Enantioselective effects of oxazol ceramide analogs applying differential gene expression analysis

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Introduction Sphingolipids and glycophingolipids are essential components of the lipid bilayer of all eukaryotic cell membranes. Whereas initially they were regarded as inert structural components of cell membranes, it has now become clear that they play an important role in the regulation of numerous cellular processes including cell recognition, differentiation, cell-cell contact and cell growth. Among bioactive sphingolipid metabolites, ceramides act as second messengers of sphingolipid signalling and mediate apoptosis (programmed cell death through various mechanisms. Induction of apoptosis with synthetic analogs offers a valuable strategy in particular for the treatment of colon and prostate cancer or for enhancing or retaining the potency of well known cancer drugs.

We are using microarray analysis for compound profiling. Both enantiomers of the bioactive oxazol compounds 1(R)-(E)-(2-methyl-oxazol-4-yl)-hexadec-2-en-1-ol and 1(S)-(E)-(2-methyl-oxazol-4-yl)-hexadec-2-en-1-ol (structures see **Fig. 2**) were synthesized and analyzed for apoptosis-inducing activities. The results prompted us to anlayze differences using microarrays.

Experimental

Cell culture Human colon carcinoma cells HT-29 (DSMZ, Braunschweig, Germany) were seeded semi-confluently in 5.8 cm2 petri dishes, equilibrated at 37°C over night in McCoy´s 5A Medium with 1% human AB serum, supplemented with 2mM L-Glutamin, 50 units/ml Penicillin, 50 µg/ml Streptomycin and stimulated with The cells were stimulated with 1-10 µM of oxazol analogs 1(R)-(E)-(2-methyl-oxazol-4-yl)-hexadec-2-en-1-ol and 1(S)-(E)-(2-methyl-oxazol-4-yl)-hexadec-2-en-1-ol and samples prepared after 2, 4 and 8 h.
RNA sample preparation

Apoptosis was quantitated by counting YO-PRO®-1 Iodide stained cells and confirmed by PARP-fragmentation and densitometric quantifikation of fragment formation.
Monolayers were washed with ice-cold PBS and cells were scraped in 3 ml lysis buffer and total RNA from HT-29 cells was extracted with RNeasy Mini Spin Columns (Qiagen, Hilden, Germany) or TriPure® Isolation Reagent (Roche Diagnostics, Mannheim) according to the manufactures instructions. RNA yields were determined spectrophoto-metrically on a NanoDrop ND-1000 Spectro-Photometer (NanoDrop Technologies, Wilmington, USA) by measuring the absorbance at 260 and 280 nm. All RNA samples used for microarray analysis were analyzed by ethidium bromide-stained RNA agarose gel to confirm purity of RNA.

Oligonucleotide Microarray hybridization
Experiments were performed using the Lab-Arraytor®-60-1 bis 60-6 combi oligonucleotide microarray (SIRS-Lab, Jena Germany), comprising 539 probes (each made as as triple replicates) addressing 519 transcripts corresponding to inflammation as well as 22 reliable control probes.
10 µg totalRNA were reverse transcribed using Superscript-II reverse transcriptase from Invitrogen (Karlsruhe, Germany) in the presence of aminoallyl-dUTP from Sigma (Taufkirchen, Germany) and labelled using the AlexaFluor 555/647 system.
AlexaFluor 548-labeled cDNA from cells treated with one enantiomer were co-hybridized with AlexaFluor 647-labeled cDNA obtained from the same amount of total RNA isolated from cells treated with the other enantiomer. After incubation in a hybridisation apparatus (HS 400, TECAN, Crailsheim, Germany, for 10 h at 42 °C, formamide-based hybridisation buffer system) arrays were washed according to the manufacturers instructions, dried and hybridisation signal intensities were measured immediately using an Axon 4000B scanner (Axon Instruments, Foster City, CA). Microarray data pre-processing of hybridisation signals included i) spot detection and background subtraction, ii) spot flagging according to defined signal-to-noise threshold values, iii) normalization and transformation of the signals obtained from different channels. For the former two steps, the GenePix® 5.0 Analysis Software 5,0 (Axon Instruments, Foster City, CA) was used; for the third step we applied the approach from Huber and colleagues (W. Huber, A. von Heydebreck, H. Sültmann, A. Poustka, M. Vingron, Bioinformatics, 2002, 18, 96-104
W. Huber, A. von Heydebreck, H. Sültmann, A. Poustka, M. Vingron, 2003, Statistical Applications in Genetics and Molecular Biology, 2, 1, Article 3; http://www.bepress.com/sagmb/vol2/iss1/art3) including variance-stabilised transformation. Expression of genes with invalid spots were labelled as missing values.
Alternatively, Illumina station employing Human-6 v2 Expression BeadChips containing 6 arrays with 48 000 probes each was used.

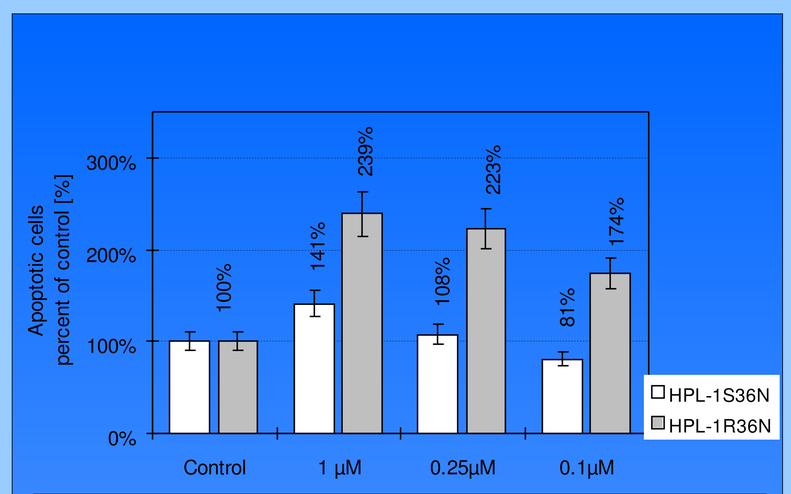
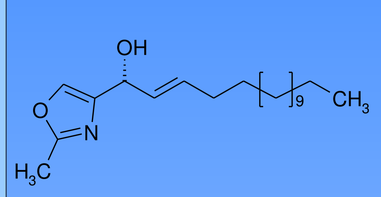
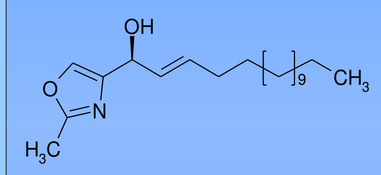


Fig. 1. Apoptosis in HAT-29 cells treated with the enantiomers of the ceramide analog. Cells were incubated with the depicted concentrations of the agents for 4 h and percentage of apoptosis determined by staining with YO-PRO®-1 Iodide (ratios confirmed by additional methods)



HPL-1R36N (1R)-(E)-(2-Methyl-oxazol-4-yl)-hexadec-2-en-1-ol



HPL-1S36N (1S)-(E)-(2-Methyl-oxazol-4-yl)-hexadec-2-en-1-ol

Cohybridized tRNA of HT-29 cells, incubated with 10µM HPL-1R36N (green) / HPL-1S36N (red)

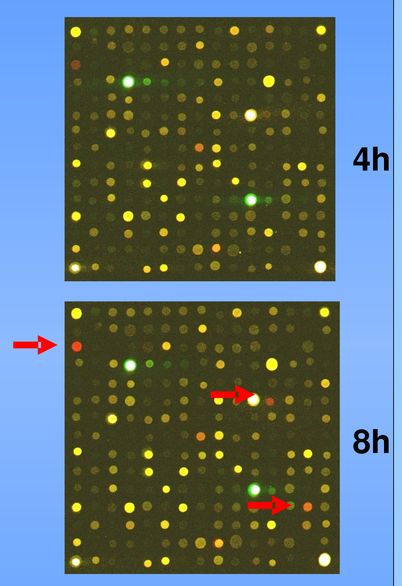


Fig. 2. Structures of the compounds under investigation and typical micrarray data abtained at indicated conditions / concentrations; arrows indicate chenges in expression

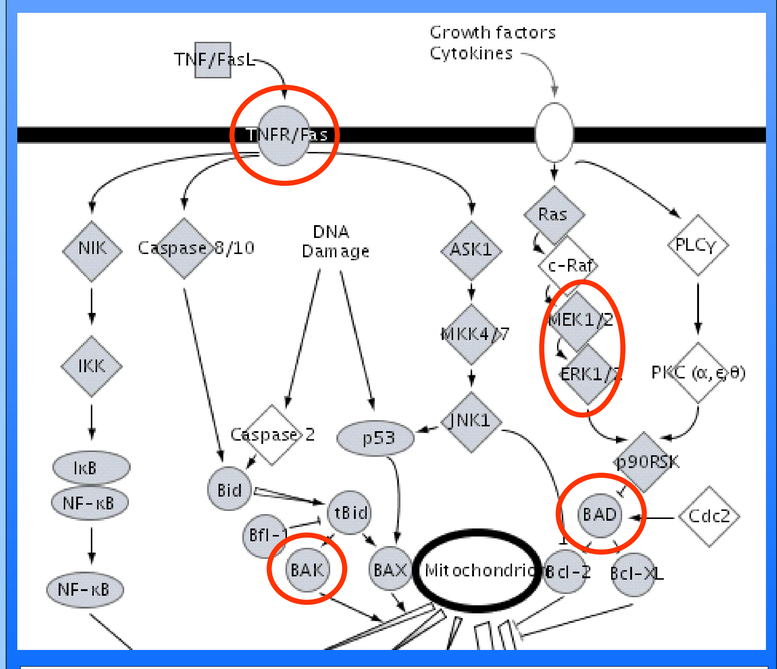


Fig. 3. Selected transcriptional changes (detail) with relevance to apoptosis (genes with changes in expression denoted by red circles) displayed by Ingenuity® software.

Results

Apoptosis induction was investigated in HT 29 cells. As depicted in **Fig. 1**, there was a marked difference in the potency of the compounds. The effect of the R-enantiomer was markedly stronger compared to the S congener.

As identical concentrations of the agents produced different levels of apototic cells, we analyzed transcription to identify potential differences in signaling. The cells were stimulated with 1-10 µM of oxazol analogs 1(R)-(E)-(2-methyl-oxazol-4-yl)-hexadec-2-en-1-ol and 1(S)-(E)-(2-methyl-oxazol-4-yl)-hexadec-2-en-1-ol,samples prepared after 2, 4 and 8 h, RNA extracted, labelled and subjected to microarray analysis.

Expression changes induced by the individual enantiomers differed markedly comprising 282 probes indicated expression changes higher than factor 3, 17 were identified which showed more than 8-fold changes (selected gene activities displayed in **Fig. 3**) Time-dependent differences in differential expression comprise gene activities relevant to apoptosis signalling such as BAD, TRAF1 and BAK1. Networks with potential interactions were displayed using Ingenuity® software to analyze enantiospecific differences with potential relevance to apoptosis signalling (**Fig. 3**). Additional analyses (not shown) using a pan-genomic array and alternative cell lines as well as preliminary PCR-analyses confirmed that the enantiomers induce differential effects with regard to transcription.

Conclusions

These data demonstrate that with regard to interactions of low molecular weight agents with cells, **structural differences in stereochemistry can confer significant changes on the transcriptional level.**

We found that **enantiomers of a synthetic ceramide analogue induced different degrees of apoptosis.**
Microarray analysis revealed that differences in molecular mechanisms of enantiomers can be detected on the transcriptional level, **key players of apoptotic pathways are affected differently and apparently reflect pro-apoptotic potency.** The method thus allows fingerprinting of biological activities and clues to mechanisms of action.

Thus complex biological effects due to different stereochemistry are efficiently detected by highly parallel transcription analysis affording an excellent starting point for further targeted analyses.
As for synthetic ceramide analogs the data show that tiny structural changes may be crucial and transcriptomics appears valuable for compound characterisation and optimisation of efficacy.