Can we study biological behaviour of bilirubin photoproducts?


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What do bilirubin photoproducts originate from?

- Phototherapy (PT) is golden standard in treatment of neonatal jaundice and Crigler-Najjar syndrome since 1950's (Dobbs & Cremer, 1975; Hansen, 2010)

- Photochemical reactions which leads to formation of bilirubin photoproducts (PP):
  - Photooxidation
  - Photocyclization
  - Configurational photoisomerization

- PT helps to reduce prevalence of kernicterus and to decrease need of exchange transfusion use

- Side effects of PT (Xiong, 2011):
  - Aggressive PT may increase neonatal mortality
  - Changes in thermal environment of infants
  - Bronze baby syndrome
  - Alteration of normal dark-light circadian rhythms
  - Degradation of bilirubin may increase oxidative stress → during PT formation of highly reactive bilirubin oxidation products (BOXes) may occur
Why to study bilirubin photoproducts?

- There are virtually no reports of biological effects of PP, main reason lies in methodological issues.
- Thus we want to study whether bilirubin PP per se exert any protective effects.

**Major aims of our project:**
1. To establish robust HPLC method for determination of bilirubin PP.
2. To isolate and separate PP for *in vitro* experiments.
3. *In vitro* experiments on SH neorublastoma cell line exposed to UCB/PP.
1) Phototherapeutic device in our lab

- We use phototherapeutic lamp Dräger Photo-Therapy 4000
- This system has 4 blue and 2 white lamps, effective intensity of blue ones is 2.4 mWcm$^{-2}$, emission maximum of lamp is 460 nm
- For optimal preparation of PP samples we tested:
  - distance between sample and the light source
  - length of irradiation
- Ten cm distance was found the most effective with no generation of biliverdin
- Optimal length of light exposure was established to 30 min.
- Due to certain limitations of the current system we are about to use phototherapeutic device developed by Vreman et al. (Stanford Univ.)
1) Determination and stability of bilirubin photoproducts

- Two groups established HPLC method for determination of bilirubin PP:
  - McDonagh et al. (1989)
  - Onishi et al. (1979)

  McDonagh’s method: SP: C18 column, isocratic elution by MP: 0.1 M di-n-octylamine acetate in methanol and water (92/8 v/v), bilirubin PP verified by NMR (1982)

  Onishi’s method: SP: Zorbax ODS column, gradient of acetonitrile (5-20% v/v), MP: mixture of acetonitrile, 0.01 M sodium phosphate buffer, pH 6.8, dimethylformamide

- Half-time of bilirubin PP:
  - 15 h. for lumirubin (Okada, 2004), generally believed to be a stable compound
  - 65 min. in dark, 20°C for lumirubin and 438 min. in dark, 37°C for ZE- and EZ- bilirubin (Onishi, 1986) were reported to revert to maternal substrate

- Both methods have limitations and can not be used for isolation of bilirubin PP
2) Isolation of single bilirubin photoisomers

- We finally adopted a method according to Spivak & Carey, 1985 (SP: C18 column, MP: gradient elution of 1% ammonium acetate buffer pH 4.5 and methanol, gradient of MeOH 60-100% in 20 min.) which was primarily invented for determination of bilirubin conjugates and may be used for MS.

- In addition, resolution of peaks is better than when using McDonagh's method.

- Since E-isomers are thermolabile, both isolated ZE-/EZ- PP were rapidly transformed whole back to native UCB; surprisingly, also lumirubin was found extremely unstable.

- To conclude, due to their instability single bilirubin PP can not be used for *in vitro* studies.
2) Isolation of single bilirubin photoisomers
3) *In vitro* experiments

1. Bilirubin PP stability aspect
   1. Due to their instability, only experiments with bilirubin PP mixture possible
2. Determination of relative neurotoxicity of UCB vs. PP *in vitro*
   1. Human neuroblastoma cells SH-SY5Y incubated with photo-irradiated and non-irradiated UCB in the presence of physiological concentrations of HSA
   2. Assessment *in vitro* cytotoxicity in the presence of photoproducts
   3. Determination of BOXes and studies of their potential biological effects
   4. Analyses on the effect of bilirubin PP on cell cycle and mitochondrial metabolism
3. Determination of the effect of photoproducts on the binding of UCB HSA (planned in collaboration with Claudio)
3) **In vitro experiments**

The effects of photo-irradiated UCB on human neuroblastoma cell line SH-SY5Y

- UCB dissolved in DMSO - final calculated UCB concentration = 100, 50, 10 and 0 μM (final concentration of DMSO was 0.6%)
- One half of UCB sample irradiated (10 cm distance, 30’), then pigments added to the cells
- Time-points of incubation were set 1h, 4h and 24h
- UCB/PP measured in media at time 0 and 24h of incubation (McDonagh’s and Zelenka’s method)
3) *In vitro* experiments

Photo-irradiated UCB before adding to cells

\[ c=10 \text{ mM} \]

Photo-irradiated UCB after incubation for 24h with cells, the same concentration
3) *In vitro* experiments

- Measurement of real concentrations of UCB in solutions before and after incubation (24h) with cells (Zelenka, 2008)
- Surprisingly, cells exposed to photo-irradiated UCB were less viable compared to non-irradiated cells
- At 1h and 4h, no change in cell viability was observed

*MTT test*

*CV test*

* P < 0.05 vs. Control

CV = crystalline violet staining
3) *In vitro* experiments

**Decrease in UCB concentrations after light exposure**

- After 24h of incubation
- Decrease of UCB concentrations 14-45%

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<th>c [µM]</th>
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3) *In vitro* experiments

**Decrease in UCB concentrations after light exposure**

- Efficiency of photo-irradiation differs between 15 and 25%
Bilirubin oxidation products (BOXes)

- During oxidative stress, reactive oxygen species can oxidize UCB onto BOXes (biopyrrins and BOX A/B)
- BOXes (A and B) were reported in cerebrospinal fluid during subarachnoid and intracerebral hemorrhage (Clark, 2008)
- Theoretically, it is highly likely that these BOXes can originate also during phototherapy
- Overproduction of BOXes, serving as markers of increased oxidative stress, may be the reason of observed photo-irradiation effects on cell viability
Determination of BOXes

- We established method for determination of BOXes, which is a modification of Wurster's (2008) and Kranc's methods (2000) - NMR and MS data also available here
- Both used SP: C18 column and as MP mixture of water/acetonitrile in different ratios
- We use SP: C6-phenyl column and as MP: 70/30 (v/v) water/acetonitrile
- BOXes are believed to be very unstable, however in our observations:
  - BOXes are stable for 4 hrs and can be detected even 2 days after preparation
  - Thermostability study also performed and BOXes do not seem to be affected by high temperature range (from -32°C to 50°C)
- MS analyses of BOXes confirmed their identity
- First analyses to confirm whether BOXes can originate during light exposure have been performed
Determination of BOXes

Boxes stability assay

- **Time 0h**
  - BOX A: 229
  - BOX B: 140

- **Time 2h**
  - BOX A: 224
  - BOX B: 129

- **Time 4h**
  - BOX A: 226
  - BOX B: 130
Determination of BOXes

HPLC of BOXes standard detection 310 nm

BOX A and B ??

Use of quadrupole analyser

HPLC analysis of PI detection 453 nm

UCB

lumirubin

ZE-EZ-PI
Determination of BOXes

Simultaneous HPLC of BOXes and PI
detection 310 nm

- BOX A and B ???
- luminarin
- ZE-/EZ-PI
- UCB
Conclusions

1. Two methods for bilirubin PP determination were established:
   1. McDonagh’s method
   2. Spivak & Carey method – this method could be also used for isolation of photoproducts and for determination of BOXes (must be studied in more detail)

2. We set optimal conditions of photo-irradiation for our experimental studies

3. Method for BOXes determination was also established

4. In contrast to literature data, lumirubin seems to be very unstable, whereas stability of BOXes is good enough for research purposes

5. Photo-irradiated UCB seems to be more harmful for neuroblastoma cells than non-irradiated UCB – effect of PP/BOXes/Bf alteration???
Future plans

- study other biological effects of photoproducts in vitro
- study biological effects of BOXes in vitro
- validate HPLC method for determination of photoproducts and BOXes within one analysis
- develop alternative ways of isolation of bilirubin PP
- study the role of Bf during phototherapy
- perform in vivo experiments (Gunn rats/mice?)
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