Assessment of toxicological properties and establishment of risk profiles - genotoxic properties of selected spice compounds

Frankfurt/Main, September 25th 2012

V.J. Koller[1], V. Auwärter[2], G. Zlabinger[3] and S. Knasmüller[1]

[1]Institute of Cancer Research, Medical University of Vienna
[2]Institute of Forensic Medicine, University Medical Center Freiburg
[3]Institute of Immunology, Medical University of Vienna

siegfried.knasmueller@meduniwien.ac.at

Contents

Part I

- Aim of the study
- Test compounds
- Cytotoxicity tests
- Impact on cytokine release
- Hormonal effects

Part II

- Genotoxicity tests
- Conclusions

siegfried.knasmueller@meduniwien.ac.at

Aim of the study

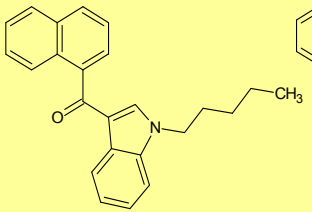
- Data on toxic properties of spice drugs are scarce.

- We conducted the first comprehensive study with six different representatives of synthetic cannabinoids and established their toxicological profiles.

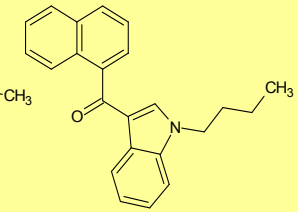
siegfried.knasmueller@meduniwien.ac.at

Test compounds

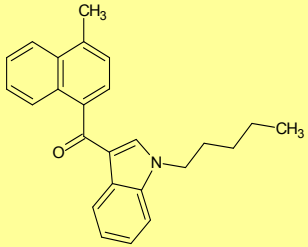
Rationale for their selection was based on their use and their chemical structures.



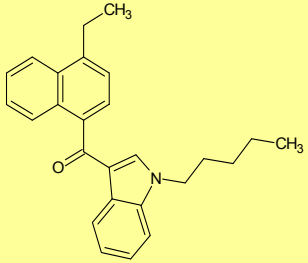
JWH-018



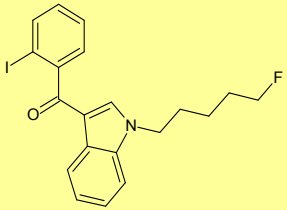
JWH-073



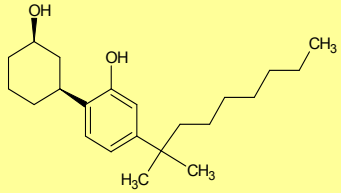
JWH-122



JWH-210

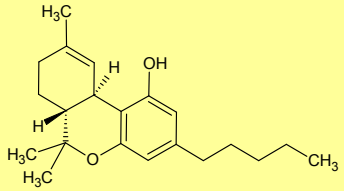


AM-694



CP-47,497-C8

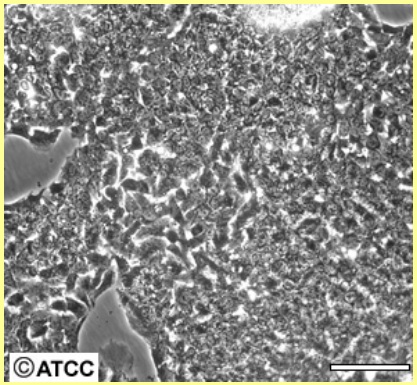
As a reference compound THC, the "classical ligand" for cannabinoid receptors, was used in all experiments.



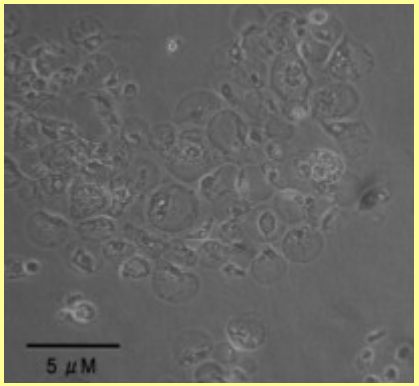
(-) - Δ^9 - THC

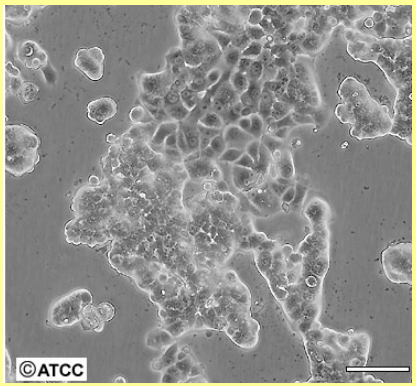
siegfried.knasmueller@meduniwien.ac.at

Cell lines used in cytotoxicity and genotoxicity tests



HepG2: Metabolically competent human derived liver cell line (expresses phase I and phase II enzymes) + cannabinoid receptors.





MCF-7: classical breast cancer cell line with estrogen receptors.

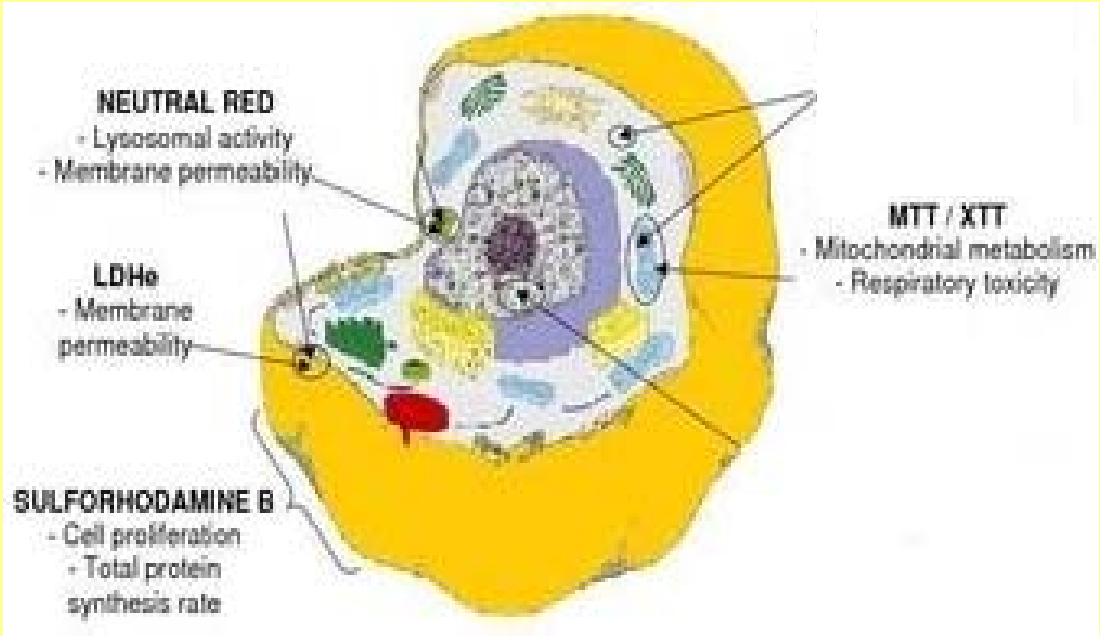
TR146: The buccal derived cell line was selected as the compounds come into first contact with mucosa cells.

siegfried.knasmueller@meduniwien.ac.at

Cytotoxicity tests: Endpoints and principles

All assays are based on colorimetric measurements

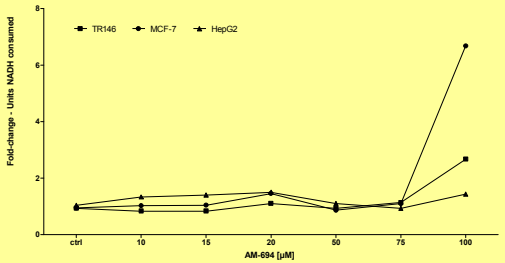
- **LDHe (extracellular lactate):** quantification of membrane integrity and cellular viability
- **Neutral Red:** quantification of the membrane permeability and lysosomal activity
- **Sulforhodamine B (SRB):** quantification of the total protein synthesis rate
- **XTT – Tetrazolium Hydroxide:** quantification of the mitochondrial metabolism and of the respiratory chain activity

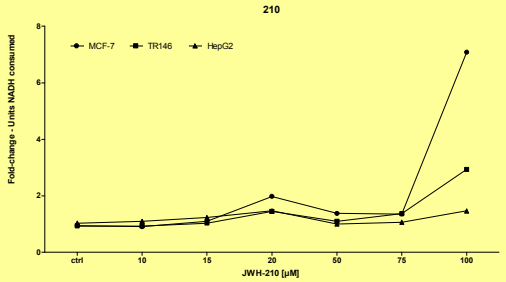


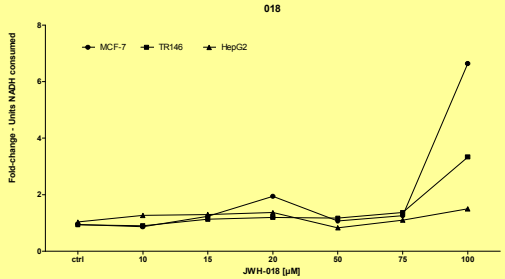
siegfried.knasmueller@meduniwien.ac.at

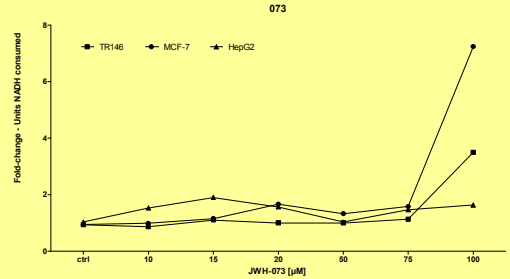
Results of the acute toxicity tests

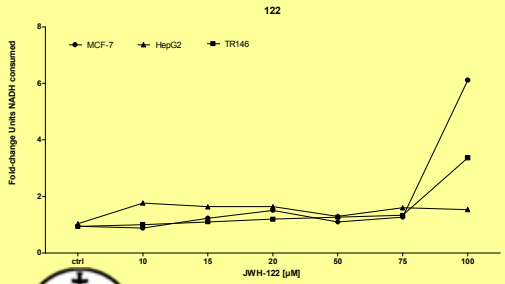
Compounds were tested over a broad dose-range up to 100 µM.

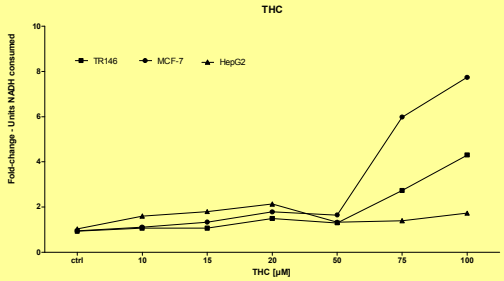












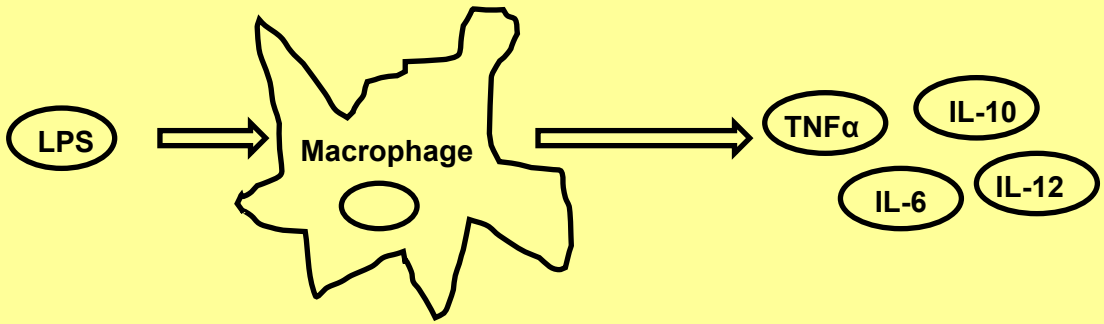
No remarkable effects were seen in HepG2 cells. Some toxic effects were observed at high dose levels in the LDHe-assay (indicative for membrane damage) in TR146 and MCF-7 cells

EC20 Values	LDH	
Compound	MCF	TR146
AM-694	~ 87.99	220,5
JWH-210	~ 91.13	80,87
JWH-018	~ 86.83	~ 117.2
JWH-073	~ 92.43	~ 87.92
JWH-122	~ 86.12	~ 114.6
THC	59,16	64,63

siegfried.knasmueller@meduniwien.ac.at

Impact on cytokine production

- Cytokines are cell signalling molecules and immune modulating agents which are secreted by numerous cells in the body (e.g. as a consequence of infections and inflammation).
- In the present study we investigated the impact of the drugs on LPS-induced responses in human peripheral blood mononuclear cells (PBMCs). The model reflects the consequences of a bacterial infection.



siegfried.knasmueller@meduniwien.ac.at

Results: Effects on cytokine release

Compound [µM]		IL-10 [pg/ml]	IL-6 [pg/ml]	IL12p40 [pg/ml]	TNFα [pg/ml]
LPS	0.0	99,0 ± 82,9	3000 ± 2066	594,2 ± 451,7	607,9 ± 239,0
AM694	0.1	93,9 ± 69,6	3326 ± 2259	667,0 ± 434,1	638,3 ± 238,1
	0.3	124,5 ± 89,7	3091 ± 1851	715,6 ± 454,6	607,5 ± 197,4
	1.0	142,9 ± 129,0	3507 ± 2317	600,8 ± 367,2	587,0 ± 207,1
	3.0	141,8 ± 124,4	3209 ± 1949	653,9 ± 380,3	583,2 ± 198,3
	10.0	164,4 ± 171,7	3221 ± 2087	556,3 ± 337,9	605,8 ± 228,0
JWH-210	0.1	112,0 ± 91,0	2983 ± 1781	604,9 ± 362,4	569,6 ± 182,7
	0.3	167,7 ± 193,5	3240 ± 1924	552,6 ± 275,7	513,4 ± 160,7
	1.0	145,8 ± 124,6	3215 ± 2089	624,0 ± 371,9	519,7 ± 146,4
	3.0	135,7 ± 132,9	2870 ± 1716	549,1 ± 286,6	506,7 ± 152,4
	10.0	130,7 ± 97,0	2492 ± 1630	395,3 ± 161,2	**499,9 ± 198,4***
JWH-122	0.1	123,3 ± 91,7	3040 ± 1721	567,7 ± 295,8	552,2 ± 180,4
	0.3	138,5 ± 116,0	3358 ± 2140	621,0 ± 416,9	529,2 ± 167,7
	1.0	129,7 ± 117,8	3180 ± 2090	535,5 ± 386,7	513,5 ± 175,9
	3.0	161,8 ± 168,9	2800 ± 1862	527,0 ± 221,9	**503,7 ± 167,0***
	10.0	147,1 ± 157,2	2583 ± 1659	**388,2 ± 290,8***	**501,1 ± 170,6***
JWH-018	0.1	127,5 ± 112,3	3112 ± 1787	590,4 ± 269,5	553,3 ± 179,8
	0.3	123,5 ± 94,7	3127 ± 1838	586,0 ± 363,3	525,9 ± 158,6
	1.0	212,6 ± 232,8	3346 ± 2166	605,0 ± 449,2	538,0 ± 203,7
	3.0	206,4 ± 246,5	3031 ± 1917	522,7 ± 251,9	527,4 ± 185,3
	10.0	124,1 ± 117,6	3062 ± 2126	460,6 ± 299,7	514,9 ± 178,8
JWH-073	0.1	111,3 ± 85,4	3025 ± 1618	613,6 ± 292,2	574,0 ± 198,3
	0.3	207,7 ± 254,5	2979 ± 1732	663,2 ± 427,7	525,1 ± 172,5
	1.0	231,3 ± 270,0	3130 ± 2061	534,0 ± 353,8	**466,6 ± 136,3***
	3.0	145,4 ± 134,7	2810 ± 1840	559,6 ± 383,2	518,1 ± 191,3
	10.0	151,2 ± 178,1	2793 ± 1881	536,4 ± 462,4	510,6 ± 207,1
THC	0.1	123,6 ± 100,9	3331 ± 1656	704,9 ± 327,8	602,9 ± 190,7
	0.3	142,1 ± 145,4	3096 ± 1870	661,0 ± 378,3	538,0 ± 196,6
	1.0	116,0 ± 90,7	3281 ± 1879	726,0 ± 436,5	549,1 ± 177,3
	3.0	126,8 ± 105,8	3286 ± 2269	639,2 ± 494,3	534,7 ± 182,8
	10.0	83,0 ± 37,4	3173 ± 2215	536,5 ± 327,6	542,7 ± 188,2

Compound [µM]		IL-10 [pg/ml]	IL-6 [pg/ml]	IL12p40 [pg/ml]	TNFα [pg/ml]
LPS		974,7 ± 224,8	12300,2 ± 1107,5	642,1 ± 305,3	1015,4 ± 349,9
CP-47,497-C8	0.1	845,4 ± 207,6	12233,8 ± 4678,2	713,0 ± 269,2	1076,5 ± 414,5
	0.3	1018,5 ± 284,8	10155,1 ± 1317,2	643,3 ± 224,0	911,3 ± 284,5
	1.0	953,5 ± 110,9	10640,7 ± 1765,1	568,4 ± 302,6	932,1 ± 275,3
	3.0	922,2 ± 129,9	14456,4 ± 1154,7	694,9 ± 264,6	1039,6 ± 306,8
	10.0	**784,6 ± 222,8***	11836,6 ± 2104,3	**916,6 ± 260,0***	**1467,2 ± 392,8***

Only with three compounds (JWH-210, JWH-122 and CP-47,497-C8) significant effects were seen at dose levels ≥ 3µM (induction of TNFα, IL-12 and IL-6).

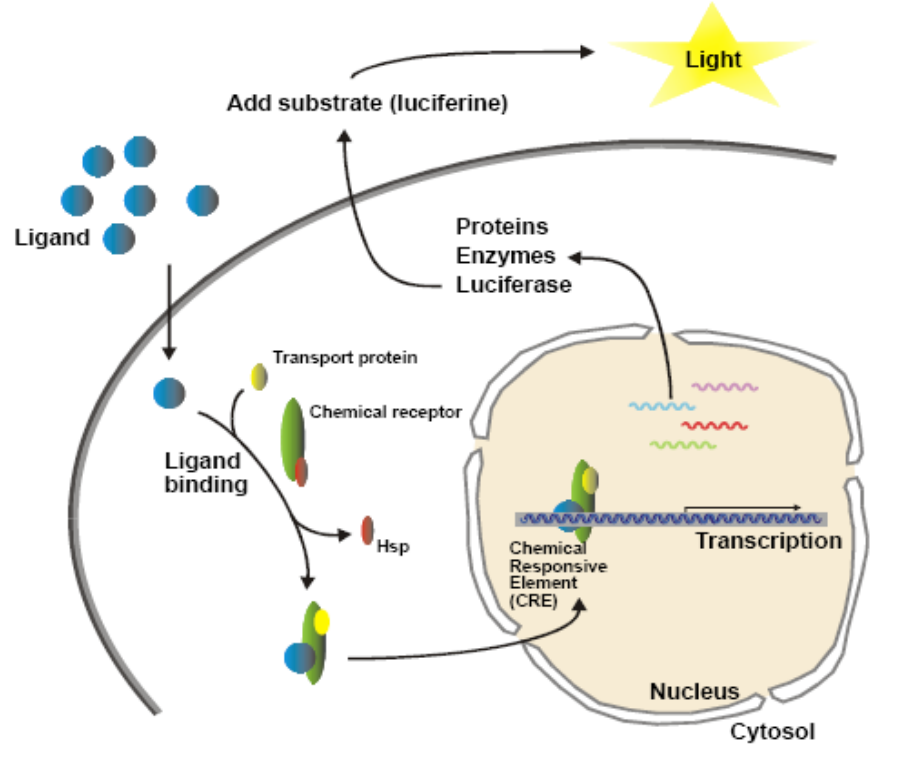
siegfried.knasmueller@meduniwien.ac.at

Hormonal effects

- **Estrogenic and anti-estrogenic effects were measured in an established model which is based on the emission of light by the use of a firefly luciferase coupled to responsive elements (REs) as a reporter gene in a human bone cell line U2-OS.**
- **17β-estradiol and tamoxifen were used as reference compounds.**
- **Estrogenic effects are associated with adverse effects such as induction of breast cancer; anti-estrogenic effects may have an impact on fertility.**

siegfried.knasmueller@meduniwien.ac.at

Principle and results of the CALUX ER assays



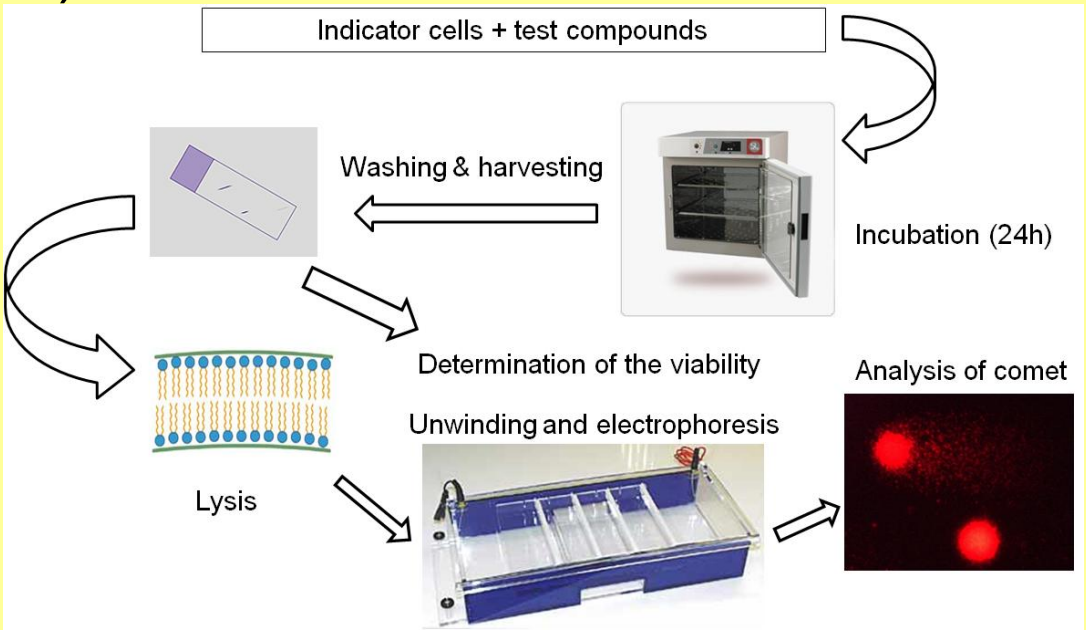
No estrogenic but anti-estrogenic activities were detectable!

Substance	IC_{20} (M)
Tamoxifen	$3.6*10^{-12}$
JWH-210	$9.3*10^{-6}$
JWH-018	$2.8*10^{-6}$
JWH-122	$2.1*10^{-6}$
AM-694	$2.3*10^{-5}$
JWH-073	$1.0*10^{-5}$
CP-47,497-C8	No activity
THC	$2.0*10^{-5}$

siegfried.knasmueller@meduniwien.ac.at

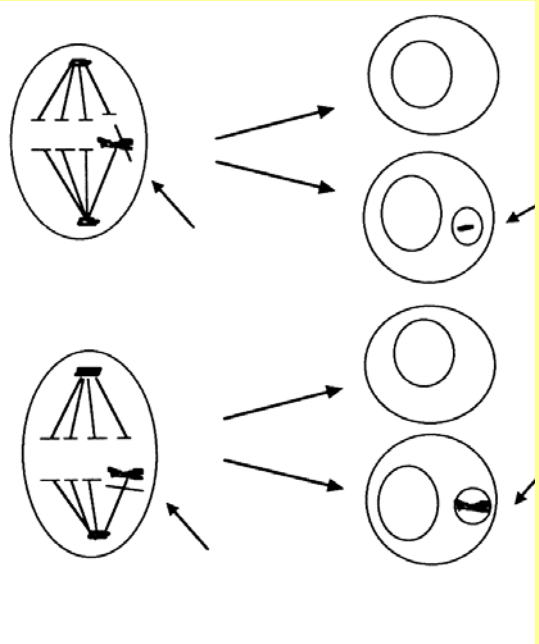
Genotoxicity tests I
Comet assay

- Comet or SCGE (Single-Cell Gel Electrophoresis) assays reflect transitory single/double strand breaks.
- They are “indicator tests” – primary lesions may be repaired without manifestation of persisting DNA-damage (i.e. mutations).

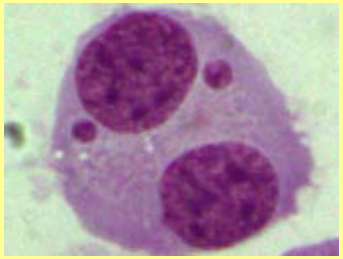


siegfried.knasmueller@meduniwien.ac.at

Genotoxicity tests II
Micronucleus (MN) assay



Micronuclei originate from **acentric fragments** (chromosome fragments lacking a centromere) or **whole chromosomes** which are unable to migrate with the rest of the chromosomes during the anaphase of cell division.

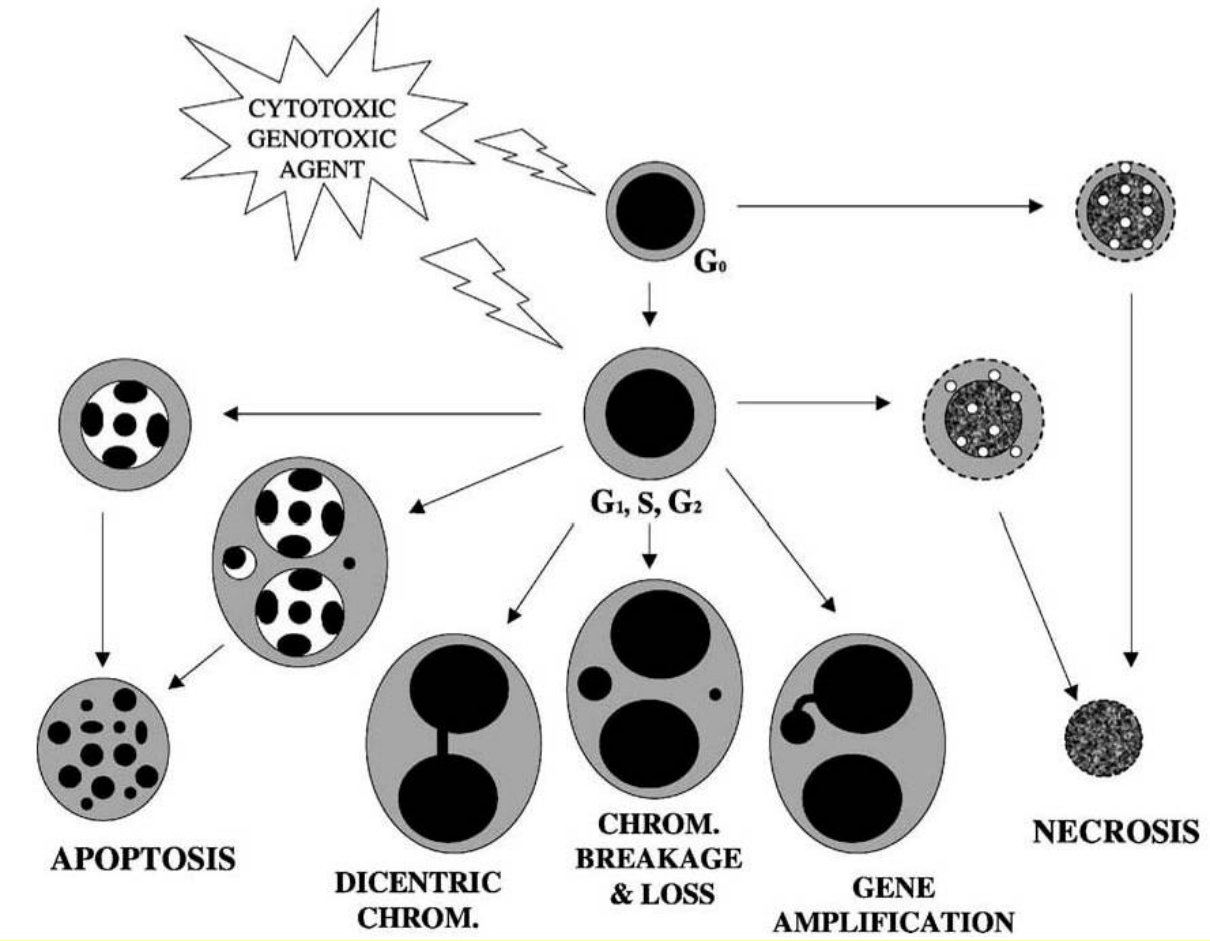


MN assay enables the assessment of **chromosome breakage** (clastogenicity) and **chromosome loss** (aneuploidy).

siegfried.knasmueller@meduniwien.ac.at

Principle of MN-Formation

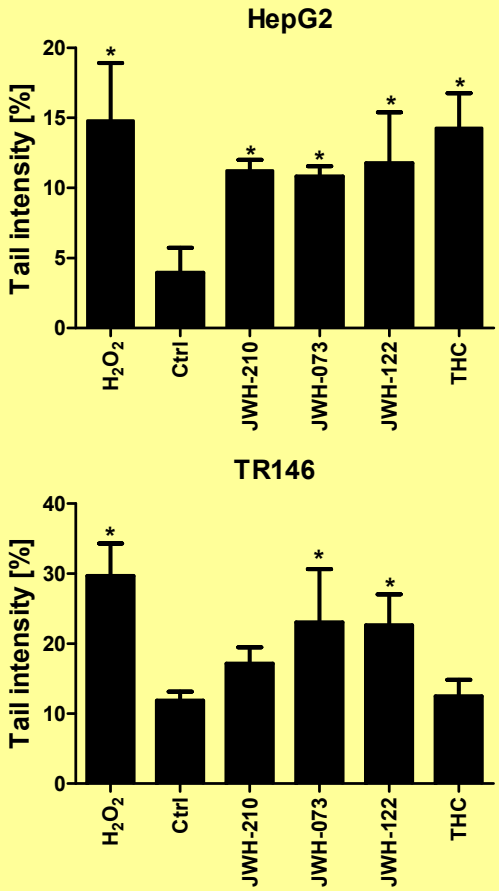
Cytokinesis Block Micronucleus (CBMN) Assay (M. Fenech et al. 2003)



siegfried.knasmueller@meduniwien.ac.at

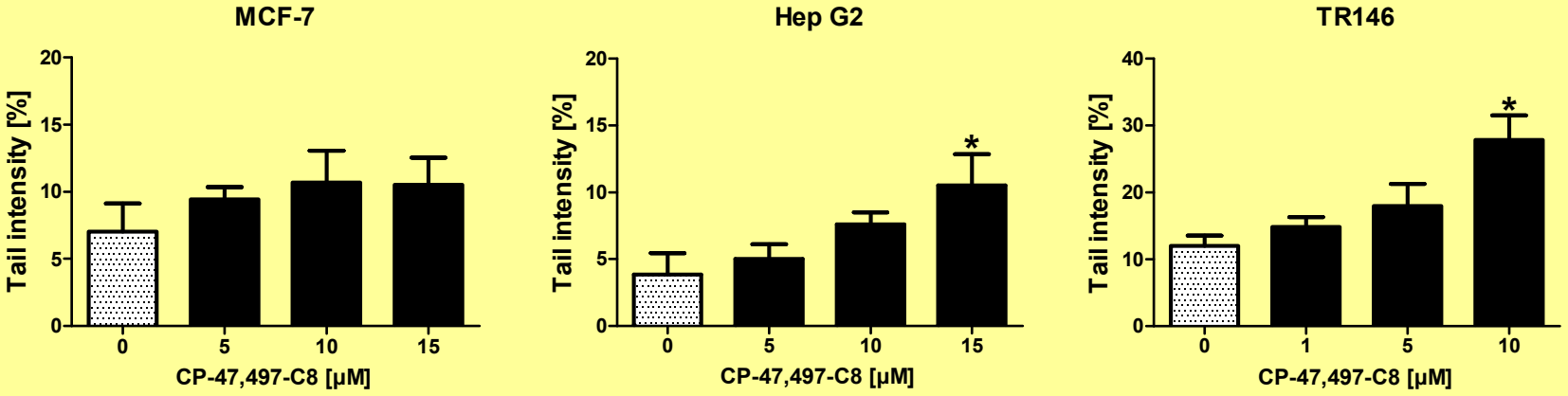
Results of the SCGE experiments

Compound		MCF-7	TR146	HepG2
Medium		7,82 ± 4,09	11,21 ± 3,28	3,35 ± 1,12
H_2O_2	50 µM	25,16 ± 3,28	25,84 ± 2,21	11,67 ± 1,81
AM-694	50 µM	6,17 ± 1,77	7,51 ± 4,53	3,66 ± 1,55
	75 µM	3,69 ± 1,24	9,18 ± 4,98	5,88 ± 2,61
	100 µM	4,27 ± 1,72	9,04 ± 3,91	4,96 ± 2,18
JWH-210	50 µM	4,18 ± 0,96	11,49 ± 7,86	2,75 ± 1,19
	75 µM	3,88 ± 1,89	8,34 ± 4,17	3,45 ± 1,41
	100 µM	4,12 ± 2,47	7,39 ± 4,37	**6,70 ± 1,42***
JWH-018	50 µM	5,92 ± 2,63	6,57 ± 1,40	3,69 ± 1,05
	75 µM	6,51 ± 3,08	8,29 ± 0,67	4,59 ± 2,32
	100 µM	4,26 ± 2,10	13,17 ± 2,67	3,22 ± 1,79
JWH-073	50 µM	5,77 ± 1,40	13,81 ± 5,61	4,54 ± 1,17
	75 µM	4,67 ± 1,63	15,94 ± 3,99	6,81 ± 2,55
	100 µM	8,55 ± 1,52	**21,82 ± 2,89***	**9,40 ± 4,22***
JWH-122	50 µM	6,90 ± 1,58	10,19 ± 3,36	9,33 ± 1,81
	75 µM	4,94 ± 3,03	13,15 ± 2,36	10,17 ± 5,56
	100 µM	6,37 ± 2,06	**15,60 ± 2,77***	**19,16 ± 8,78***
THC	50 µM	4,57 ± 1,70	19,72 ± 2,91	6,21 ± 5,01
	75 µM	8,01 ± 1,74	16,50 ± 5,68	4,58 ± 1,14
	100 µM	8,27 ± 3,02	13,98 ± 1,90	**9,20 ± 3,92***



siegfried.knasmueller@meduniwien.ac.at

CP-47,497-C8

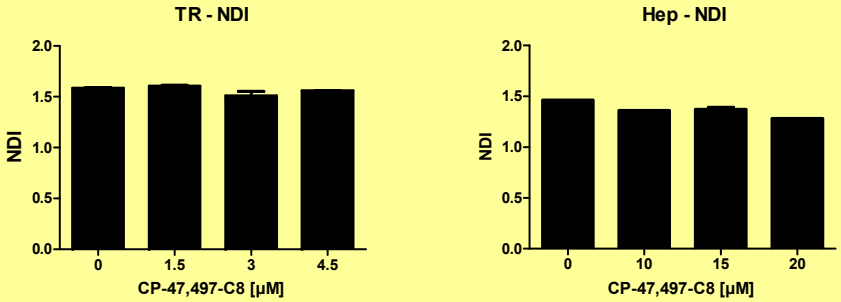


The compound CP-47,497-C8 induced comet formation already at lower doses ≤ 15 µM and was further investigated in MN assays with HepG2 and TR146 cells.

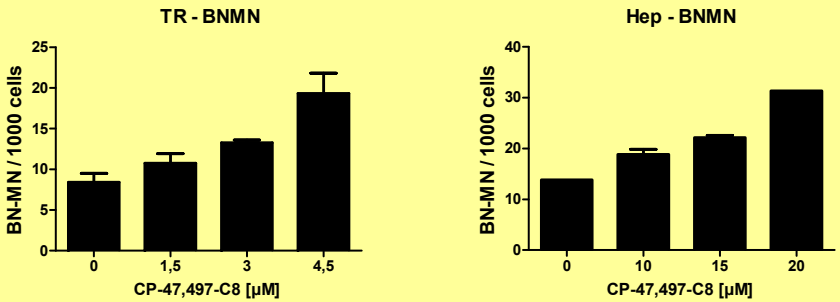
siegfried.knasmueller@meduniwien.ac.at

MN-Formation

CP-47,497-C8 induced formation of MN in both cell lines.



No effect on cell division



Clear MN-induction

This finding shows that comet formation leads to chromosomal aberrations which is indicative for increased human cancer risks.

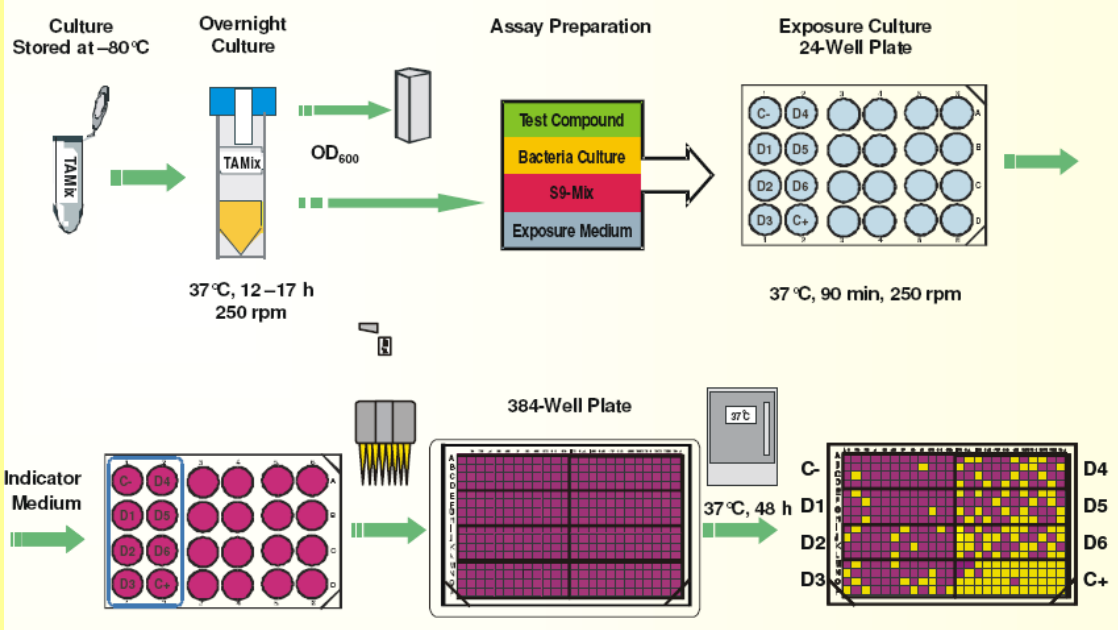
siegfried.knasmueller@meduniwien.ac.at

Genotoxicity tests III
Principle of the Ames II assay

The Salmonella/microsome assay is based on the detection of the formation of his$^+$ revertants due to gene mutations.

In the present study the microplate version was used.

(Scheme from G. Engelhardt, E. Jacob, R. Jäckh; Ames II assay: Results of a validation study)



Negative results were obtained under all experimental conditions.

This indicates that the compound causes no induction of gene mutations in bacterial cells.

siegfried.knasmueller@meduniwien.ac.at

Conclusion I

- **Compounds were only toxic at high dose levels which exceed the plasma levels found in a human study with the naphthoylindole compound JWH-018 by several orders of magnitude.**
- **However, acute toxic effects occur as a result of membrane damage in human derived cells and anti-estrogenic effects were seen with the drugs.**
- **Most interestingly DNA-damaging properties were obtained with four of the compounds. In the case of the JWH compounds, these effects may be due to formation of epoxides, which are known to be DNA-reactive molecules.**

siegfried.knasmueller@meduniwien.ac.at

Conclusion II

- It is assumed that the effects of genotoxic compounds are not linear over a broad dose range. Therefore, it can be not excluded that the DNA is damaged in drug users.
- With one representative of the cyclohexylphenoles it was found that it causes chromosomal damage leading to MN-formation.
- It is known that MN formation in human is a valid marker for cancer risks.

siegfried.knasmueller@meduniwien.ac.at

Thank you for your attention

siegfried.knasmueller@meduniwien.ac.at