Disease Relationships</li> <li>Expression Localization</li> <li>Knock-Out Analysis</li> <li>De-orphanization</li> <li>Patents (prior art)</li> <li>Alternative Pathways</li> </ul>	<ul style="list-style-type: none"> <li>Target-to-Pathway-to-Known Drugs-to-Lead Candidate</li> <li>New Leads from Alternative Pathways</li> <li>Assay Development</li> </ul>	<ul style="list-style-type: none"> <li>Mechanism of Action Understanding</li> <li>Dose Response</li> <li>Predictive Toxicology</li> <li>Polymorphisms</li> </ul>	<ul style="list-style-type: none"> <li>Support IND/NDA</li> <li>ADME/Tox Profiling</li> <li>Disease Subtypes</li> <li>Patient Stratification</li> <li>Pharmacogenomics</li> </ul>	<ul style="list-style-type: none"> <li>Alternative Indications</li> <li>Personalized Medicine</li> </ul>

Mechanism of Action Understanding  
Dose Response  
Predictive Toxicology  
ADME/Tox Profiling

## Ingenuity Pathways Analysis Provides Safety Assessment of Lead Compounds and Insight Into Molecular Mechanisms of Hepatotoxicity

### Introduction

Many of the drug candidates that fail in clinical trials are withdrawn because of unforeseen toxic effects on human metabolism. The ability to understand potential toxicity associated with a lead compound, and confident elimination of such compounds early in the drug discovery and development process is one of the biggest challenges facing researchers today. Traditional toxicity endpoints may be insufficient for identification of latent toxicity and early elimination of toxic compounds. As a result, toxicology researchers have turned to high throughput “omics” technologies such as microarrays to identify gene expression profiles that accurately capture adverse and toxic effects of lead compounds. This toxicogenomics approach has the potential to identify surrogate biomarkers of toxicity, pathways perturbed by a compound, and pathways involved in a lead compound’s mechanism of action.

This field is making progress toward the eventual utilization of gene expression as an endpoint to define toxicity, as a tool for predictive risk assessment, and for elucidation of mechanisms of toxicity

(2, 3). However, bringing the promise of toxicogenomics to fruition is dependent on the ability to analyze large amounts of expression data in the context of regulatory networks, cellular pathways, and biological functions. Ingenuity Pathways Analysis allows scientists to concurrently analyze multiple datasets (genomic and proteomic datasets, dose response datasets, etc.) to identify the key functions and pathways that are activated or disrupted upon treatment with a compound. This web-delivered application makes use of the Ingenuity Pathways Knowledge Base, the world’s largest curated database consisting of millions of individually modeled relationships between proteins, genes, complexes, cells, tissues, drugs, and diseases.

### Case Study

Compound A-277249, a thienopyridine, is a compound being designed as a potential therapeutic for inflammatory disorders. It was expected to downregulate the expression of adhesion molecules by interfering with the NF-κB pathway<sup>1</sup>. Unfortunately, at high doses, a hepatotoxic effect was observed by classical means in early pre-clinical animal studies<sup>1</sup>.

Can toxicogenomic analysis of expression data at several dosage levels, coupled with biological function analysis, provide insights into how to proceed with this lead compound, and with this project? In particular, this case study will answer the following questions:

1. What was the observed effect of high doses of this lead compound on the target NF- $\kappa$ B pathway?
2. What does this gene expression profile reveal about unintended effects on off-target pathways? And what molecular mechanisms may be driving toxicity observed with histopathology?
3. Are there other clues to help a “go – no go” decision on this project?
4. Could biomarkers be developed from the data to assist in future studies?

## Methods

### *Gene expression profile background*

This analysis was performed using a gene expression dataset published previously by Waring et al<sup>1</sup>. This dataset was generated from rats treated for 3 days with two doses (10 or 100 mg/kg per day) of the thienopyridine compound A-277249. Following treatment, cRNA from liver tissue from the high and low dose treated rats was hybridized to the Affymetrix Rat Toxicology U34 Array. The microarray analysis and clustering algorithm used to identify genes whose expression changed significantly in response to drug treatment were performed using MAS software from Affymetrix and Gene-Maths™ from Applied Maths. An expression profile containing ~100 genes significantly changed in rats treated with the high dose of A-277249 vs. control was identified. These genes and their

expression values (fold change) served as input to Ingenuity Pathways Analysis.

### *Ingenuity Pathways Analysis*

**Generating Networks:** The 100 genes regulated by high doses of A-277249, their Affymetrix identifiers and corresponding expression values (fold change) were uploaded into Ingenuity Pathways Analysis. These genes were used as the starting point for generating biological networks. To build networks, Ingenuity Pathways Analysis queries the Ingenuity Pathways Knowledge Base for interactions between genes in the expression profile and all other genes/gene products stored in the Ingenuity Pathways Knowledge Base. The interactions utilized for network building are derived from the full text of peer-reviewed literature, and involve direct relationships that describe physical (binding) and functional (phosphorylation, transcription, proteolysis, etc.) interactions between proteins and genes. Network generation is optimized for inclusion of as many genes from the expression profile as possible, and aims for highly connected networks.

**Identifying significant Functions and Pathways:** The Global Functional Analysis feature identified the biological functions most significantly associated with the high-dose gene expression profile. Results are generated by querying the Ingenuity Pathways Knowledge Base for relationships that describe a gene’s role in cellular and organismal functions (e.g. apoptosis, angiogenesis, organ morphology, diseases, metabolism, etc.). The significance value associated with a function in Global Functional Analysis is expressed as a p-value, which is calculated using the right-tailed Fisher Exact Test. This is done by comparing the number of genes from the gene expression profile that participate in a given function, relative to

the total number of occurrences of those genes in all functional annotations stored in the Ingenuity Pathways Knowledge Base.

### Results

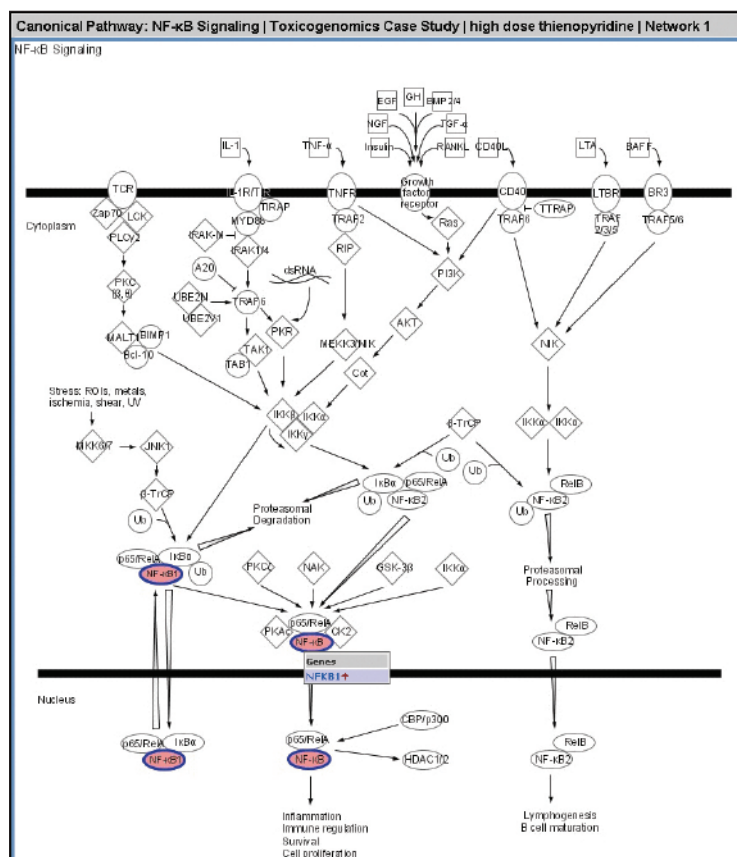
This compound was designed to down-regulate the expression of adhesion molecules by interfering with the NF-κB pathway, as a potential therapy for inflammatory disorders. So, what does this high-dose gene expression profile reveal about the observed effect on the NF-κB pathway?

This question can be quickly addressed using the Global Functions and Pathways feature in Ingenuity Pathway Analysis. This feature visualizes well-characterized signaling and metabolic pathways in the context of gene expression profiles. As seen in **Figure 1**, rather than the intended effect of disrupting the inflammation-related NF-κB pathway, it appears that a key component of this pathway, NF-κB1 is upregulated in response to high doses of the thienopyridine compound A-277249.

These results highlight additional evidence that this drug is not working as expected at high doses. The intended effect on the target pathway has not been achieved. So, what can we learn about unintended effects on off-target pathways and cellular functions? The Global Functions and Pathways feature in Ingenuity Pathways Analysis can identify the cellular functions associated with genes in the expression profile, and that are likely being targeted at high doses of A-277249.

As seen in **Figure 2**, some of the most significant cellular functions associated with the genes regulated at high doses include cell death and cell cycle regulation. Global Functional Analysis results further indicate that 19 genes significantly up or downregulated in response to high doses of A-277249 play a role in DNA replication, recombination and repair. These results provide evidence that high doses of A-277249 may be triggering DNA damage pathways, and quickly offer a possible mechanism of toxicity for this lead compound.

The potential disruption of these cellular functions can be further defined mechanistically by understanding how these 19 genes interact at the molecular level.



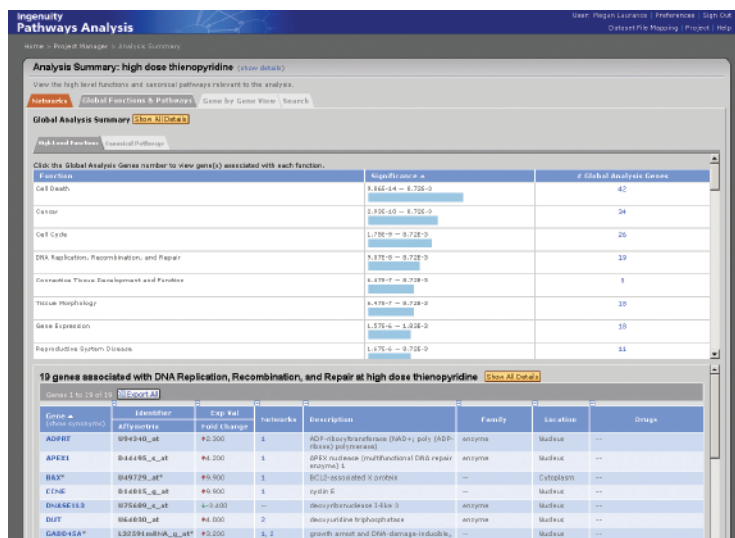
**Figure 1: Ingenuity Pathways Analysis graphical representation of NF-κB signaling in the context of the high-dose gene expression profile.** Genes upregulated by 100 mg/kg doses of A-277249 are shaded red, suggesting that rather than disruption of NF-κB signaling leading to repression of inflammatory pathways, high doses of this compound appear to upregulate NF-κB1.

The Global Functional Analysis feature (See **Figure 2**) provides direct links to computationally generated networks, as well as the ability to merge networks related by the function DNA damage and repair. Ingenuity Pathways Analysis generates networks by querying the Ingenuity Pathways Knowledge Base for direct relationships that describe how these genes bind to and regulate each other's activity. All relationships used to build networks are supported by experimental evidence captured from the full text of peer-reviewed literature.

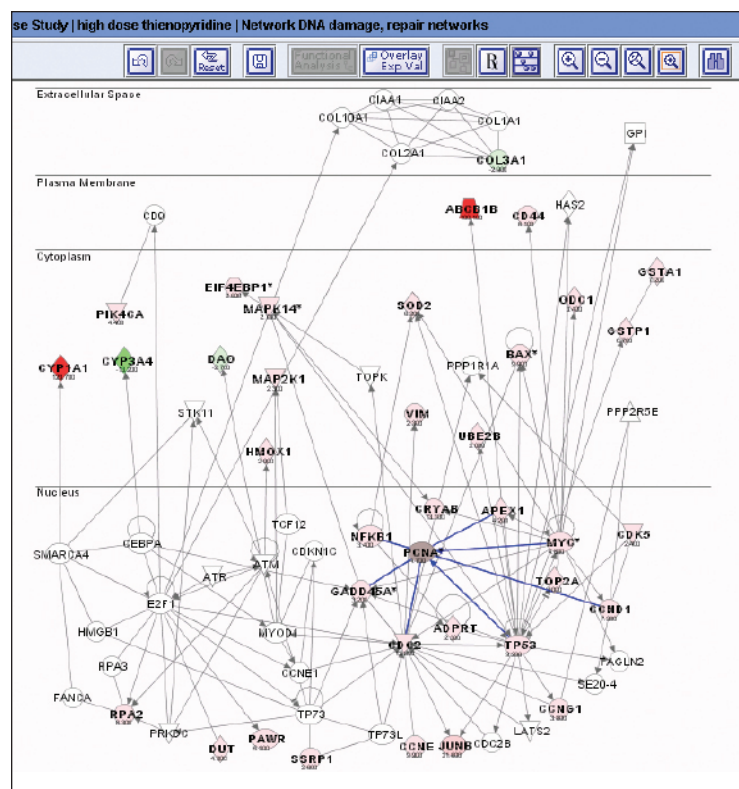
**Figure 3** represents a molecular network merged from two functionally related networks. This network focuses on the genes involved in DNA damage and repair, as highlighted in **Figure 2** below. One of the key regulators of this network, PCNA, is upregulated in response to high doses of A-277249. Interestingly, several of PCNA's direct neighbors were also upregulated in response to high doses, suggesting that this cluster of genes and their interactions may reflect a key trigger or activation of DNA damage pathways.

The network in **Figure 3** elucidates a potential molecular mechanism that may be the driving force behind the hepatotoxicity observed with more traditional toxicity methods such as histopathology. Does the gene expression pattern from lower doses of A-277249 suggest that this network of DNA damage and repair genes was already being perturbed? Was there any indication at low doses we were headed in this direction? Can we identify a gene expression signature that precedes the toxic outcome measured with histopathology? And would the genes in that signature make good markers of toxicity?

The Overlay Ranks feature in Ingenuity Pathways Analysis enables a quick assessment as to whether the gene expression levels of any of the genes involved in this DNA damage and repair network were also affected by low doses (10 mg/kg) of this compound. Indeed, as seen in **Figure 4**, both PCNA and APEX1 were mildly upregulated by low doses of A-277249.



**Figure 2: Table of High Level Functions most significantly associated with the high-dose gene expression profile.** "DNA Replication, Recombination and Repair" has a significance of 1.53E-7. Significance is expressed as the negative exponent of the p-value calculated for each function. A significance of < .05 indicates that the associations between the genes from the gene expression profile and the function did not occur by random chance alone. The 19 genes in this gene expression profile associated with DNA Replication, Recombination, Repair are also displayed.

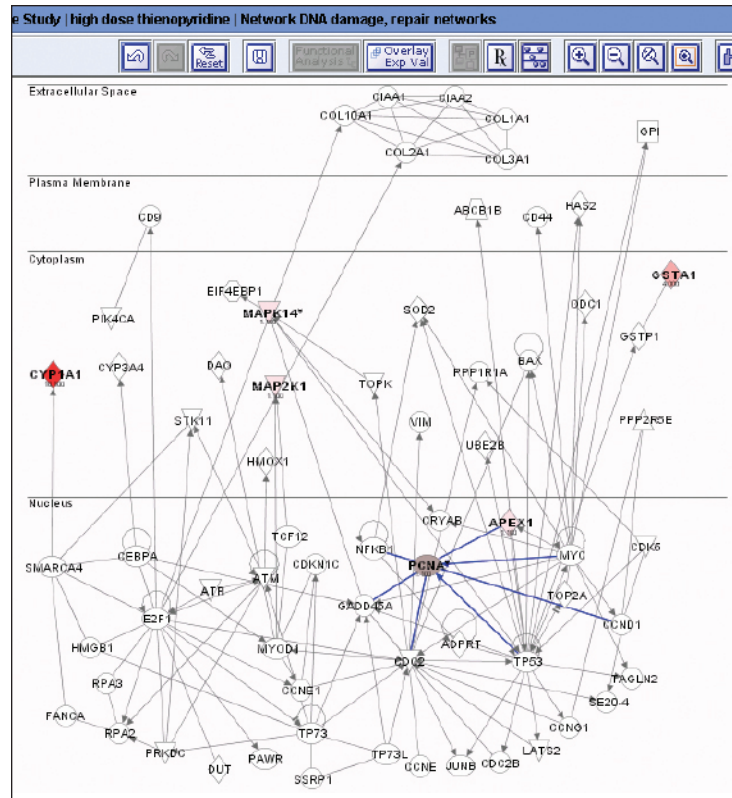


**Figure 3: Genes upregulated in response to high doses of A-277249 are shaded red. Downregulated genes are shaded green. Expression values (fold change) are displayed below each gene name. Genes with a white background were not part of the high-dose gene expression profile, but were integrated into the computationally generated networks based on evidence stored in the Ingenuity Pathways Knowledge Base indicating a strong biological relevance to that network. Direct relationships between PCNA and other DNA damage and repair genes, namely Cyclin D1 (CCND1), CDC2, GADD45A and APEX1, are highlighted in blue.**

Ingenuity Pathways Analysis results identified a molecular network of genes regulated by high doses of A-277249, that play a role in DNA damage and repair and that may be disrupted even at low doses of this compound.

Correlating gene expression profiles with a potential molecular mechanism of toxicity, as shown in **Figures 3** and **4**, helps identify biomarkers that can be used to monitor drug safety and establish early attrition of undesirable lead

compounds. Would PCNA and APEX1 make good candidates as biomarkers of toxicity? Does it make sense that APEX1 would be upregulated in response to treatment with a potential hepatotoxin? In what other cellular systems does APEX1 play a role? What else modifies the expression of APEX1? Answers to these questions will help qualify APEX1 and other genes in the network as to their utility as biomarkers. These questions are quickly addressed using the Ingenuity Pathways Analysis feature Node View.

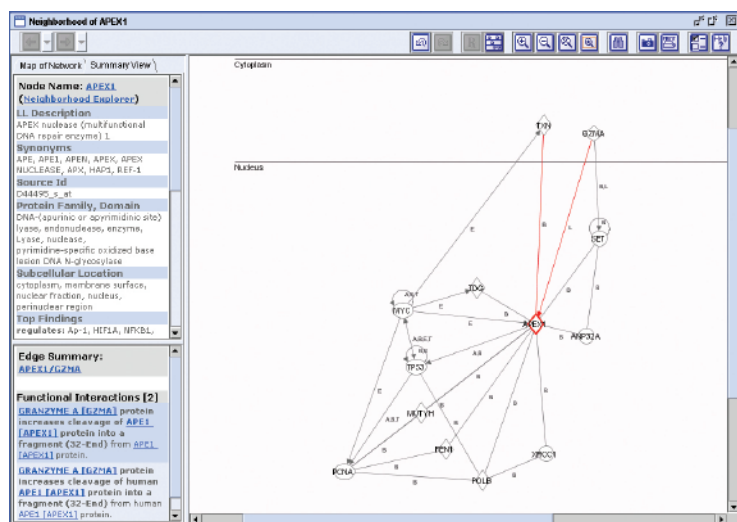


**Figure 4: Network of DNA damage and repair genes in the context of gene expression changes induced by low doses of A-277249. Upregulated genes are shaded red.**

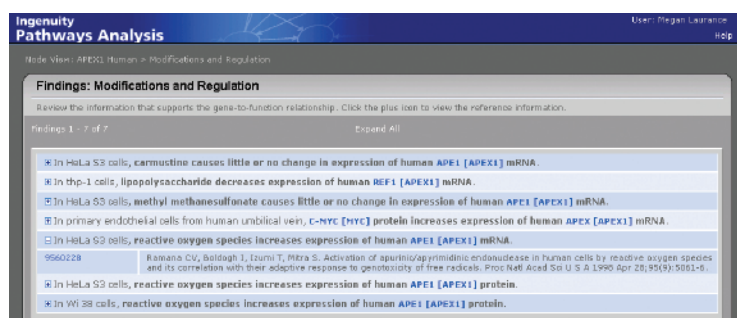
The screenshot shows the 'Node View: APEX1' in the Ingenuity Pathways Analysis software. The interface includes a search bar, a user profile, and a help button. The main content area displays the following information for APEX1:

- LL Description:** APEX nuclease (multifunctional DNA repair enzyme) 1
- Synonyms:** APE, APE1, APEN, APEX, APEX NUCLEASE, APX, HAP1, REF-1
- Source ID:** D44495\_u\_at
- Protein Family, Domains:** DNA (apurinic or apyrimidic site) lyase, endonuclease, enzyme, Lyase, nuclease, pyrimidine-specific oxidized base lesion DNA endonuclease
- Subcellular Location:** cytoplasm, membrane surface, nuclear fraction, nucleus, perinuclear region
- Top findings from Ingenuity Knowledge Base (show all 215 categorized literature findings):**
  - regulates:** Ap-1, HIF1A, NFKB1, SMUG1, TDG, TP53
  - regulated by:** Gk2, GHI, GRX2, GZMA, HMGDB2, MYC, TP53
  - binds:** ANP32A, CPAS1, FEN1, Hsp70, PRUTH, PCNA, POLD, Rps, SET, TDG, TP53, TRN, XRCC1
  - role in cell:** --
  - disease:** --
- Descriptions from External Databases:**
  - LocusLink Summary:** Apurinic/apyrimidinic (AP) sites occur frequently in DNA molecules by spontaneous hydrolysis, by DNA damaging agents or by DNA glycosylases that remove specific abnormal bases. AP sites are pre-mutagenic lesions that can prevent normal DNA replication so the cell contains systems to identify and repair such sites. Class II AP endonucleases cleave the phosphodiester backbone 5' to the AP site. This gene encodes the major AP endonuclease in human cells. Splice variants have been found for this gene; all encode the same protein.

**Figure 5: Ingenuity Pathways Analysis Node View for the gene APEX1 highlights APEX1's role in DNA repair in response to damaging agents.**



**Figure 6: Neighborhood Explorer for APEX1 highlights role for human APEX1 (APE1) in immune system cell death through its relationship with Granzyme A (GZMA).**



**Figure 7: Modifications and Regulation section of the human APEX1 Node View indicates that its expression is regulated by reactive oxygen species.**

As shown in **Figures 5 - 7**, each Node View in Ingenuity Pathways Analysis contains comprehensive information on a gene's function, how that gene is regulated, its direct neighbors, synonyms, protein family membership, as well as a link to all results from the peer-reviewed literature stored in the Ingenuity Pathways Knowledge Base that involve that gene. Results specific to the human, mouse or rat ortholog of each gene are available under the appropriately labeled tab.

### Conclusions

Ingenuity Pathways Analysis of gene expression profiles from rats treated with A-277249 indicates that this compound is not working as expected. The intended therapeutic effect of this lead compound (downregulation of key components of the NF-κB signaling pathway to disrupt inflammatory processes) was not observed at high doses.

Rather, it is likely that a toxic effect was brought on by activation of DNA damage and repair programs. Ingenuity Pathways Analysis of the gene expression profile from rats treated with different doses of the thienopyridine compound A-277249 indicate that several genes upregulated in high dose treated rats (PCNA, CCND1, CDC2, GADD45A, APEX1) are key regulators of DNA damage and repair programs. APEX1 and PCNA were also mildly upregulated at lower doses, suggesting that upregulation of this pair of genes and their neighbors might make a good marker of potential toxicity.

Using Node View to further qualify these markers as to their expression and localization patterns, modifications and regulation may result in useful efficacy and toxicity biomarkers for this series of compounds.

□ 1: Proc Natl Acad Sci U S A. 1998 Apr 28;95(9):5061-6. Related Articles, Links

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**Activation of apurinic/apyrimidinic endonuclease in human cells by reactive oxygen species and its correlation with their adaptive response to genotoxicity of free radicals.**

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Apurinic/apyrimidinic (AP) endonuclease (APE, EC 4.2.99.18) plays a central role in repair of DNA damage due to reactive oxygen species (ROS) because its DNA 3'-phosphoesterase activity removes 3' blocking groups in DNA that are generated by DNA glycosylate/AP-lyases during removal of oxidized bases and by direct ROS reaction with DNA. The major human APE (APE-1) gene is activated selectively by sublethal levels of a variety of ROS and ROS generators, including ionizing radiation, but not by other genotoxins-e.g., UV light and alkylating agents. Increased expression of APE mRNA and protein was observed both in the HeLa S3 honor line and in WI 38 primary fibroblasts, and it was accompanied by translocation of the endonuclease to the nucleus. ROS-treated cells showed a significant increase in resistance to the cytotoxicity of such ROS generators as H<sub>2</sub>O<sub>2</sub> and bleomycin, but not to UV light. This 'adaptive response' appears to result from enhanced repair of cytotoxic DNA lesions due to an increased activity of APE-1, which may be limiting in the base excision repair process for ROS-induced toxic lesions.

PMID: 9560228 [PubMed - indexed for MEDLINE]

**Figure 8: Supporting evidence for the results displayed in Figure 7.** All findings in Ingenuity Pathways Analysis are directly linked to the peer-reviewed article that was the original source of the finding.

All of the analyses of pre-clinical A-277249 data suggest a strong “no go” vote for this project. However, it is important to note that Ingenuity Pathways Analysis provided an integrated approach to analyzing the signaling pathways, cellular functions, and molecular interaction networks implicated by high dose treatment with this compound.

Further, these results provided a quick understanding of the impact (if any) on the intended target pathway and the likelihood of interactions with unintended target pathways. Ingenuity Pathways Analysis identified potential markers of hepatotoxicity, identified off-target effects of this compound, and provided evidence that suggests abandoning this lead compound.

For more information on Ingenuity Pathways Analysis or other Ingenuity products, go to [www.ingenuity.com](http://www.ingenuity.com). Or, sign up for a fully functional free trial at [www.ingenuity.com/trial](http://www.ingenuity.com/trial).

## References

1. Waring JF, Gum R, Morfitt D, Jolly RA, Ciurlionis R, Heindel M, Gallenberg L, Buratto B, Ulrich RG. *Identifying toxic mechanisms using DNA microarrays: evidence that an experimental inhibitor of cell adhesion molecule expression signals through the aryl hydrocarbon nuclear receptor*. Toxicology. 2002 Dec 27;181-182:537-50
2. Kier LD, Neft R, Tang L, Suizu R, Cook T, Onsurez K, Tiegler K, Sakai Y, Ortiz M, Nolan T, Sankar U, Li AP. *Applications of microarrays with toxicologically relevant genes (tox genes) for the evaluation of chemical toxicants in Sprague Dawley rats in vivo and human hepatocytes in vitro*. Mutat Res. 2004 May 18;549(1-2):101-13.
3. Hamadeh HK, Bushel PR, Jayadev S, Martin K, DiSorbo O, Sieber S, Bennett L, Tennant R, Stoll R, Barrett JC, Blanchard K, Paules RS, Afshari CA. *Gene expression analysis reveals chemical-specific profiles*. Toxicol Sci. 2002 Jun;67(2):219-31.

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