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Flow cytometric and microarray analyses for the investigation of apoptosis inducing substances in tumor cells

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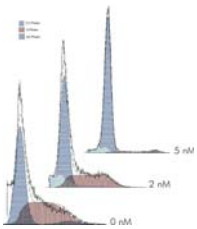
Abstract

In previous screening tests, ratjadone revealed a growth inhibitory effect on bacteria, yeast and human cancer cells. Ratjadone is also known to inhibit the cell cycle of human cancer cells by arresting the cells in the G1 Phase. For analysing changes in gene expression associated with ratjadone treatment, we performed DNA microarray analyses on ratjadone treated HEPG2 cells. 20 $\cdot 10^6$ cells were treated with 10 nM ratjadone and the RNA was extracted after 24 and 48 hours. Labelled cDNA prepared from total RNA was hybridized on a PIQOR TM cell death human microarray. The results show active apoptotic pathways. These results were compared to flow cytometric apoptosis analysis which shows after 24 h an average apoptotic rate of 27 % of the cell population and after 48 h an average apoptotic rate of 48 %.

Introduction

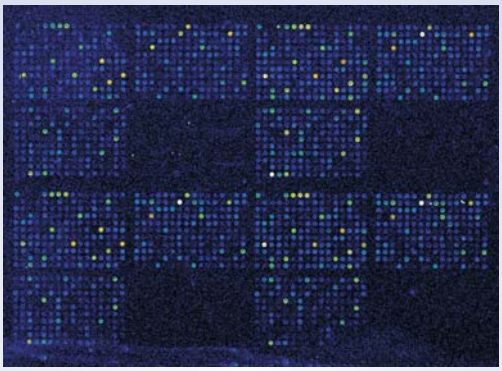
Ratjadone, isolated from the myxobacterium *Sorangium cellulosum* by Schummer et al in 1995, belongs to the family of orphan ligands. In previous screening tests, ratjadone revealed a growth inhibitory effect against bacteria, yeast and human cancer cells [Schummer, 1995].



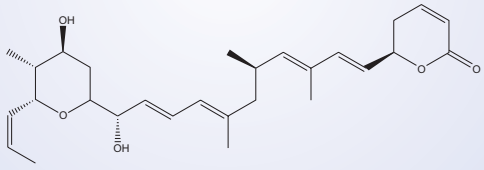
HepG2 cells after treatment with ratjadone at different concentrations; cell cycle analysis by DNA staining with propidiumiodid.

The cell cycle analysis showed that ratjadone intervenes in the cell cycle and arrests the cells in the G1-phase at remarkably low concentrations (nanomolar scale) [Burzlaff, 2003]. The gene regulation of ratjadone treated cells was analysised using DNA-microarray technology.

This results of an Apoptosis microarray, reveals an increase of the gene concentrations which are linked to apoptotic pathways of the tested cell line and an increase in cell cycle arrest relevant gene concentration. These results go can be confirmed by the flow cytometric results which show an increase of cells with phatidylserin located on the outside of the cell. The amount of these apoptotic cells nearly doubles from the 24 h to the 48 h sample.



Ratjadone



6-[11-Hydroxy-11-(4-hydroxy-5-methyl-6-propenyl-tetrahydro-pyran-2-yl)-3,5,7-trimethyl-undeca-1,3,7,9-tetraenyl]-5,6-dihydro-pyran-2-one

Materials and Methods

Cell lines: The human hepatoma cell line HEPG2 was obtained by German Collection of Microorganisms and Cell Cultures, DSMZ (catalog number ACC 180).

Medium: DMEM supplemented wih 10% newborn bovine serum. Antibiotic or Antifungal agents were not used, to avoid the potential effect of these agents on gene expression and flow cytometric assays.

Flow cytometry: About 1 $\cdot 10^5$ HEPG2 cells were treated with 10 nM ratjadone for 24 and 48 hours. Afterwards the cells were stained with AnnexinV/FITC to detect the apoptotic population and with Propidiumiodide as counterstain to determine necrotic cells. For cell cycle analysis the cells were stained only with Propidiumiodide and the DNA content of each cell was determined. All analyses were performed on a Beckman Coulter Epics XL-MCL Cytometer.

Microarrays: The RNA for analysis of gene expression after ratjadon treatment can be easily obtained using the trizol method. Subsequently a DNase I digest of the isolated RNA is performed to exclude binding of genomic DNA to the immobilized probes on the array. During reverse transcription the purified total RNA is converted into first strand cDNA using olgo dT primers. Thereby the cDNA is directly labeled with different fluorescent dyes. The differently labeled cDNAs of both samples are hybridized simultaneously in one experiment to the same array. After hybridization, the unbound and unspecifically fixed cDNA is removed by stringent washing from the array. Subsequently the array is scanned for the two distinct fluorescent dyes (Cy3 and Cy5) of the cDNA derived from treated cells and non-treated cells.

Cell Cycle

Fig. 1:down-regulated gene sets after 24 and 48 h.

Fig.2: up-regulated gene sets after 24 and 48 h.

Fig. 1 and 2 show the number of up- and the down-regulated genes in HEPG2 cells. The number of regulated gene, which were clearly up- or down-regulated, was over 100 after 24 and 48 hours. The regulated genes show a broad range, but many of the regulated genes in the 48 hours sample were related to an active apoptotic pathway. Although some up regulated genes belong to inflammatory response. The flow cytometric analysis showed after 24 hours only about 27% of the cells in an apoptotic state while after 48 hours this level increases to 48%.

Many of the up regulated genes were catalogued into the stress response/cell cycle/apoptosis related genes.

Fig. 3 shows the p53 pathway. The concentration of the cell cycle regulation gene CDKN1A increases 1,8 fold in the 24 hours sample and 20 fold in the 48 hours sample.

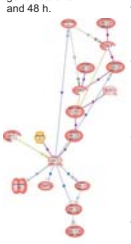


Fig. 3: Hypothetical signal transduction of ratjadone treated HepG2 cells. Numeral represents the ratio of treated cells. P53 pathway

Apoptosis Pathways

The BCL2 activity is greatly up regulated after 48 hours.The APAF1 regulation is greatly decreased and therefore many of the caspases show a decreased regulation. Caspase 7 shows an immense increase after 48 h which leads to apoptosis.

Fig. 4: Hypothetical signal transduction of ratjadone treated cells. Numeral represents the ratio of treated cells. Apoptotic pathway

In Fig. 5 the TNF pathway is described. TNF is a pro inflammatory zytokine. It's 18,3 fold up-regulated in the 48 hours sample leading to the up-regulation of TNFRSF1, TRADD and FADD which are leading towards apoptosis [Schulze-Osthoff, 1998]. Although CASP8 which is the intermediate step before the apoptotic reaction shows no up-regulation.

Fig. 5: Hypothetical signal transduction of ratjadone treated HepG2 cells. Numeral represents the ratio of treated cells. TNF pathway

Array Data processing

Signal quantification, background adjustment and other analysis were performed using the Pathwaystudio Software from Ariadne Genomics. Genes were only considered for further analysis, if their signal showed a low significance. Pair-wise comparison analysis was performed between Ratjadone treated and control cells. The signal ratio was calculated for each probe using the following formula: [Signal intensity in control cells] / [Signal intensity in Ratjadone treated cells]. If the value was below 1 then the following string was attached to the formula to show the correct ratio: 1 / [Signal intensity in control cells] / [Signal intensity in Ratjadone treated cells]·(-1). Probe sets with a value greater then 1,0 were considered up-regulated and those with a value below 1 were considered down-regulated. The signal transduction relationship between the up- and down-regulated genes were graphically displayed using the PathwayStudio Software from Ariadne Genomics with the ResNet 3.0 database installed.

Conclusion

From the gene expression analysis of the ratjadone treated cells several changes in comparison to the untreated cells are seen which suggests the induction of apoptosis. This includes the upregulation of BCL2, Caspase 7, TNF and the downregulation of APAF 1. The apoptosis of the ratjadone treated cells could be confirmed by flow cytometric analysis through the annexin V staining which showed after 24 h an average apoptotic rate of 27 % and after 48 hours an average apoptotic rate of 48 % in the cell population. The cell cycle arrest in the G1/S phase which has been observed in flow cytometry [Burzlaff, 2003] can also be detected in the chip experiment because the cell cycle regulation gene CDKN1A increased 20 fold at 48 hours.