Author's response to reviews

Title: A hepatoprotective Lindera obtusiloba extract suppresses growth and attenuates insulin like growth factor-1 receptor signaling and NF-kappaB activity in human liver cancer cell lines

Authors:

Christian Freise (christian.freise@charite.de)
Martin Ruehl (martin.ruehl@charite.de)
Ulrike Erben (ulrike.erben@charite.de)
Ulf Neumann (uneumann@ukaachen.de)
Daniel Seehofer (daniel.seehofer@charite.de)
Ki Young Kim (kkyoung@wonkwang.ac.kr)
Wolfram Trowitzsch-Kienast (trowitzsch-kienast@t-online.de)
Thorsten Stroh (thorsten.stroh@charite.de)
Martin Zeitz (martin.zeitz@charite.de)
Rajan Somasundaram (rajan.somasundaram@charite.de)

Version: 2 Date: 18 April 2011

Author's response to reviews: see over
Manuscript revision

Dear Editor,

please find attached our revised manuscript entitled “A hepatoprotective *Lindera obtusiloba* extract suppresses growth and attenuates insulin like growth factor-1 receptor signaling and NF-kappaB activity in human liver cancer cell lines” by authors Christian Freise, Martin Ruehl, Ulrike Erben, Ulf Neumann, Daniel Seehofer, Kiyoung Kim, Wolfram Trowitzsch-Kienast, Thorsten Stroh, Martin Zeitz and Rajan Somasundaram.

Hoping to meet the reviewer’s objections, we answered the questions point-to-point (see below) and carefully adapted the manuscript.

Response to the reviewer:

We would like to thank the reviewer for their efforts and some valuable suggestions and hints. We carefully read and discussed the reviewer’s reports and like to give a point-by-point response to the concerns as requested. Changes in the manuscript are highlighted blue.

Reviewer #1:

1. The figures of the Western Blot are unclear, and based on these figures, it is difficult to interpret results. Better representation with good figures is needed. Further, no quantitative estimation of band expression has either been provided.

The reviewer picks up a similar problem as the editorial team did prior to sending out the manuscript for review. Since several probes and substances were tested simultaneously, single bands in the blots were not directly neighboured in each case. Thus, bands were cut and positioned next to each other while preparing the figure. Blots in figure 2 are shown as a representative only. As proposed by the reviewer we therefore integrated parts of the quantitative data from table 3 into figure 2. Nevertheless, to give even more detailed information we also show the data in table 3.
2. Why didn’t the authors provide any data of another arm of control preferably taken from the culture of normal liver cells? A comparison with say, PBMC, could have enriched the findings and thus provide more convincing data. The data on normal basal level of activity of enzymes is important to know to ascertain if it is actually increased or decreased than the basal level.

This is a very good point of discussion. We intensely discussed this fact in the beginning of our experiments. Based on earlier reports we decided not to use other cell types like PBMC because the hepatoma cell line HepG2, a standard cell line for liver cytotoxicity studies (Kim et al. `JNK- and p38 kinase-mediated phosphorylation of Bax leads to its activation and mitochondrial translocation and to apoptosis of human hepatoma HepG2 cells´. J Biol Chem 2006;281:21256–65), is routinely used to screen candidate pharmaceuticals for intrinsic hepatotoxicity.

Further, as also mentioned in point 4, one aim of our study was to distinguish between effects of the extract on well and poorly differentiated HCC cells such as Hep3B and SK-Hep1 cells, respectively. Nevertheless, we re-emphasized this in the manuscript.

3. For the transfection experiment of HCC, it was stated that a plasmid was used, but description of the type/orientation of the plasmid was not provided. There was no mention of the transfection efficiency of the plasmid either.

In this context we added two additional citations by our co-author Thorsten Stroh who established the electroporation method in the lab and also used the NF-κB reporter plasmid in earlier studies. The citations along with a note regarding the transfection efficacy (~75%) were inserted in the respective chapters in the methods section.


4. Several cell lines were mentioned, like HepG2 (ATCC HV-8062), Hep3B (ATCC HV-8064), Huh-7 (JCRB 0403; Tokyo, Japan) and SK-Hep1 (ATCC HTB-52), but the data provided appeared to deal mainly with Hep3B and SK-Hep1 for Western Blot analysis. What’s about the other cell lines?. What was the rationale behind studying different aspects using different cell lines? Why the authors preferred to study one parameter in one cell line and another in another cell line?

In our study we investigated anti-tumor effects of the L.obtusiloba extract using human HCC cell lines that represent diverse stages of differentiation and aggressiveness. Our first experiments regarding proliferation, apoptosis and invasion revealed that notably the aggressive and poorly differentiated SK-Hep1 cells display a high invasion and migration (see also figure below which shows unpublished data of a wound-healing assay). Here, the effects of the extract are more pronounced than in the other three less aggressive cell lines.
Effects of *L. obtusiloba* extract on migration of HCC cell lines. For migration experiments 5x10^4 cells of each HCC cell line were seeded into 24-well plates. After reaching confluency artificial wounds were created using a sterile yellow 100 µl pipette tip. After washing with PBS remaining cells were treated with or without 100 µg/ml *L. obtusiloba* extract. After 0, 8 and 24 h migrated cells were photographed using a digital camera coupled to a phase contrast microscope. Wound diameters were analyzed using Image J (version 1.41; National Institutes of Health, Bethesda, MA) software.

In our manuscript we set a clear focus on aspects affecting angiogenesis and tumor cell invasion and aimed to understand mechanisms of action of *L. obtusiloba* extract. Thus, to investigate signaling pathways involved with western-blots specific for (p)IGF-1R and the activation states of its target proteins we on the one hand focused on the poorly differentiated SK-Hep1 cells and on the other hand on Hep3B cells as one out of the three less aggressive cell lines used as shown by invasion and migration assays (see fig. above). We made this point more clear in the manuscript.

5. Providing supportive evidence of microscopic study indicating profibrotic activity or conformational change of cells should be more convincing.

This is an important aspect, but not focus of our work. In the context of studies on epithelial to mesenchymal transition this would be a good supplement to the data. But in our case no such morphological changes were observed (microscopic control during routinely cell culture or treatment of the cells) or were intended, respectively.

6. There has been no or very little mention of the ingredients or main bioactive components present in the aquatic extract of *L. obtusiloba*, and their possible action. Suitable references of this aspect should be added.

The reviewer is right. Indeed, this aspect is under intensive investigation by us and our collaboratives. Currently, a manuscript dealing with the technical aspects - such as HPLC analysis and NMR-studies - of the isolation and structural identification of a compound from *L. obtusiloba* extract is in preparation. Namely (+)-episesamin was found and preliminary data suggest comparable anti-fibrotic activity of this compound like *L. obtusiloba* extract. We added this point to the discussion section.

Minor points:
1. IC 50 values not mentioned, and how the exact dose and time period was selected should be stated briefly but more clearly.

The IC50 values for the HCC cell lines were assessed once during the proliferation assays (see results section in the chapter dealing with proliferation). This concentration ~100 µg/ml was used in all adjacent experiments. The initial required range of concentration of the extract for the proliferation studies was educed from our preceding publications dealing with anti-fibrotic and anti-inflammatory activities of *L. obtusiloba* extract. There, using hepatic stellate cells and (pre)adipocytes a concentration of 135 µg/ml worked well. The same holds for the time periods of the experiments which were also adopted from former studies. We now mention this in the results section of the manuscript.

2. There are a few typos: P.3, TGF #, Page 4, ...7,9 and 10,4 ..? P6, ..ovar...

We corrected the text passages accordingly.

3. Conventionally, cell nos. in Y axis are represented as 1x103, 2x103 etc.

We changed the formation of the given cell nos. for the Y axis in Fig. 1C accordingly.

Reviewer #2:
1) Results - Fig.2: Authors applied β-actin as a house-keeping protein for Western-blot. However, band of β-actin was not shown. Authors should add the band of β-actin in Fig. 2.

We added the respective bands of β-actin to figure 2.

2) Discussion - Authors should discuss about the active compounds in *Lindera obtusiloba* extract, if possible. There are several reports about the isolation and structural characterization from *Lindera obtusiloba* extract.

*L.obtusiloba* extract contains lignans and butanolides. This aspect is under intensive investigation by us and our collaboratives. Currently, a manuscript dealing with the technical aspects - such as HPLC analysis and NMR-studies - of the isolation and structural identification of a compound from this extract is in preparation. Namely (+)-episesamin was found and preliminary data suggest similar anti-fibrotic activity of this compound like *L.obtusiloba* extract. As suggested, we added a small chapter to the discussion section of the manuscript.

Minor comments:

Abstract

Methods (Line 3) TGF-#-expression # TGF-#-expression

The manuscript was changed accordingly.

We thank the reviewer for the efforts and the really helpful comments and for considering our manuscript to be published in your Journal *BMC Complementary and Alternative Medicine*.

Sincerely,

Rajan Somasundaram, MD
Dept. of Gastroenterology and Hepatology,
Charité, Campus Benjamin Franklin
Hindenburgdamm 30
12203 Berlin, Germany
Tel: (+4930)8445 4017, Fax: (+4930)8445 4017
E-Mail: rajan.somasundaram@charite.de