New Biomarkers to Monitor the Dietary Consumption of Isothiocyanates

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Introduction: Isothiocyanates in the diet?

Glucosinolate (GL) + Glucose + KHSO₄ → Isothiocyanate (ITC)

**Glucosinolate (Precursor)**
- Glucotropaeolin
- Gluconasturtiin
- Sinigrin
- Glucoraphanin

**Isothiocyanate**
- Benzyl-Isothiocyanate (BITC)
- Phenethyl-Isothiocyanate (PEITC)
- Allyl-Isothiocyanate (AITC)
- Sulforaphane (SFN)

**Food Sources**
- Cabbage, garden cress, Indian cress
- Watercress
- Cabbage, horseradish, mustard
- Broccoli sprouts, broccoli, Brussels sprouts, cabbage
INTRODUCTION

Are isothiocyanates (ITC) good for you?

- ITCs found in cruciferous vegetables have demonstrated cancer preventive activity in animals, and increased dietary intake of ITCs has been shown to be associated with a reduced cancer risk in humans.
- ITCs exert their cancer chemopreventive action by modulating the activities of phase I and phase II drug metabolism enzymes.
- ITCs and their thiol conjugates inhibit the cell cycle and cause apoptotic cell death, possibly by activation of vital signal transduction pathways.
- ITC-protein adducts account for 87% of total cellular ITC-uptake after 4h of treatment. The time course of this protein binding correlated well with the inhibition of proliferation and the induction of apoptosis. This suggests that cellular protein adducts of ITC may be an early event for apoptosis induction.

OBJECTIVES

• Most epidemiological studies on the relation of diet and cancer have relied on the information collected with questionnaires to monitor the food intake. This is not very accurate.

• Urinary metabolites might provide the exposure history of the last 24h, if the urine of the full next day is collected (spot urine samples are not a reliable exposure marker). However, this is not feasible in large epidemiological studies. Furthermore the mercapturic acids of ITC in urine are not stable.

• Stable biomarkers are needed which reflect a larger time span of the ITC-exposure history. Hemoglobin adducts and albumin adducts have a lifetime of 120 days and a half-life of 20-25 days, respectively

• Thus, we propose to develop a method to determine stable reaction products of ITCs with albumin and hemoglobin in human.

• The method will be tested on human subjects after controlled cruciferous vegetables intake.

• The applicability of the method for epidemiological studies will be applied to a group of 85 people.
Lifetime of Biomarkers

The paradigm of biomonitoring

- **EXTERNAL DOSE**
  - Air, water, food
    - Individual Susceptibility

- **INTERNAL DOSE**
  - Urinary metabolites
    - Individual Susceptibility

- **BIOLOGICALLY EFFECTIVE DOSE**
  - Protein-, DNA-Adducts
    - Individual Susceptibility

- **EARLY BIOLOGICAL EFFECTS**
  - Micronuclei, p53-mutations
    - Individual Susceptibility

- **LATE BIOLOGICAL EFFECTS**
  - Altered cell structure/function
    - Individual Susceptibility

- **TUMOR**
Urinary metabolites of Isothiocyanates

Glutathione (=GSH) + R-N=C=S → ITC-GSH

ITC-GSH → ITC-Cys-Gly → ITC-Cys

AcHN

ITC-NAC mercapturic acid

R-N=C=S + NH-S-R1 + 2 x 1,2-Benzenedithiol → 2 x 1,3-Benzo-dithiole-2-thione + 2 x R-NH2 + R1-SH

In vitro

Amino acids → ITC → Amino acid-adducts

Hemoglobin (Hb) → ITC → Hb-adducts

Albumin → ITC → Albumin-adducts

Digestion with pronase, + IS

Amino acid-adducts

In vivo

Hemoglobin → Digestion with pronase, + IS → Unmodified and adducted amino acids

Enrichment by solid phase extraction, or HPLC

Albumin

Amino acid-adducts

Internal Standards (IS): ITC-[\(^{13}\)C\(_6\)^{15}\)N\(_2\)]Lys

LC-MS/MS-analyses: ITC-Lys, ITC-[\(^{13}\)C\(_6\)^{15}\)N\(_2\)]Lys;

Synthesis: Reaction products of isothiocyanates with amino acids

Isothiocyanate adducts with cysteine = Trojan Horse

EXPERIMENTAL PROCEDURE

Determination of albumin adducts of isothiocyanates

ALBUMIN

in vitro or in vivo modified

IS1:BITC-[^{13}C_6^{15}N_2]-Lys
IS2:PEITC-[^{13}C_6^{15}N_2]-Lys
IS3:AITC-[^{13}C_6^{15}N_2]-Lys
IS4:SFN-[^{13}C_6^{15}N_2]-Lys

Digestion with pronase

Unmodified and adducted amino acids, IS1-IS4

Enrichment by solid phase extraction

Amino acid-adducts, IS1-IS4

LC-MS/MS-ANALYSES

BITC-Lys, PEITC-Lys, AITC-Lys, SFN-Lys, IS1-IS4

EXPERIMENTAL PROCEDURE

LC-MS/MS analyses of the albumin adduct of SFN

Control

Mouse exposed to PEITC-AcCys

Human exposed to watercress

PEITC-[\textsuperscript{13}C\textsubscript{6}\textsuperscript{15}N\textsubscript{2}]Lys

PEITC-Lys

RESULTS: Albumin (Alb) and hemoglobin (Hb) adducts of ITCs in a human subject eating garden cress and watercress on day 0 and eating regularly broccoli

<table>
<thead>
<tr>
<th>Days</th>
<th>1</th>
<th>30</th>
<th>121</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEITC–Lys pmol/mg Alb</td>
<td>2.365</td>
<td>0.823</td>
<td>0.046</td>
</tr>
<tr>
<td>BITC–Lys pmol/mg Alb</td>
<td>1.011</td>
<td>0.480</td>
<td>0.029</td>
</tr>
<tr>
<td>SFN–Lys pmol/mg Alb</td>
<td>2.203</td>
<td>3.306</td>
<td>1.372</td>
</tr>
<tr>
<td>PEITC–Lys pmol/mg Hb</td>
<td>0.050</td>
<td>0.020</td>
<td>a)</td>
</tr>
<tr>
<td>BITC–Lys pmol/mg Hb</td>
<td>0.023</td>
<td>0.021</td>
<td>a)</td>
</tr>
<tr>
<td>SFN–Lys pmol/mg Hb</td>
<td>0.140</td>
<td>0.203</td>
<td>a)</td>
</tr>
</tbody>
</table>

Albumin-adduct kinetics after a Single dose of BITC and/or PEITC

$\frac{1}{2}t_{1/2}=21.3 \text{ and } 23.2 \text{ days resp.}$

a) Below limit of quantitation


RESULTS: Albumin adducts of ITCs in a group of 85 Italian male smokers from Torino, Italy

The feasibility of the method was tested on biological samples collected previously for a diet-study. The volunteers were randomly assigned to three groups:

1. Group A (n=29): the diet was rich in flavonoids, but not supplemented.
2. Group B (n=29), the diet was a normal iso-caloric diet with an adequate administration of fruit and vegetables.
3. Group C (n=27): the diet was based on supplementation of the normal diet with additional flavonoids in the form of green tea and soy products.

Blood samples were collected at the beginning (0) and after 4 weeks (4).

After enzymatic digestion of albumin we determined the adducts of the isothiocyanates (ITC)s with lysine (Lys) using LC-MS/MS and isotope dilution method

## RESULTS

**Albumin adduct levels of ITCs in the different diet groups**

<table>
<thead>
<tr>
<th>Set</th>
<th>Subjects (n)</th>
<th>AITC-Lys</th>
<th>BITC-Lys</th>
<th>PEITC-Lys</th>
<th>SFN-Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>85</td>
<td>4.7%</td>
<td>48%</td>
<td>6%</td>
<td>25%</td>
</tr>
<tr>
<td>4 weeks</td>
<td>85</td>
<td>2.4%</td>
<td>35%</td>
<td>11%</td>
<td>24%</td>
</tr>
</tbody>
</table>

**Correlation of the albumin adduct levels of ITCs after 4 weeks**

<table>
<thead>
<tr>
<th></th>
<th>AITC-Lys-4</th>
<th>BITC-Lys-4</th>
<th>PEITC-Lys-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>BITC-Lys-4</td>
<td>0.238* (0.028)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEITC-Lys-4</td>
<td>0.474** (&lt;0.001)</td>
<td>0.104 (0.342)</td>
<td></td>
</tr>
<tr>
<td>SFN-Lys-4</td>
<td>0.328** (0.002)</td>
<td>0.205 (0.060)</td>
<td>0.480** (0.001)</td>
</tr>
</tbody>
</table>

The levels of BITC-Lys and SFN-Lys and the total cruciferous vegetable levels determined with questionnaire were compared among the 3 diet groups and the two different time points (beginning of the study = set 0, and after 4 weeks= set 4). The significant differences found between 2 groups using the Mann-Whitney test were marked with a pair of equal numbers 1-8: \(^1\text{-}6\) p < 0.05; and \(^7\text{-}8\) p < 0.1. In addition the percentage of subjects found positive for all adducts (AITC-Lys, BITC-Lys, PEITC-Lys, SFN-Lys) were listed.

<table>
<thead>
<tr>
<th>Set</th>
<th>Group (n)</th>
<th>AITC-Lys</th>
<th>BITC-Lys</th>
<th>PEITC-Lys</th>
<th>SFN-Lys</th>
<th>BITC-Lys [fmol/mg] Median (25(^{th}),75(^{th}),90(^{th}))</th>
<th>SFN-Lys [fmol/mg] Median (25(^{th}),75(^{th}),90(^{th}))</th>
<th>Total cruciferous vegetables g/day Median (25(^{th}),75(^{th}),90(^{th}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 A</td>
<td>n=29</td>
<td>3.4%</td>
<td>69%</td>
<td>10.3%</td>
<td>13.8%</td>
<td>5.39 (^1,7) (0, 7.07, 7.98)</td>
<td>0 (^3) (0, 0, 71.7)</td>
<td>4.9 (^8) (3.5, 8.7, 12.2)</td>
</tr>
<tr>
<td>4 A</td>
<td>n=29</td>
<td>6.9%</td>
<td>34.5%</td>
<td>17.2%</td>
<td>31%</td>
<td>0 (^7) (0, 6.18, 8.30)</td>
<td>0</td>
<td>68.8 (^5) (29.3, 110, 145)</td>
</tr>
<tr>
<td>0 B</td>
<td>n=29</td>
<td>6.9%</td>
<td>55.2%</td>
<td>0%</td>
<td>17.2%</td>
<td>5.86 (^2) (0, 8.26, 12.7)</td>
<td>0 (^4) (0, 0, 98.9)</td>
<td>2.4 (n=17) (^8) (1.5, 8.9, 12.7)</td>
</tr>
<tr>
<td>4 B</td>
<td>n=29</td>
<td>0%</td>
<td>37.9%</td>
<td>0%</td>
<td>10.3%</td>
<td>0 (0, 7.95, 11.3)</td>
<td>0 (0, 0, 38.7)</td>
<td>6.3 (n=17) (^5,6) (3.0, 16.7, 32.7)</td>
</tr>
<tr>
<td>0 C</td>
<td>n=27</td>
<td>3.7%</td>
<td>18.5%</td>
<td>7.4%</td>
<td>44.4%</td>
<td>0 (^1,2) (0, 0, 9.56)</td>
<td>0 (^3,4) (0, 43.3, 196)</td>
<td>4.5 (n=16) (2.0, 8.7, 18.1)</td>
</tr>
<tr>
<td>4 C</td>
<td>n=27</td>
<td>0%</td>
<td>33.3%</td>
<td>14.8%</td>
<td>29.6%</td>
<td>0 (0, 5.74, 9.05)</td>
<td>0 (0, 21.3, 169)</td>
<td>38.7 (n=16) (^6) (26.5, 73.4, 109)</td>
</tr>
</tbody>
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## RESULTS
Correlation of the albumin adduct levels of ITCs after 4 weeks

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## RESULTS

Comparison of the diet with the albumin adduct levels of ITCs

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<tr>
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<th>BITC-Lys-0</th>
<th>SFN-Lys-0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cruciferous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vegetables (4 weeks)</td>
<td>0.061</td>
<td>-0.090</td>
<td>0.203</td>
<td>0.264*</td>
</tr>
<tr>
<td></td>
<td>(0.639)</td>
<td>(0.485)</td>
<td>(0.114)</td>
<td>(0.038)</td>
</tr>
<tr>
<td>Total cruciferous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vegetables (4 weeks)</td>
<td>0.306*</td>
<td>0.190</td>
<td>0.403**</td>
<td>0.399**</td>
</tr>
<tr>
<td></td>
<td>(0.016)</td>
<td>(0.14)</td>
<td>(0.001)</td>
<td>(0.001)</td>
</tr>
</tbody>
</table>

CONCLUSIONS

1. Isothiocyanates (ITCs) (AITC, PEITC, BITC, SFN) react with lysine present in proteins such as albumin: ITC-Lys

2. Using LC–MS/MS, ITC–Lys could be determined in up to half of the study subjects.

3. Since cell protein adducts are involved in the chemopreventive effects of ITCs, blood protein adducts are probably not only a biomarker of exposure but also a potential surrogate marker for the effects of ITCs at the cellular level.

4. This new method will enable to quantify ITC adducts in blood proteins from large prospective studies about diet and cancer, overcoming the limitations of questionnaire estimates.
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