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US Environmental Protection Agency
Research Triangle Park, NC

Poor water sanitation and a lack of safe drinking water take a greater human toll than war, terrorism and weapons of mass destruction combined…… *The Lancet*

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The Development of a Normal Human Colon Cell Culture to Assess Toxicities of Drinking Water Disinfection Byproducts and Mixtures

Anthony DeAngelo

US EPA Office of Research and Development
National Health and Environmental Effects Research Laboratory
Environmental Carcinogenesis Division
Research Triangle Park, NC 27711

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NCM460 Cell Cultures

normal human colon cells
Grow as a mixed monolayer/suspension culture

Multi-lineage capability for in vitro differentiation: stem cell component In the population

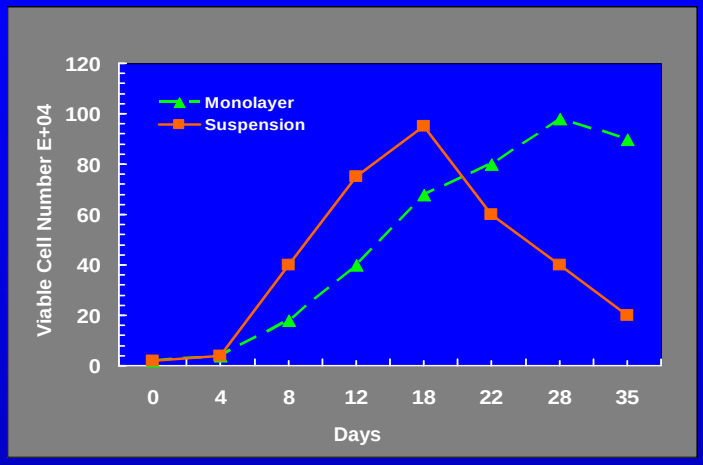
Cells in suspension are in various stages of differentiation, enter apoptosis and die

>90% + for epithelial cell and intestinal epithelial markers

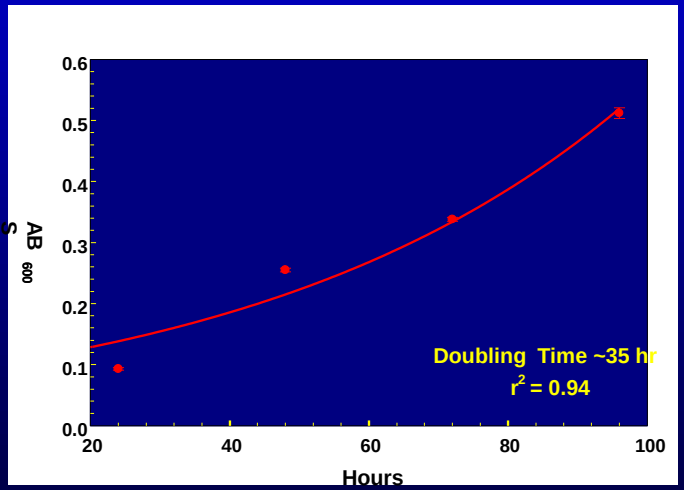
Cells unable to grow in soft agar ****
Nontumorigenic in nude mice

****Recent reports and studies from our laboratory indicate that NCM460 (Stem Cell Component) cells can grow in soft agar….a problem we had to solve.

Growth of NCM460 Cells



Moyer, MP et al., In Vitro Cell Biol.,---Animal, 32:315-317, 1998



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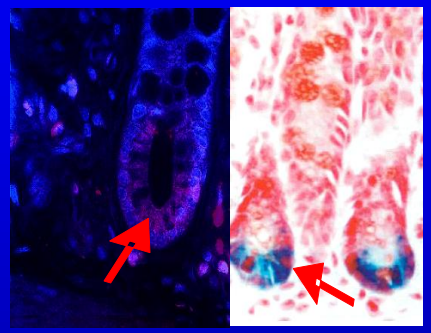
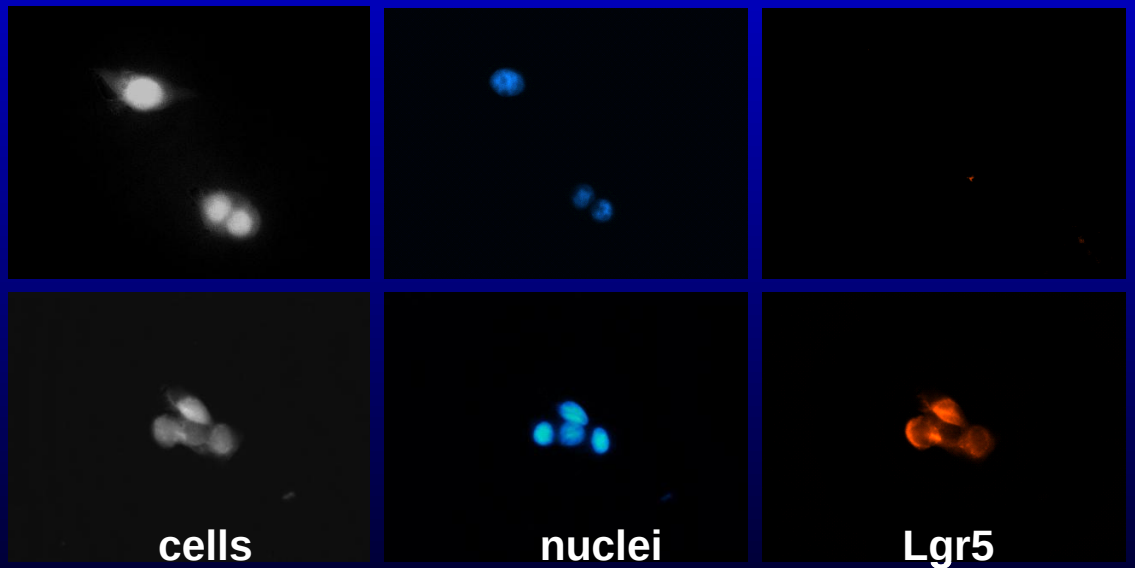
Lgr5 Colon Stem Cell Marker

Rat Colon



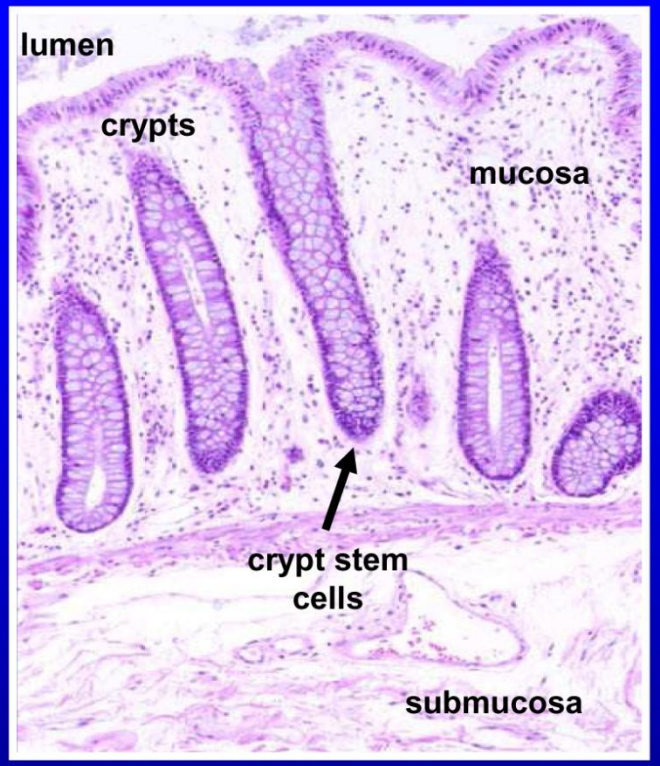
Mouse
Small Intestine
Barker, N (2007) *Nature, 449*:1003-1008



Lgr5 -
NCM460 Cells

Lgr5 +
NCM460 Stem Cells

Human Colon

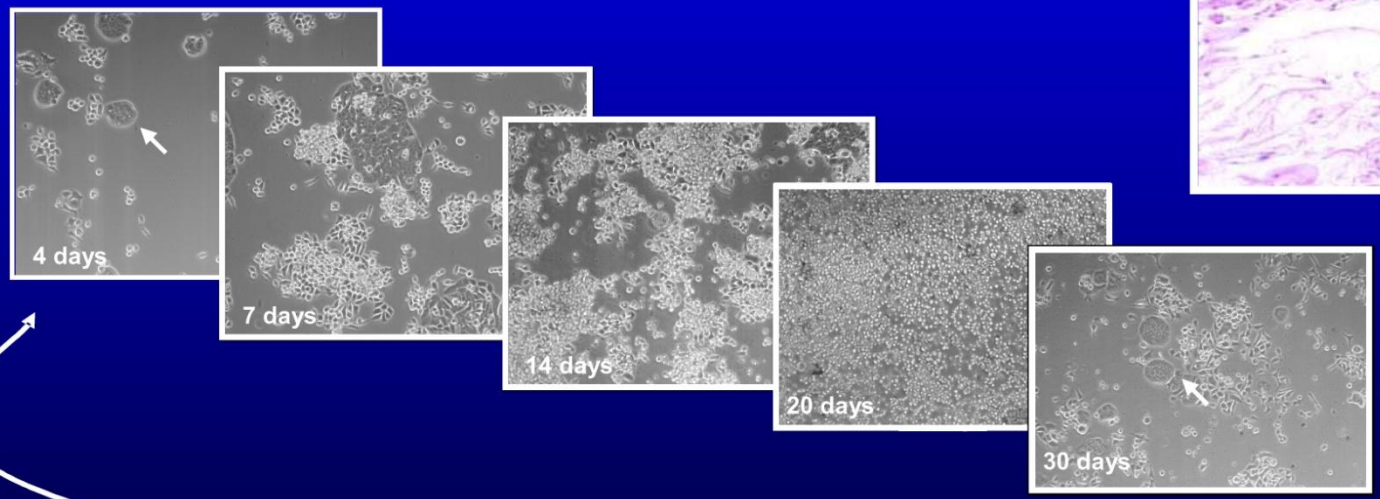


NCM460 – Normal Human Colon Cell Cultures

Vs

Human Colon Crypts Cells

NCM460 Cells



The Distribution and Concentration of P450 Enzymes Can Differ Among Tissues

McKinnons, RA, Burgess, WM, Gonzalez, FJ, and McManus, ME (1993). Metabolic differences in colon mucosal cells. *Mutation Research*, 290:23.

Bergheim, I, Bode, C, and Parlesak, A (2005). Distribution of cytochrome P450 2C, 2E1, 3A4, and 3A5 in human colon mucosa. *BMC Clinical Pharmacology*, 5:4.

Martignoni, M, Groothuis,G, and de Kanter, R (2006). Comparison of mouse and rat cytochrome P450-mediated metabolism in liver and intestine. *Drug Metabolism and Distribution*. 34:10471054

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Ratio of GSTT -1 and *p* - Nitrophenol Activities in Normal Human Colon Mucosal Cells and Liver and Colon *In Situ*

GSST-1 Activates Trihalomethanes

TISSUES/CELLS	(Cytosol GST) GST-ENPP conjugation activity (nmol min-1/mg-1 cytosol protein)	Microsomes (*p*NP) Ratio *p*-nitrophenol hydrolase activity (nmol min-1/mg-1 microsome protein)	GST/*p*NP
NCM460	21.4 ± 4.8[a] 31.2 ± 8.1		
NCM460	43.3[b] 41.4	0.04 0.17	1082 242 662
Liver*	33.6 ± 5.0 a	0.93 ± 0.02	80
Large Intestine*	21.01 ± 6.6	0.06 + 0.02	778

* Ross, MK and Pegram , RA (2003). *Chem. Res. Toxicol*. 16, 216-226.

a. Mean ± SD

b. 3-4 replicates/flask

McKay JA, Murray JI, Weaver RJ, Ewin SW, Melvin WT, Burke MD (1997). Xenobiotic metabolizing enzyme expression in colonic neoplasia *GUT* 34:1234-1239.

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NCM460 – Normal Human Colon Cell Cultures Cytotoxicity Studies

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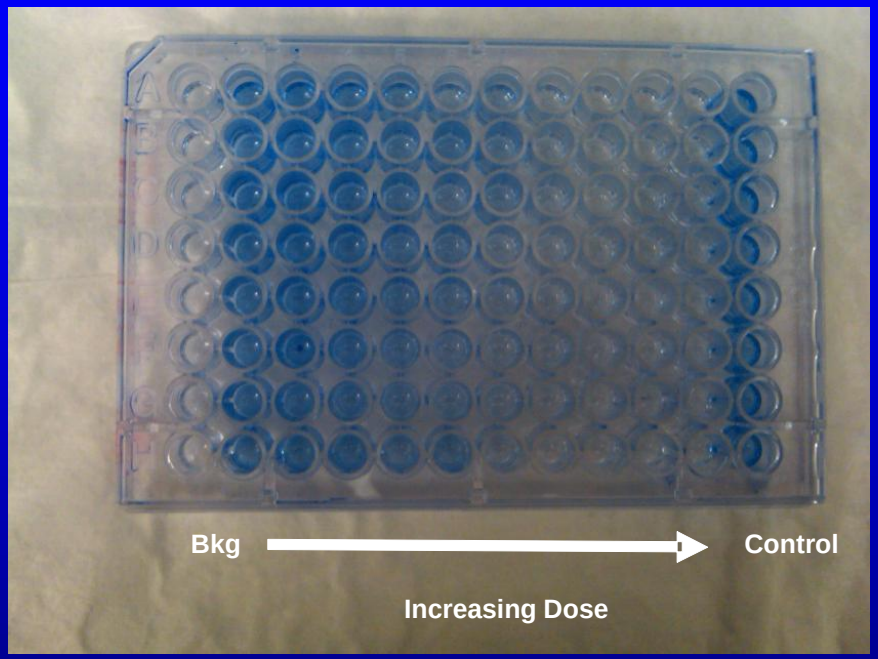
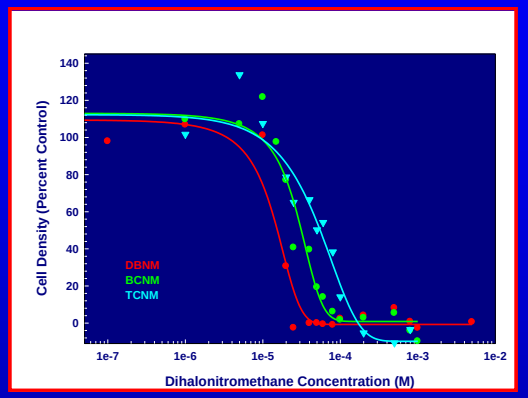
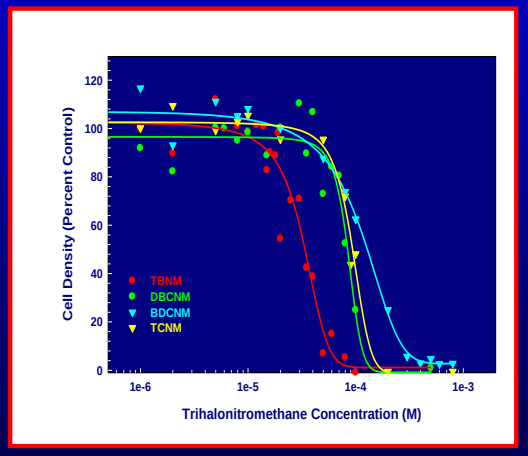
Inhibition of NCM460 Cell Growth Assay







Haloacetamides	12
Haloacetonitriles	6
Halonitromethanes	9
Haloacetic Acids	12
Trihalomethanes	4
N-Nitrosamines	5
Misc.	5

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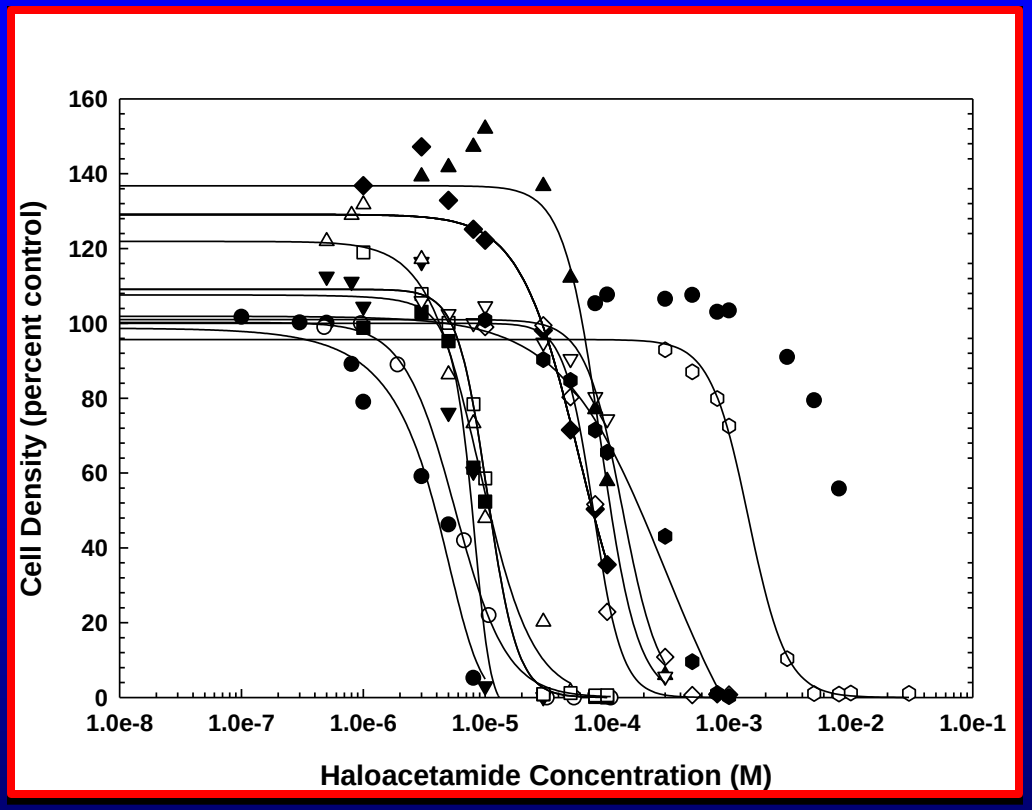

	IC50	IC50 Range	r2
CIAM	3.91E-06	3.00-4.86E-06	0.973
DIAM	5.70E-06	5.26-6.13E-06	0.999
BDCA	7.79E-06	6.13-9.26E-06	0.959
BAM	9.85E-06	9.17E-06-1.08E-05	0.996
BIAM	1.08E-05	8.20E-06-1.72E-05	0.951
IAM	1.09E-05	9.86E-06-1.36E-05	0.992
DBAM	7.64E-05	5.40E-05-1.28E-04	0.901
TBAM	3.27E-05	2.58-4.35E-05	0.961
BCAM	1.07E-04	8.42E-05-1.76E-04	0.913
DBCAM	1.34E-04	1.18-1.63E-04	0.981
TCAM	1.40E-04	1.28-1.56E-04	0.997
CAM	1.88E-04	1.44-2.48E-04	0.988

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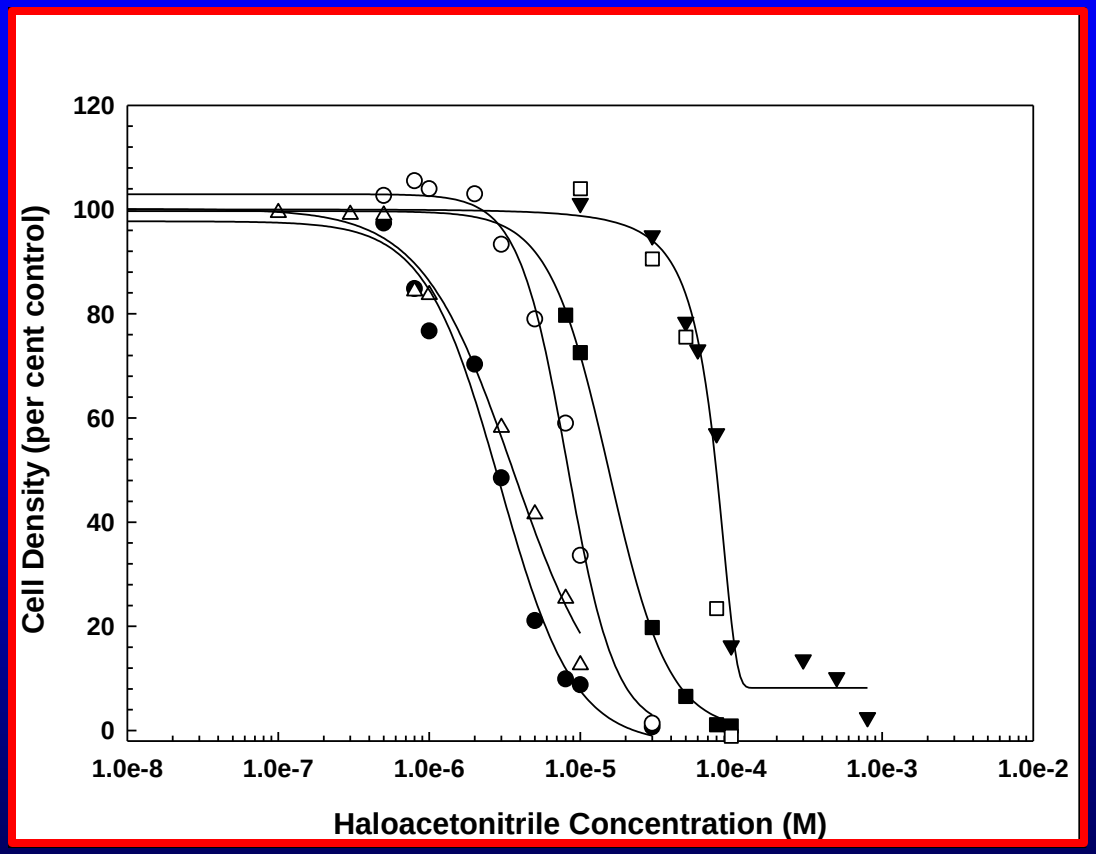

	IC50	IC50 Range	r2
IAN	2.78E-06	2.30-3.31E-06	0.988
DBAN	3.60E-06	3.06-4.21E-06	0.989
BAN	8.35E-06	7.71-9.10E-06	0.992
CAN	8.00E-05	7.35-8.66E-05	0.987
DCAN	1.54E-05	1.48-1.65E-05	1.000
TCAN	6.18E-05	5.31-7.03E-05	0.988

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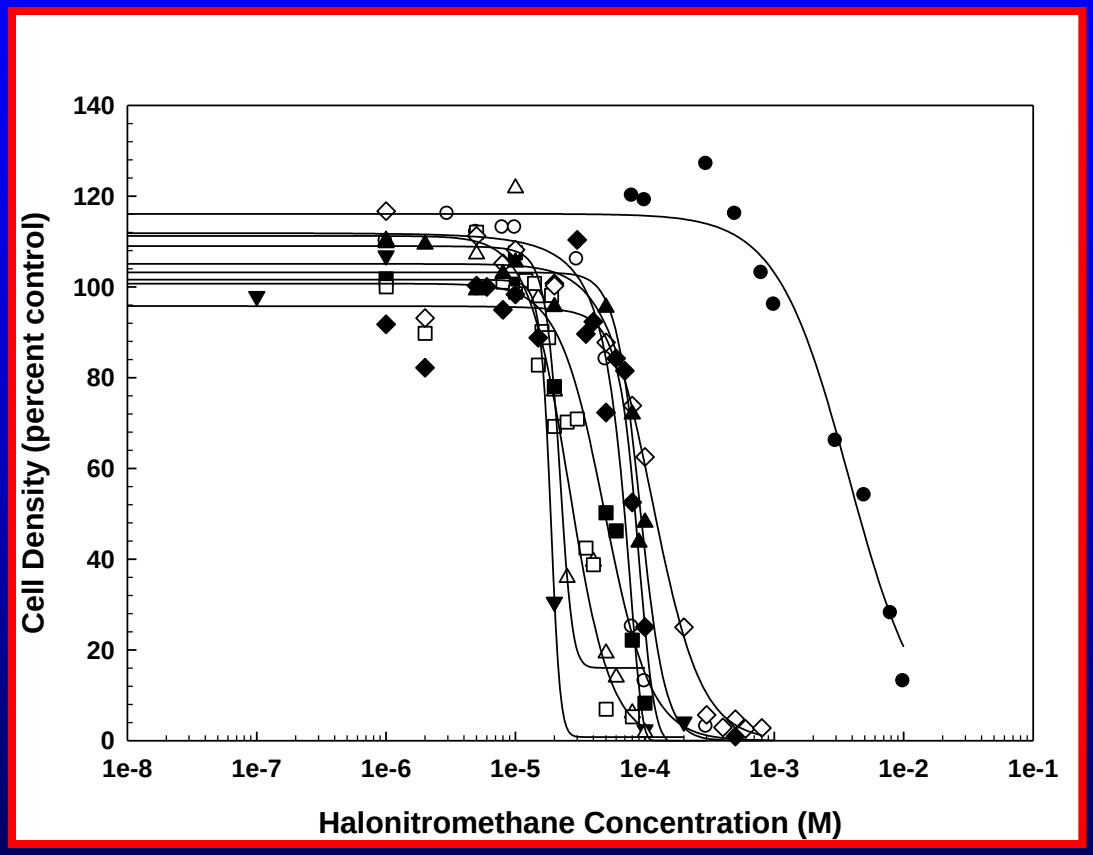

	IC50	IC50 Range	r2
DBNM	1.86E-05	1.36-1.97E-05	0.997
BCNM	2.81E-05	2.28-4.83E-05	0.946
TBNM	3.38E-05	2.99-3.87E-05	0.936
DCNM	4.93E-05	3.52-5.97E-05	0.969
BNM	6.98E-05	6.31-7.57E-05	0.984
DBCM	8.33E-05	7.80-9.26E-05	0.948
TCNM	9.29E-05	8.75-9.98E-05	0.989
BDCNM	1.20E-04	1.05-1.37E-04	0.987
CNM	4.51E-03	3.31-5.96E-03	0.956

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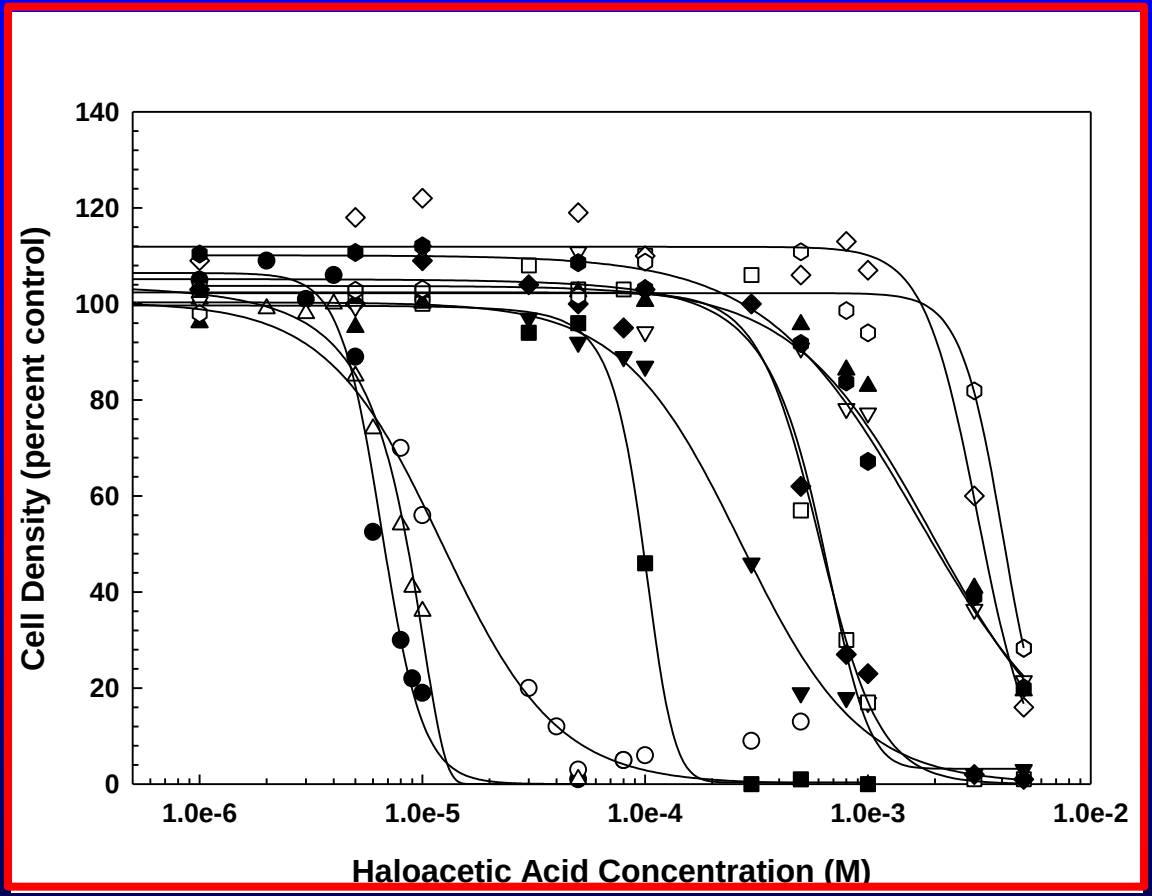

	IC50	IC50 Range	r2
IAA	6.73E-06	7.21-7.28E-06	0.980
DIAA	8.54E-06	8.17-9.04E-06	0.987
BAA	1.24E-05	9.79E-06-1.53E-05	0.973
BIAA	9.62E-05	9.09e-05-1.03e-04	0.998
CAA	2.65E-04	2.29-3.14E-04	0.992
BCAA	6.10E-04	5.55-6.68E-04	0.990
DBAA	6.27E-04	5.42-7.23E-04	0.979
BDCAA	1.98E-03	1.71-2.31E-03	0.991
DBCAA	2.07E-03	1.63-2.64E-03	0.973
TBAA	2.57E-03	2.26-2.94E-03	0.991
DCAA	3.27E-03	2.74-3.83E-03	0.962
TCAA	4.06E-03	3.61-4.58E-03	0.959

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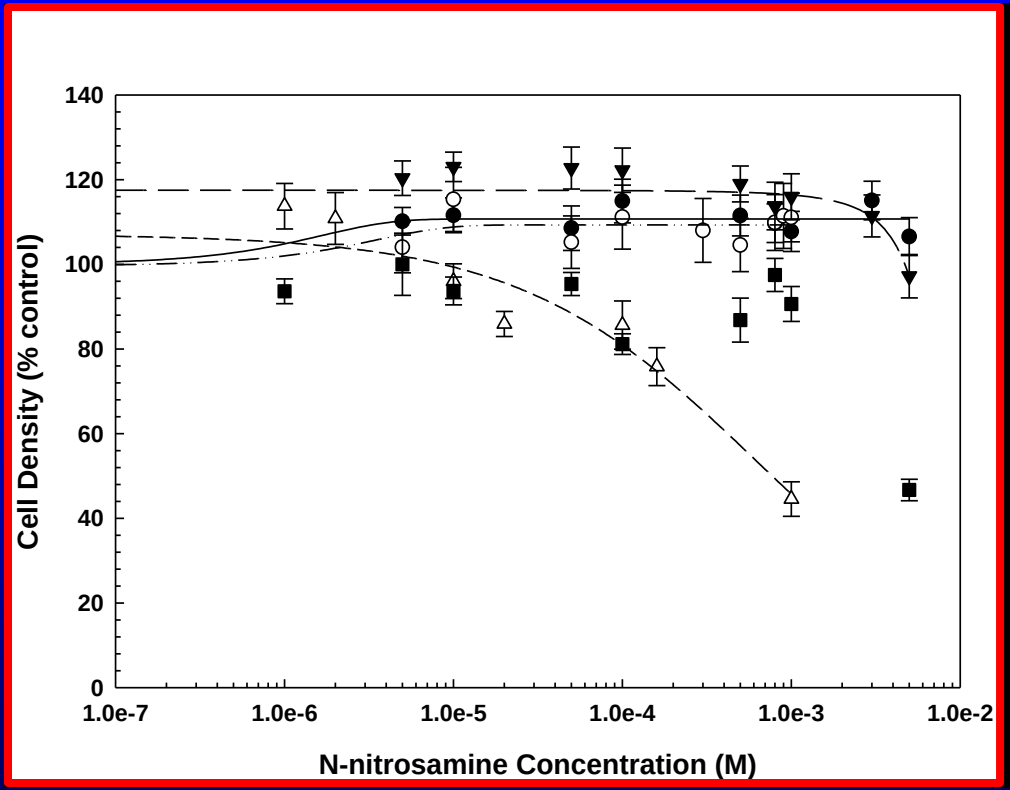

N-nitrosodimethylamine	r2=0.59
N-nitrosopyrrolidine	r2=0.45
N-nitrosodiethylamine	r2=0.47
N-nitrosodiphenylamine	r2=0.91; IC50=7.88E-04 M
N-nitrosodipropylamine	r2=0.87; IC50=4.76E-03M

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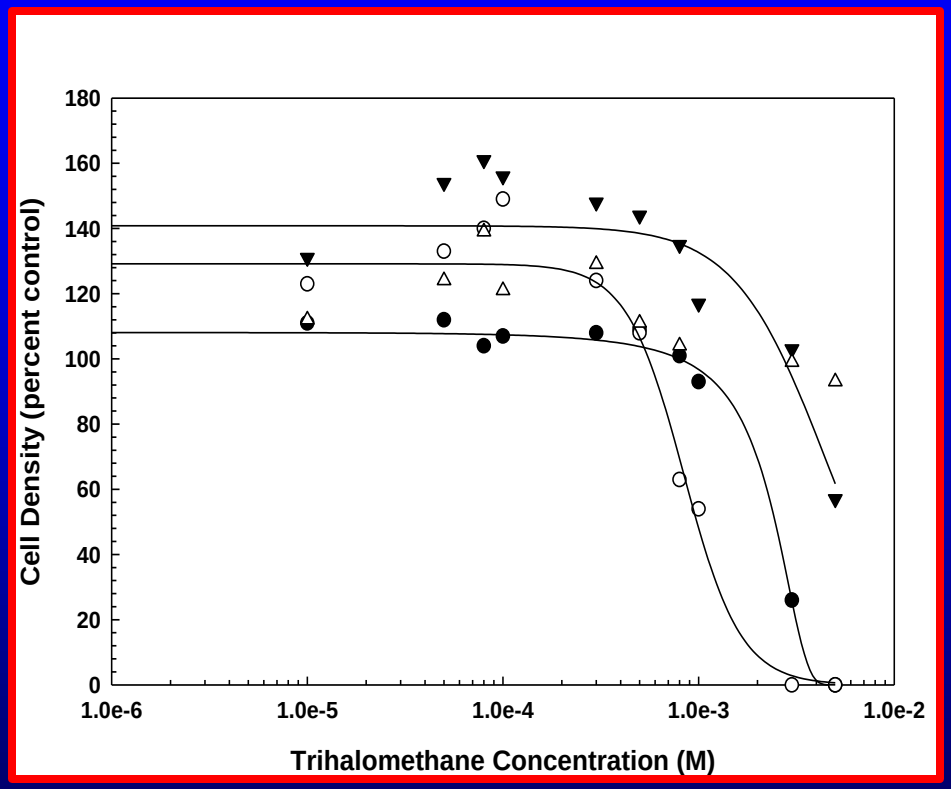
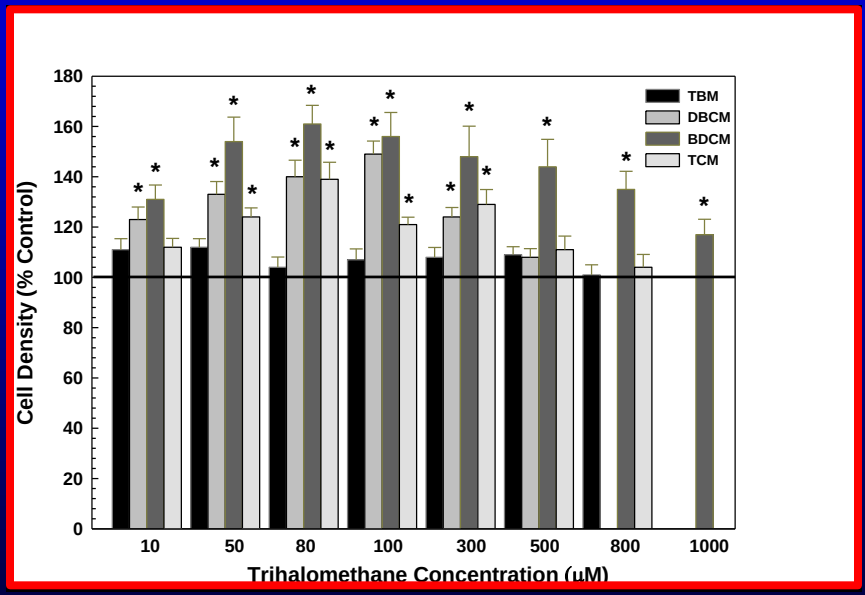


	IC50	IC50 Range	r2
DBCM	9.82E-04	7.87E-04-1.45E-03	0.947
TBM	2.70E-03	2.16-2.69E-03	0.990
BDCM	ND	ND	0.693
TCM	ND	ND	0.416



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Comparative Cytotoxicity between NCM460 Colon Cells and Chinese Hamster Ovary Cells

	Pearson Coefficient	r2
Total DBPs	0.58	P<0.05
Haloacetamides	0.90	p<0.05
Haloacetonitriles	0.76	p>0.05
Halonitromethanes	0.57	p>0.05
Haloacetic Acids	0.56	p>0.05

M. Plewa

Comparative Cytotoxicity between NCM460 Colon Cells and Mouse Neurotube Dysmorphogenesis

	Pearson Coefficient	r2
Haloacetic Acids	0.70	p<0.05

S. Hunter

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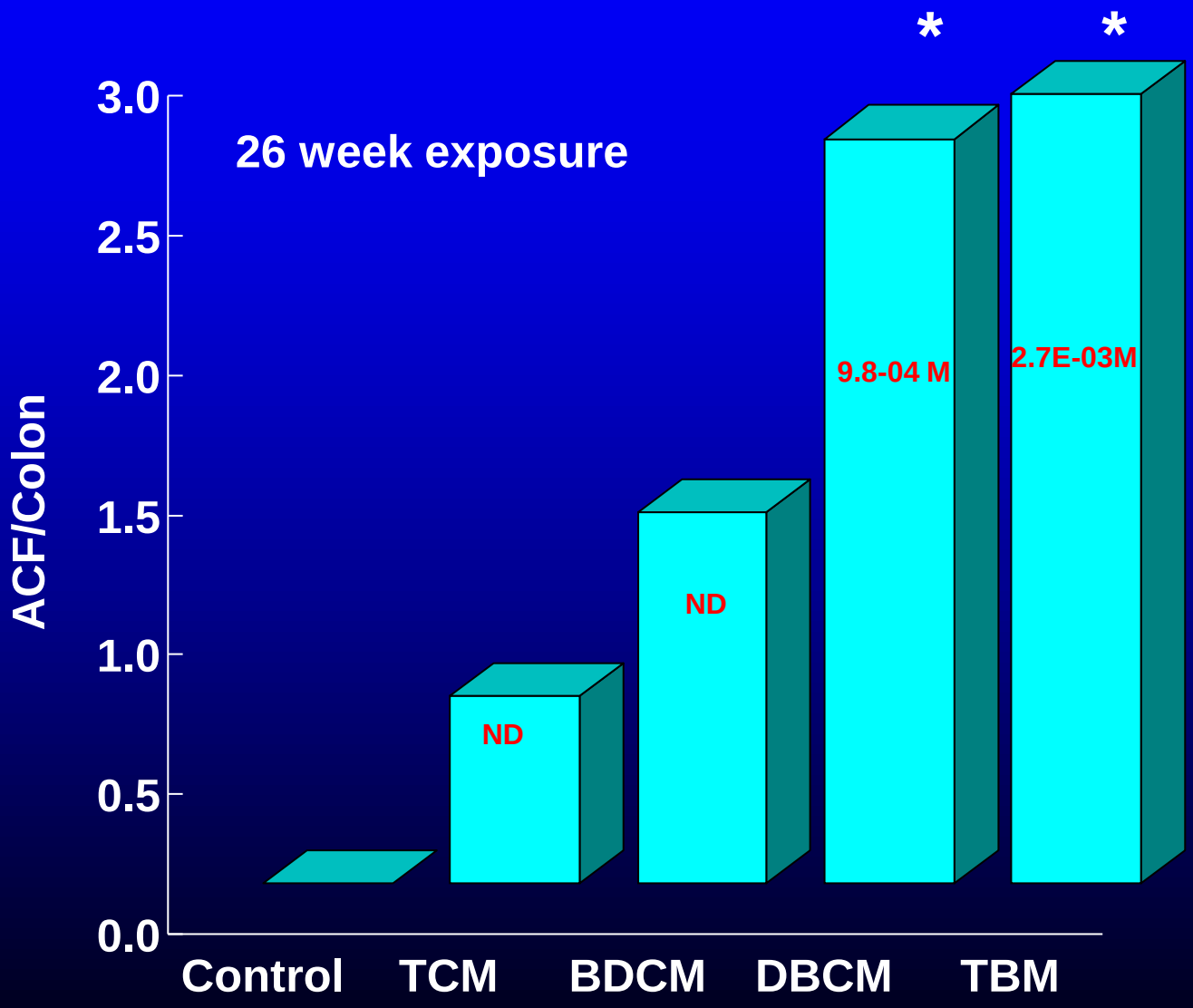
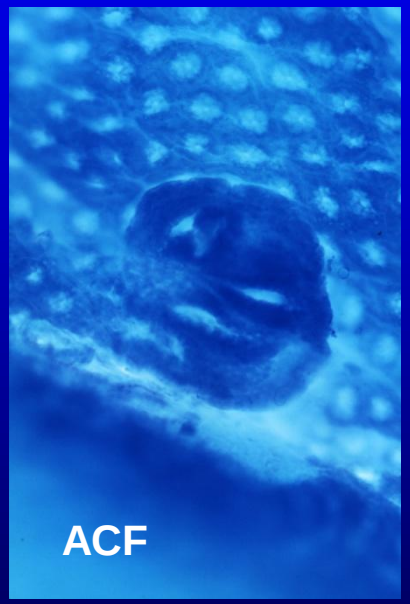
The Induction of ACF by Isomolar Concentrations of THMs in the Drinking water





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Transfrormation of NCM460 Colonocytes by DBPs

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Risk of Rectal Cancer

µg/day

Bromoform	OR (CI 95%); 1.85 (1.25 – 2.74) *p = 0.002*
Dibromochloromehane	OR (CI 95%) ; 1.78 (1.00 - 3.19) *p = 0.052*
Bromodichloromethane	OR; (CI 95%); 1.15 (1.00 - 1.32) *p = 0.047*
Chloroform	OR; (CI 95%); 1.00 (0.98 - 1.02) *p = 0.908*

Bromoform Quartile µg/day.

0.09 – 0.64	--------	--------	--------
0.65 – 0.97	OR (CI 95%) ; 1.43 (0.73 – 2.74) *p = 0.42*		
0.97 – 1.68	OR; (CI 95%); 1.63 (0.85 – 2.69) *p = 0.10*		
1.69 – 15.43	OR; (CI 95%); 2.32 (1.22 – 4.39) *p = 0.01*		

Linear trend *p = 0.002*

Bove Jr GE, Rogeson, PA, Vena Je (2007) Case control study of the geographic variability of exposure to disinfectant byproducts exposure and risk for rectal cancer. Internat. J. Health Geographics. 6:16.

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Experimental Design

NCM460 normal colon cell culture

Seed adherent monolayer → 7 or 10 days → Suspend culture → 14 days → Split suspension (S0) → 14 days → Halt suspension (S1) Begin soft agar plate → 28 days → Halt agar colonies

Begin DBP exposures (10^{-6}M)

Halt exposure

↓

Real-time qRT-PCR of β-catenin targets (Figure 2)

↓

Gene expression microarray & ICC stain (Figures 1 & 2, Table 1)

↓

Transformed colony count & ICC stain (Figures 2 & 3)

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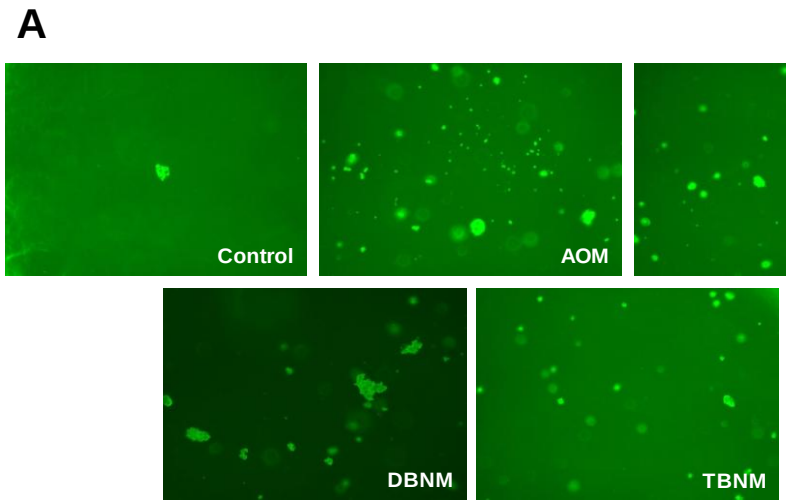

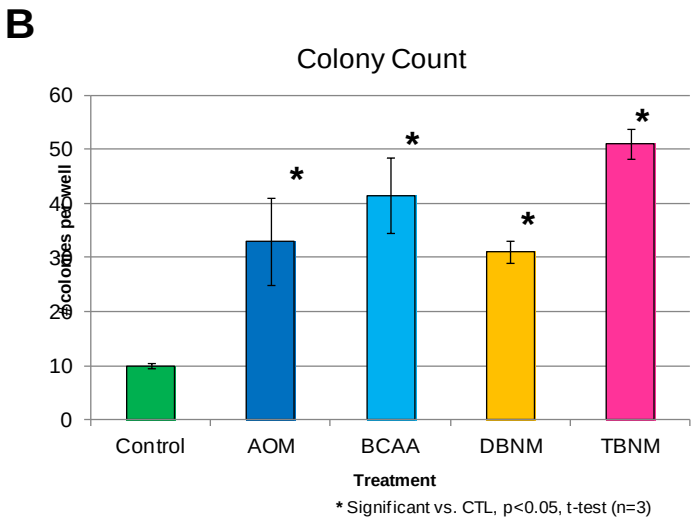

Figure 3. Colony transformation assay. (A) S1 fraction cells were mixed 0.4% low melting agar per well and stained with iodonitrotetrazolium chloride after 28 days of growth. Growth in soft agar is an indication of anchorage independent growth and a hallmark of cellular transformation. Colony formation was demonstrated with AOM, BCAA, DBNM and TBNM exposure in NCM460 cells. **(B)** Transformed colonies were quantified using ImageJ software (NIH).

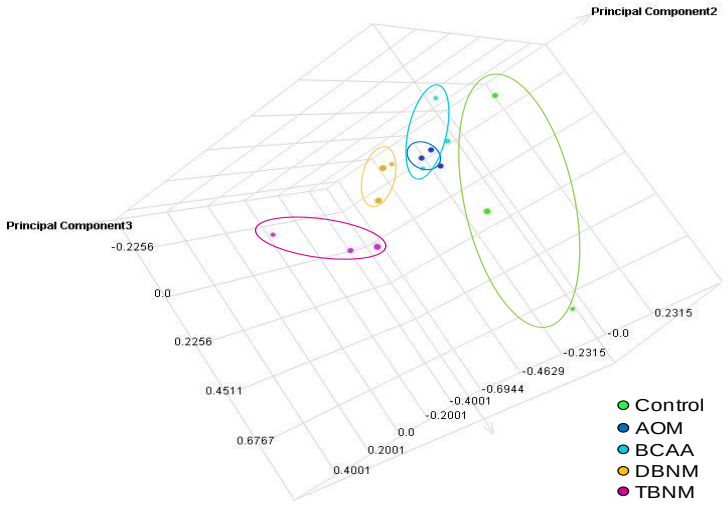

Figure 1. Microarray gene expression analysis of NCM460 cells treated with DBPs. 3-way Principal Component analysis of microarray expression data. S1 phase NCM460 were treated for 10 days using 10^{-6}M of each treatment (see Experimental Design). PC analysis indicates that the AOM control and DBP treatments alter gene expression from vehicle-treated, control cells.

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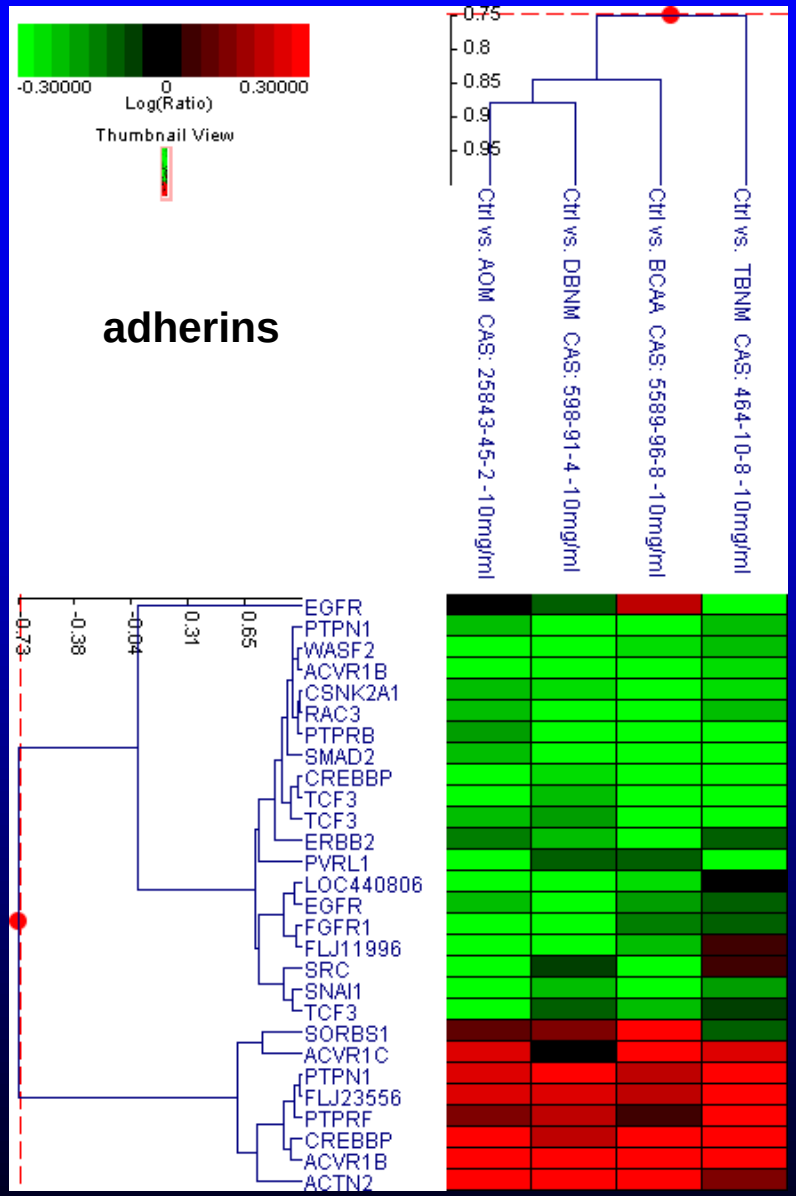
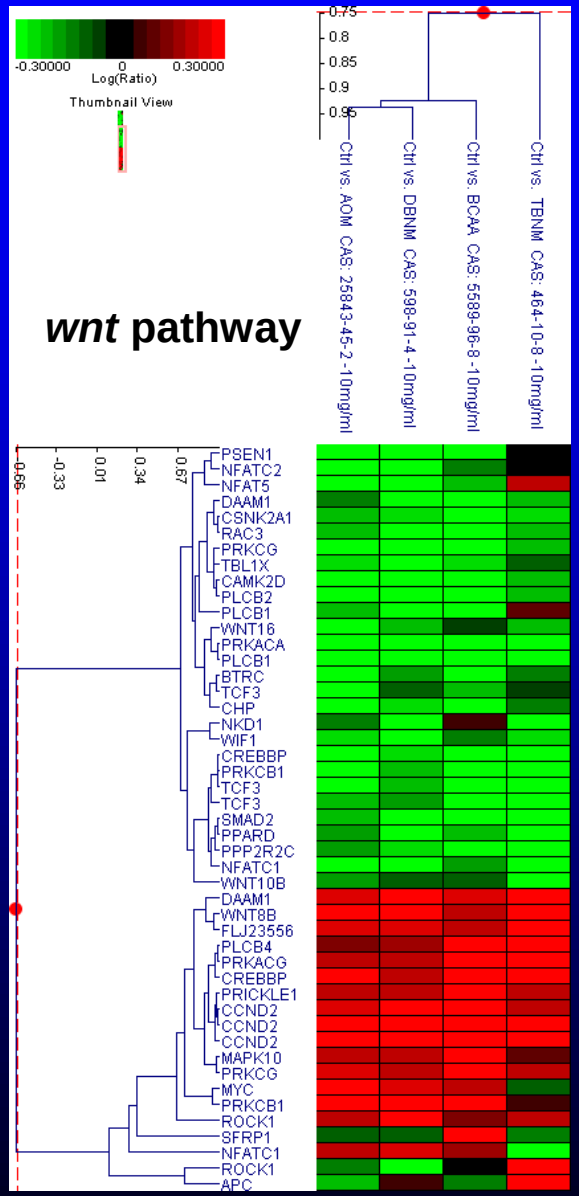
Common and unique pathways for AOM, BCA, DBNM, and TBNM from genomic analysis

	AOM	BCA	DBNM	TBNM
Common to 4 chemicals	Adherens Junction	Adherens Junction	Adherens Junction	Adherens Junction
Common to 3 chemicals		Metabolism of Xenobiotics by cyp 450	Metabolism of Xenobiotics by cyp 450	Metabolism of Xenobiotics by cyp 450
	WNT signaling		WNT signaling	WNT signaling
	Androgen and Estrogen metabolism	Androgen and Estrogen metabolism	Androgen and Estrogen metabolism	
	Starch and Sucrose metabolism	Starch and Sucrose metabolism	Starch and Sucrose metabolism	
	Ubiquitin mediated proteolysis		Ubiquitin mediated proteolysis	Ubiquitin mediated proteolysis
	Pentose and Glucuronate interconversions	Pentose and Glucuronate interconversions	Pentose and Glucuronate interconversions	
Common to 2 chemicals	Tight Junction			Tight Junction
			Leukocyte Transendothelial migration	Leukocyte Transendothelial migration
			Glycoshpingolipid metabolism	Glycoshpingolipid metabolism
Unique to each chemical	Insulin Signaling pathways	RB tumor suppressor/Checkpoint signaling in response to DNA damage	Antigen processing and presentation	TGF-beta signaling
	Biosynthesis of Steroids	Glycolysis/gluconeogenesis		Notch signaling

Genomic Analysis Heatmap





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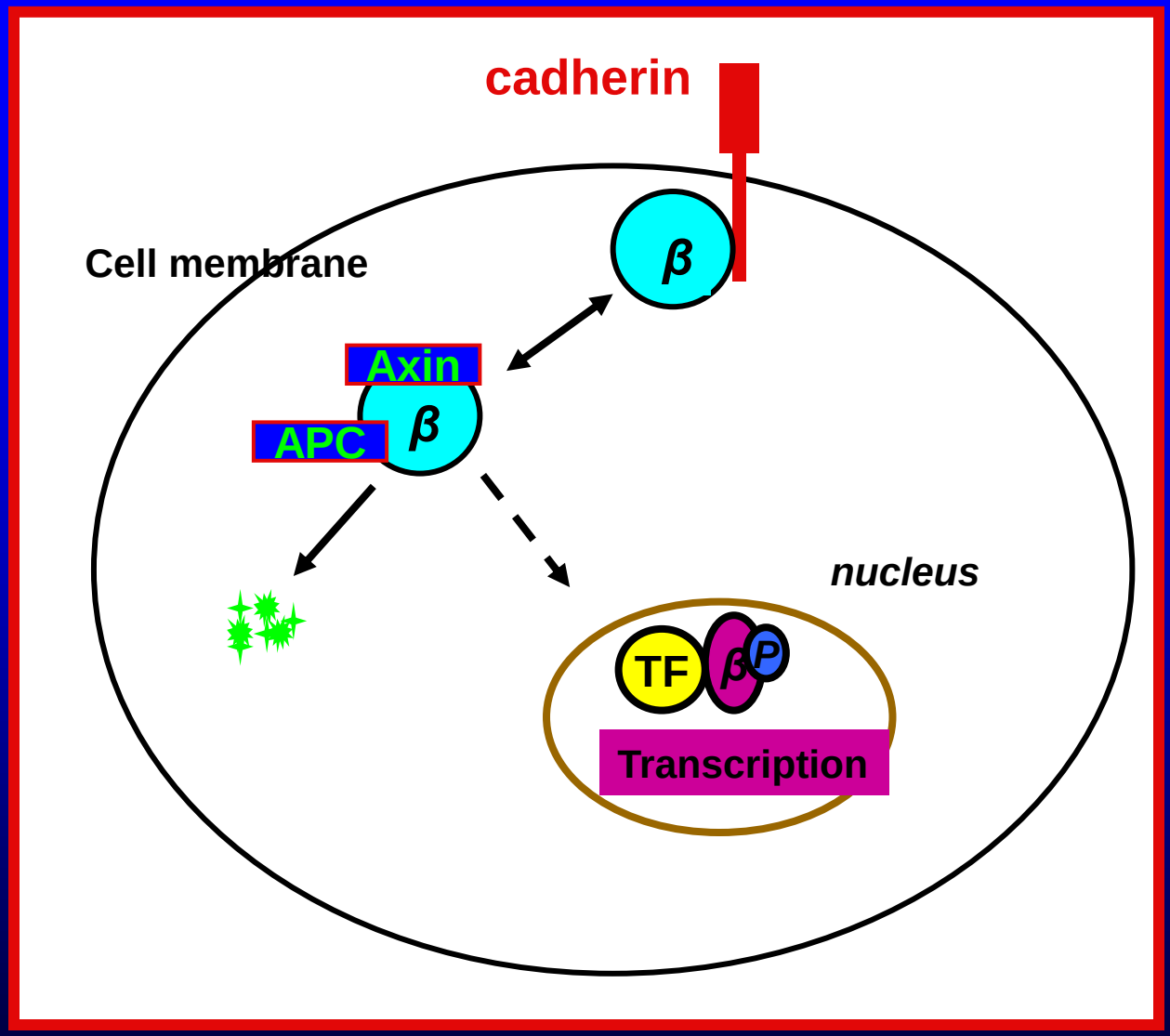
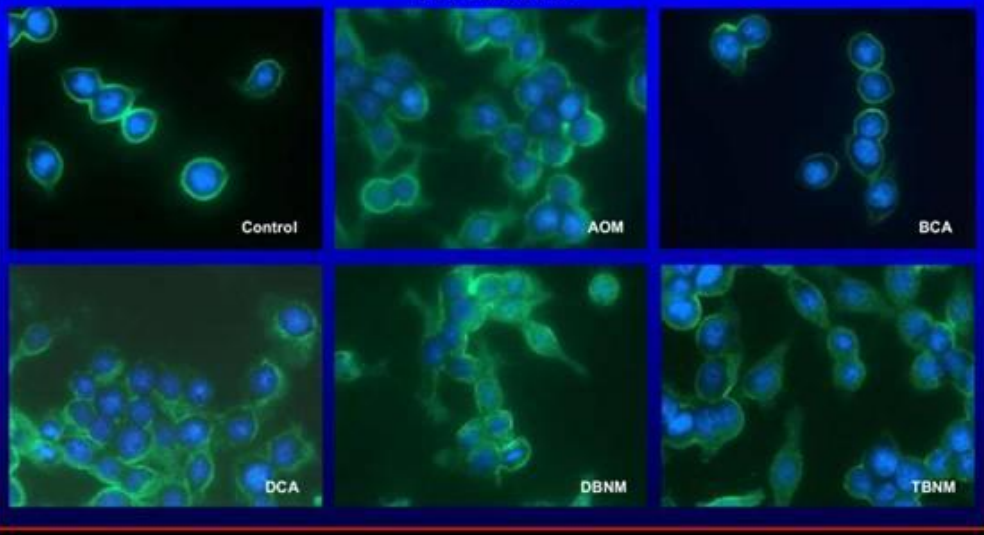
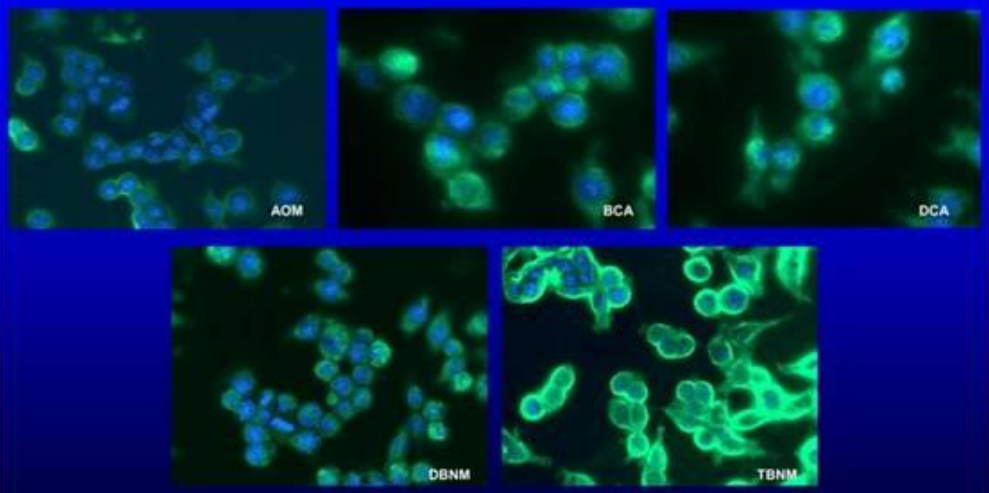


***Wnt* Pathway: activated in >95% of colon adenocarcinomas**

β-catenin S1 Fraction
NCM460 Cells



β-catenin transformed NCM460 cells
from Soft Agar



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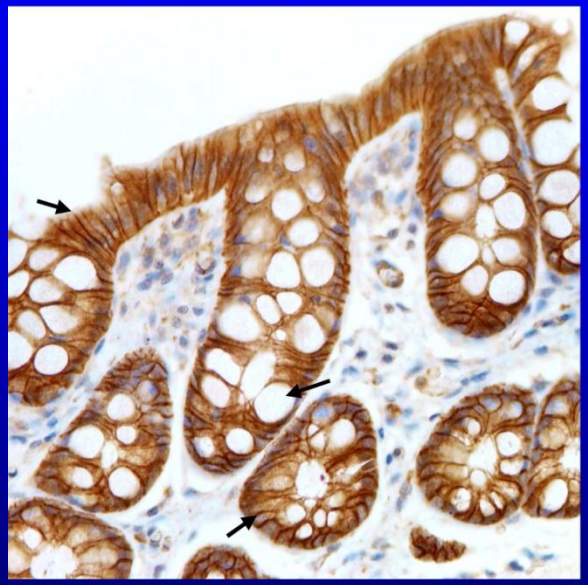
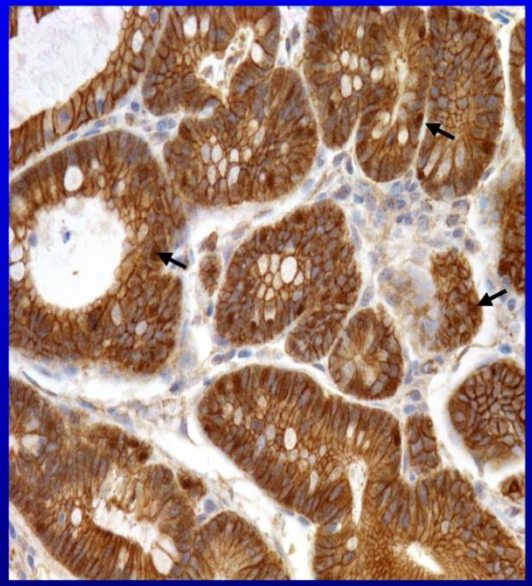
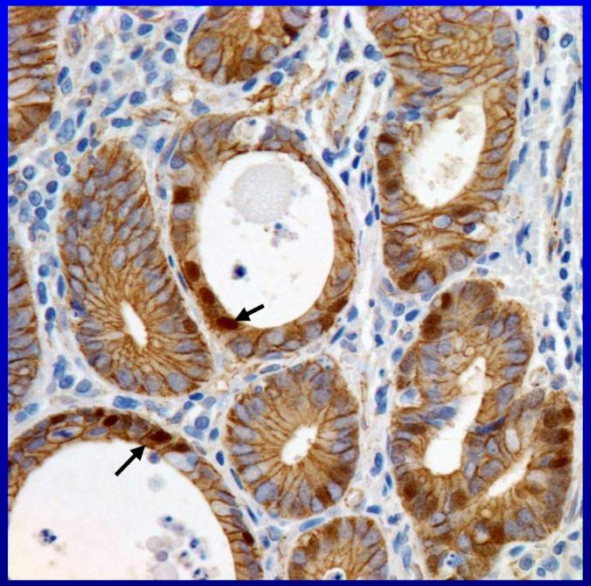
β-catenin Localization in Neoplastic Colon Crypts Induced by Trihalomethane Treatment of Rats



Control



Adenoma



Adenocarcinoma

Two Important Molecular Alterations the Development of Colon Cancer Were Activated

Wnt Signaling Pathway
Adherens Junction

The Wnt/-catenin pathway is important during embryogenesis and carcinogenesis.
β-Catenin interaction with E-cadherin (Adherins Junction) is crucial in cell-cell adhesion.

Subcellular distribution of β-catenin regulates its function. Membrane-bound β-catenin mediates cell–cell adhesion, Increased cytoplasmic and nuclear pool of β-catenin is associated with oncogenic function.

β-Catenin translocates from the cytoplasm to the nucleus, where it serves as a transcriptional factor to stimulate tumour formation.

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Acknowlegements

USEPA

Susan Richardson
Sheau-Fung Tsai
Bill Ward
Brian Chorley
Sid Hunter

Carlton Jones
Michael George
Ernest Winkfield
Beena Ballanat
Steve Kilburn

University of Illinois Champaign-Urbana

Michael Plewa
Elizabeth Wagner

INCELL Corporation

Mary Moyer

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