Research Article

Cytotoxicity of Maillard reaction products determined with a peptide spot library

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The reaction of reactive carbonyl compounds (RCCs) with lysine and arginine (Maillard reaction) is a common modification of proteins in thermally processed foods. In this study, the toxicity of Maillard reaction products (MRPs) formed from defined amino acids or dipeptides (bound to a cellulose membrane) with ribose, glycerinaldehyde or methylglyoxal was investigated. Murine RAW 264.7 macrophages were cultivated on the cellulose membrane and the effect of MRPs on cell viability was determined. The toxicity of MRPs was dependent on the RCC used and increased in the order of ribose < glycerinaldehyde < methylglyoxal. The dipeptides were more cytotoxic than the amino acids, with Lys–Lys MRPs being the most toxic of all tested MRPs. Cell numbers did not fall below the starting point, indicating that the MRPs rather inhibited proliferation than actually caused cell death. To develop an assay, in which whole membranes with multiple peptide spots could be tested simultaneously, we measured cell numbers on larger cellulose membranes using image analysis of the intracellularly formed formazan crystals. Although this method was technically feasable, it appears that uneven cell attachment on the membrane would require a way to detemine starting cell number by a non-destructive assay to yield more robust data.

Keywords: Cell viability / Glycation / Maillard products / Peptide library / Proliferation

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1 Introduction

The reactions of reducing sugars with the N-terminus and acid side chains of amino acids (the ‚Maillard reaction') is one of the most common post-translational modifications of amino acids, peptides and proteins, and was described by the French scientist Louis Camille Maillard already in 1912 [1]. Subsequent rearrangements, oxidations and dehydrations yield a heterogeneous group of mostly coloured and fluorescent compounds, termed Maillard reaction products (MRPs) or advanced glycation endproducts (AGEs). AGE formation has been observed *in vivo* on long-lived proteins such as collagen, eye lens crystalline and in pathological protein deposits such as β_2-microglobulin deposits in hemodialysis patients [2–4]. In Alzheimer's disease, AGEs have been identified in the two major characteristic insoluble protein deposits, *i.e.* in neurofibrillary tangles [5, 6], amyloid plaques [7–9] and Hirano bodies [10], most likely contributing to their insolubility and protease-resistance through intra- and intermolecular crosslinking [11, 12]. Biochemical and genetic evidence suggests that AGEs are involved in the etiopathogenesis or progression of various other age-related diseases most notably in complications of diabetes (through increased levels of glucose and methylglyoxal) and end stage renal failure hemodialysis (through impaired clearance of AGEs) [13–15].

AGEs *per se* can induce production of reactive oxygen species and cause energy depletion and subsequent cell death in an oxidant-mediated manner [16–18]. Furthermore, AGEs can bind to a specific cell surface receptor, the receptor for AGEs (RAGE) and activate a variety of downstream signalling cascades including a classical redox-sen-

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Abbreviations: AGE, advanced glycation endproducts; **DMF,** dimethylformamide; **MRP,** Maillard reaction product; **RCC,** reactive carbonyl compound

sitive pathway involving NADPH-oxidase [19, 20], the transcription factor NF-κB [21], subsequently leading to the induction of various NF-κB regulated pro-inflammatory genes including MCP-1, TNF-α, IL-6 and iNOS [22, 23].

MRPs are the food analogues of AGEs [24 – 26]. Although the chemistry is essentially the same, their formation takes place at higher temperatures such as during heating of foods such as bakery products and roasted meats, often under dehydrating conditions. MRPs are responsible for aroma and flavour formation in cooked, roasted and baked food, and these compounds are under intensive investigation by the food industry to provide tailor-made aromas for processed foods. In recent years a dicussion has been arisen, whether excess consumption of such dietary AGEs might be a risk to human health [25, 27, 28]. However, it is quite likely that food-derived MRPs can directly trigger inflammatory processes in the intestine, or – after resorption – can contribute to a systemic inflammatory response in the whole human body [29]. Macrophages which are important mediators of the innate immune response are of particular interest for MRPs induced pro-inflammatory responses. They are widely distributed in the intestine, modulating the intestinal immune response. In the pathogenesis of inflammatory bowel disease, a large number of activated macrophages can be detected in the intestinal mucosa, triggering an inflammatory response. Thus, particularly in inflammatory bowel disease, the activation of macrophages by MRPs could lead to an enhanced inflammatory reaction.

In addition to their pro-inflammatory actions, AGEs and MRPs have also been shown cause direct effects on cell viability. At low concentrations, AGEs cause cellular proliferation of neuroblastoma (Neuro2A) by increasing cyclin D(1)/cdk4 and decreasing the cdk inhibitor p16 expression, and promoting the transition of cells into the S-phase [30]. At high concentrations, AGEs cause significant cell death in a dose-dependent manner [16], which increases with the degree of AGE modification [18, 31]. AGEs also decrease cellular ATP levels and increase glucose consumption and lactate production [32]. However, it is not clear which of the many AGEs/MRPs identified so far can exert cytotoxic properties. Such information would be valuable to optimize cooking and roasting procedures to minimize the toxic and maximize flavoursome MRPs in a food preparation. MRPs are formed by a reaction between a reactive carbonyl compound (RCC) and one or maximal two reactive side chains or the α-amino group. However, liquid phase synthesis, deprotection and purification of a large number of MRPs might be a quite labour intensive task and – to our knowledge – has not been attempted so far in a systematic manner. We have attempted to narrow down the possible amino acid/RCC combinations and simplify the purification procedures by using amino acids and dipeptides bound to a solid support *via* their C-terminus using a peptide library.

Simultaneous synthesis of compounds on solid supports has been used to develop technologies that allow hundreds to thousands of compounds to be synthesized in parallel. These large collections of compounds (combinatorial libraries) have been shown to be useful to search for lead compounds of biotechnological and pharmacological interest. Combinatorial peptide libraries synthesized on paper sheets (SPOT libraries), initially introduced by Frank (GBF, Braunschweig), can also be used to address questions about structural and functional properties of proteins with respect to their molecular composition [33]. We have shown previously used a 20×20 dipeptide library and shown that the reactivity of the 20 proteinogenic amino acid side chains in the Maillard reaction can simultaneously investigated using a membrane-bound spot dipeptide library [34]. In a similar fashion, we have identified the amino acid specificity of various AGE-antibodies [35].

In this study, we have used the AGE/MRP modified spot mini-library in a cell-based cytotoxicity assay. We show that cells can be cultivated on the cellulose-based spot library and that the influence of MRPs acids on cell viability can be investigated.

2 Materials and methods

2.1 Materials

Cell culture flasks and 96-well plates were from Sarstedt (Adelaide, SA). Cell culture reagents were purchased from Invitrogen (Melbourne, VIC). All other chemicals were from Sigma – Aldrich (Castle Hill, NSW). A Wallac 1420 Victor3TM V (Perkin Elmer) fluorescence and absorbance reader was used for the microplate assays. Image analysis was performed with Image MasterTM 2D Platinum (Amersham Biosciences).

2.2 Synthesis of the amino and dipeptide spot library

The assembly of peptides forming an array of spots of membrane-bound peptides follows essentially the methods described by Frank [33], using an automated pipetting system (Auto-Spot Robot ASP 222, ABIMED, Langenfeld, Germany). All other steps were performed manually. The following side chain-protected Fmoc amino acid derivatives were used: Arg (Pmc) and Lys (Boc). ‚Amino-functionalized' membranes were prepared from Whatman 540 filter-paper sheets (9 cm × 3 cm) by esterification with activated Fmoc-βAla (2.5 mL/membrane of 0.2 M Fmoc-βAla 0.24 M *N*,*N*′ diisopropylcarbodiimide (DIC), 0.4 M *N*-methylimidazole in dimethylformamide (DMF), followed by Fmoc deprotection (15 mL 20% piperidine in DMF for 10 min). Spotwise distribution (0.2 mL) and coupling of a second βAla provides an anchor and at the same time an array of spots on which amino acid building blocks are added by cycles of coupling and Fmoc deprotection. Fmoc amino acid derivatives (0.25 M amino acid, 0.375 M

1-hydroxybenzotriazole and 0.3 M DIC in *N*-methyl-pyrrolidinone) were incubated for 30 min before dispensing aliquots on the membrane. Residual amino functions on and between the spots formed by incomplete coupling as well as underivatized hydroxyl groups on the cellulose membrane were capped by acetylation with 2% acetic anhydride in DMF. Following the final cycle of elongation, the N-terminus was set free. Deprotection of the amino acid side chains was achieved by treating the membranes in 50% TFA, 45% dichloromethane, 3% triisobutylsilane and 2% water for 2 h, and by careful washing in DMF and ethanol, while the amino acids or peptides remained attached to the membrane.

2.3 Modification of amino acids and peptides with RCCs

The cellulose membranes carrying the amino acid or dipeptide spots were washed three times with water, sterilized in 70% ethanol for 5 min, and dried in a sterile laminar air flow. For the preparation of MRPs, the membranes were cut in strips containing 6–8 spots and then incubated with ml solution of 0.5 M ribose, methylglyoxal or glyceraldehyde in PBS for 2 wk at 60°C under sterile conditions. The dipeptide/amino acid spot membranes were also incubated with PBS under the same conditions to serve as a negative control. Membrane strips (not containing any amino acids or dipeptides) were incubated with ribose, methylglyoxal, glyceraldehyde or PBS under the same conditions to serve as a further control. Finally, the solutions were removed and cellulose membranes were washed three times with distilled water and sterilized as described above.

2.4 Cultivation of RAW 264.7 macrophages

RAW 264.7 cells were cultivated in DMEM containing 3% FCS in a cell incubator at 37°C in a humidified atmosphere containing 5% CO_2 in 5 mL (25 cm^2 flask) or 10 mL medium (75 cm^2 flask). The media were supplemented with penicillin (100 IU/mL) and streptomycin (100 µg/mL). Confluent cells were mechanically detached from the flask by scraping, resuspended in medium and split 1:10. For use in experiments, cells were suspended as described above, counted with a Neubauer counting chamber and the indicated amounts of cells described in the experiment were seeded in 96-well plates.

2.5 Determination of cell proliferation on MRP-modified amino acid/dipeptide spots

The squares (side length 0.4 cm) containing the MRP-modified amino acid/dipeptide (which could be visualized under a UV lamp or by observing the brown colour) were cut out from the membrane strips. The peptide squares containing one single spot of MRP-modified or PBS-incubated

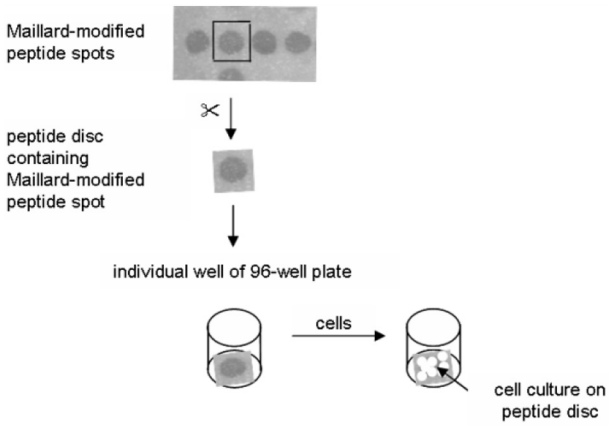

Figure 1. Schematic illustration of the cellulose-based membrane-based cell viability assay. The diagram shows the assay used to investigate the influence of membrane-bound MRPs on cell viability. Amino acid or dipeptides were synthesized on a cellulose membrane and then incubated with RCCs such as ribose, glyceraldehyde or methylglyoxal to form spots of amino acid or dipeptide MRPs. Squares containing the amino acid or dipeptide spots were then cut from the membrane and placed in an individual well of a 96-well plate. The cells were directly seeded on the membrane squares and allowed to grow. Relative cell numbers were monitored Alamar Blue or MTT cell viability assays.

amino acid/dipeptide or negative controls were placed in a 96-well plate (one square/well) under sterile conditions and 0.1 mL cell culture medium was added. The 96-well plate was incubated in the cell incubator for 10 min to soak the squares with medium prior to seeding 5.5×10^4 cells *per* well, trying to preferentially make them attach to the square. The cells were allowed to attach for 2 h before transferring each square into a new well (Fig. 1). This ensured that the number of cells growing outside the square stayed behind and all data were obtained only with membrane attached cells. Afterwards, cell viability (or relative cell numbers) were measured with the Alamar Blue assay. The number of viable cells on each square can be expressed as fluorescence intensity signal (relating to the amount of fluorescent resorufin formed from Alamar Blue). The Alamar Blue solution was them removed from the wells and the cells were further grown in 0.2 mL medium. After a 24 and 48 h, the number of viable cells on the squares was again determined. The fluorescence intensity signal after 2 h was set to 1 (100%), and the relative number of cells after 24 and 48 h were expressed a *x*-fold of this initial value. For the determination of the cytotoxic/cytostatic effects of the MRPs, cell numbers were compared to numbers determined on the PBS-incubated control squares.

2.6 Cell viability assays

The MTT and Alamar Blue assay were used for measuring cell viability. The MTT assay is based on the reduction of

MTT by mitochondrial enzymes of viable cells, which cleave the yellow tetrazolium salt to purple formazan crystals. Viable cells on the squares could be visualized with formazan crystals forming from MTT (1 mg/mL MTT in PBS), resulting in blue stained cells easily detectable on the white or yellow-coloured membranes. Briefly, the medium was removed from the cells and 0.1 mL MTT solution was added for 1 h. The colour intensity of the blue-stained cells on a spot was quantified using the Image MasterTM 2D Platinum software. The colour intensity value of the PBS-incubated control was set as 100%. For the Alamar Blue assay, the cell culture medium was carefully removed from the wells and the remaining cells attached to the squares were incubated with 0.1 mL Alamar Blue solution (2.5 mg/L resazurin sodium salt in cell culture medium) for 1.5 h in a 96-well plate. The fluorescence was read at wavelengths of 545 nm for excitation and 595 nm for emission. When required, cells were further grown by re-exchanging the Alamar blue solution with 0.2 mL medium. For that purpose the assay was performed under sterile conditions.

2.7 Data presentation and analysis

Data are expressed as mean ± SD. Statistical analysis of the results was carried out by post-hoc ANOVA using the Graphpad Prizm 5 software. Statistical significance was established at a p-value < 0.05 (*), <0.01 (**) or <0.001 (***), respectively.

3 Results

3.1 Synthesis and selective deprotection of the amino acid and dipeptide library

The use of an amino acid or dipeptide library for the purpose of synthesizing defined populations of MRPs is advantageous for various reasons. Firstly, all MRPs and AGEs identified so far are derived from reactions of one or two amino acids with one RCC. The minimal model that allows the synthesis of all possible MRPs would consist of a dipeptide library containing all 400 possible dipeptides. Secondly, attaching the amino acids and dipeptides to a solid support simplifies the washing and sterilization procedures. In preparation for the cytotoxicity assays, the amino acids or dipeptides were synthesized on filter paper sheets by an automatic synthesizer which arranges them in an array [34]. We have previously calculated that a comparable amount of peptide is present in each spot (17 ± 1 nmol), and thus would most likely allow a comparable number of cells attaching to the spots. This is also a precondition to allow the accurate comparison of the number of viable cells on the spots and calculation of the relative cytotoxicity of each individual amino acid or dipeptide MRP. We have restricted ourselves to amino acids and dipeptides which were deprotected to expose both free N-termini and free side chains for

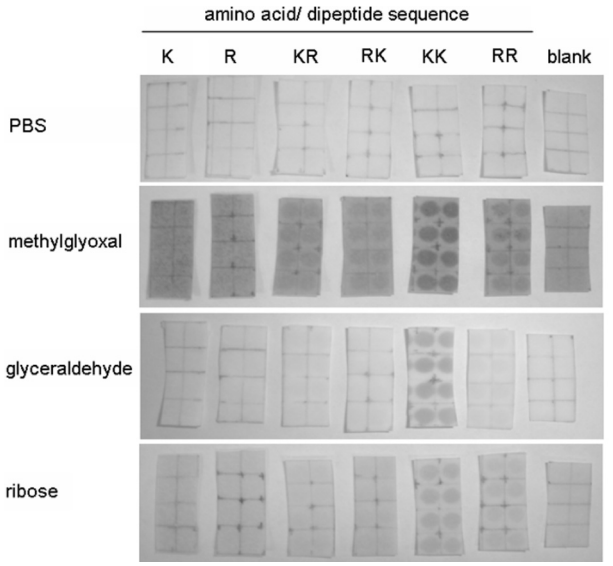

Figure 2. Formation of MRPs on amino acid and dipeptide spots on cellulose membranes. Membrane strips containing eight amino acids or dipeptides consisting of Lys (K) and Arg (R) were heated with 0.5 M methylglyoxal, 0.5 M glyceraldehyde, 0.5 M ribose or PBS at 60°C for 2 wk. Browning of spots was used as indicator of MRPs formation.

two major reasons: Firstly, hydrolysis of proteins during the high temperatures used for food preparation will create a variety of smaller peptides and thus increase the number of available N-termini in MRPs. Secondly, commercially available foods often contain protein hydrolysates which are prone to N-terminal glycation [36]. In particular, milk proteins hydrolyzates are specifically designed as hypoallergic foods for use in infant formulas [37, 38].

3.2 Preparation of membrane bound amino acid and dipeptide-MRPs

The side chains of lysine and arginine are reaction partners in the majority of identified MRPs. Therefore, we only used spots containing the N-terminally and side-chain deprotected amino acids Lys (K) and Arg (R) as well as the dipeptides Lys–Arg (KR), Arg–Lys (RK), Lys–Lys (KK) and Arg–Arg (RR) for our mini-library. In a preliminary experiment, Lys–Lys and Arg–Arg dipeptides were incubated with 0.5 M glucose 60°C but failed to produce brown peptide spots within 2 wk (data not shown). Thus, more reactive RCCs such as methylglyoxal, glyceraldehyde and ribose were used. Browning of the spots was used as an indicator of MRP formation. After two weeks, colour intensity of the spot remained constant, indicating that the amino acids and dipeptide had been maximally modified with MRPs. It appeared that the Maillard reaction proceeded much slower on the spots than has been described for reactions in solution. One possible cause for this slow reaction might the slow diffusion of reactants through the mem-

brane. As negative controls, the amino acids and dipeptide spots were also incubated with PBS under the same conditions. Further negative control used cellulose membranes only incubated with the RCCs. The Lys–Lys dipeptide appeared to be the most reactive compound towards formation of brown MRPs, with the activity of the RCCs increasing from ribose < glyceraldehyde < methylglyoxal. The Arg–Arg dipeptide showed strong formation MRP with methylglyoxal, followed by ribose and, very faint, with glyceraldehyde (Fig. 2). When the different RCCs were compared for their reactivity, methylglyoxal always caused the strongest formation of brown MRPs. The spots containing single amino acids did not generate significant amounts of coloured MRPs under all incubation conditions. It might be possible, however, that colourless MRPs (with absorption outside the visible range, *e.g.* CML) might have had formed, which still might be toxic. As expected, membranes without dipeptides or amino acids (which do not contain any free amino groups) incubated with methylglyoxal, glyceraldehyde, ribose did not form brown MRPs. Interestingly, formation of some cellulose-derived brown products could be observed on the membrane incubated with methylglyoxal.

3.3 Establishment of cell culture on cellulose membranes

Common cytotoxicity assays use cells cultured in standard cell culture plasticware, which employs surfaces optimized for cell attachment and growth. Cells are then exposed to toxins, in most cases soluble products, which are then expected to cause cell death in a dose-dependent manner. Instead of using soluble amino acids or dipeptide MRPs for the cytotoxicity experiments, we used membrane bound MRPs and had to establish cell culture methods and viability assays for this experimental paradigm. Growing cells on a cellulose membrane is not a routine cell culture method, and therefore, the suitability of the cellulose support membrane for culturing adherent RAW264.7 macrophages was investigated first.

Squares (4 mm × 4 mm), which contained the amino acid and peptide spots (or unmodified membranes used as negative controls) were cut out from the membrane, before they were placed in an individual well of a 96-well plate. The cells were seeded, allowed to attach to the membrane, and after 2 h, the square was transferred to a new well, leaving all 'escapees' behind. In the new well, all cells were attached to the square, and here the relative cell number on each spot could be conveniently monitored at various time points by the Alamar Blue assay by simply replacing the medium.

In a first experiment to analyse the growth characteristics of the RAW 264.7 macrophages, 10^4 cells were seeded on an unmodified cellulose membrane square. Cell number, monitored with the Alamar Blue assay, increased steadily in an exponential manner till day 11 (Fig. 3A). With the MTT assay, a qualitative increase in cell density on the membrane squares could also be detected over the 12 day period (Figs. 3B and C). Since, the Alamar Blue assay can be performed with the same population of cells over and over again, it was used for most of the following experiments. We also observed that the lag time for cell growth at this seeding density (10^4 cells *per* square) was to long, therefore the initial number of cells was increased to 5.5×10^4 cells *per* square for all following experiments.

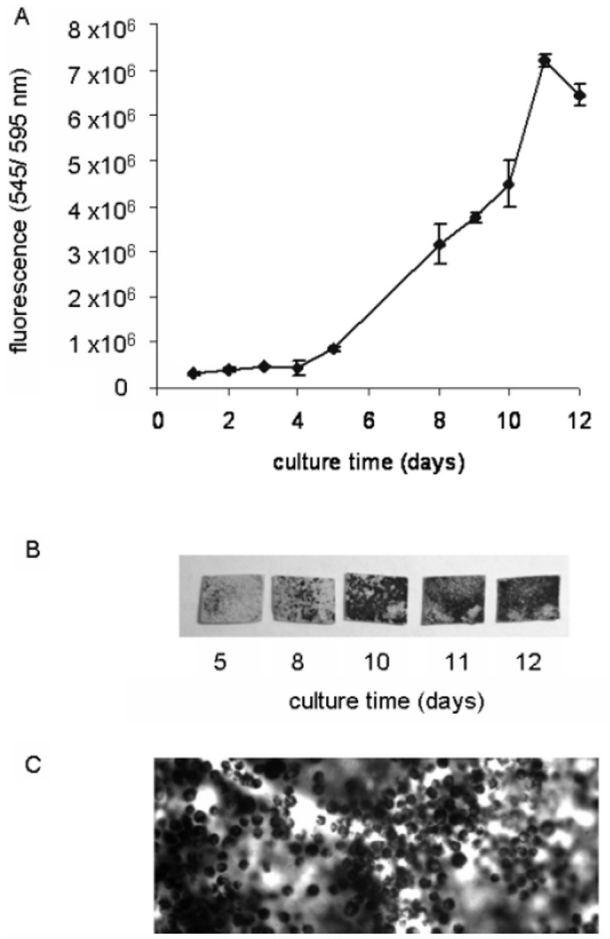

Figure 3. Establishment of cell culture and measurement of cell proliferation on cellulose membrane squares (A) RAW 264.7 macrophages were seeded at a density of 10^4 cells *per* square and cultured for up to 12 days. Relative cell numbers are determined by the Alamar Blue assay. This experiment used an unmodified cellulose membrane and shows data from two independent experiments performed in triplicate (mean ± SD). (B) The same experiment as in (A) with the difference that the cells were incubated with MTT and the membrane square was photographed. Note that the deposited formazan crystals in the cells can be observed as a dark blue stain. (C) Microscopic image of the formazan labelled cells on the membrane square.

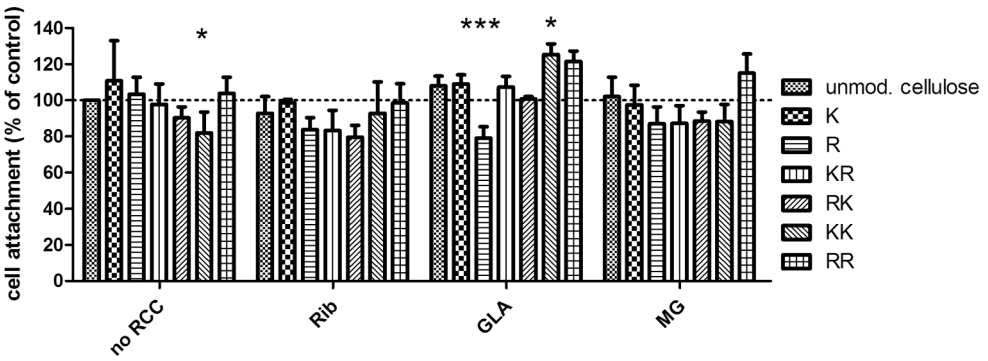

Figure 4. Comparison of the degree of attachment of cells to the spots. The cells were seeded on the membrane squares and after 2 h the square with the attached cells were transferred into new wells. Then, the amount of viable cells on the squares was determined with the Alamar blue assay. The value obtained with the unmodified cellulose membrane (no amino acid, no RCC) was set to 100%, and all other relative cell number normalized to this value. Bars show mean ± SD ($n = 4$). Significance was calculated by two way ANOVA, whereby the amino acids and peptide-MRPs were compared to the unmodified cellulose which had been incubated with the same RCC (left column in each set of bars). Significance is indicated by *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$).

3.4 Attachment of the cells to the cellulose membrane

One precondition for experiments comparing cytotoxicity of different MRPs is that a similar number of cells are present on each square at the start of the experiment. Whereas the number of seeded cells is likely to be quite similar (within the margins of pipetting errors), it is not clear if the same percentage of cells attaches to the different spots. This question was addressed by seeding the exact number of cells (5.5×10^4) on each membrane square, allowing them to attach for 2 h, transferring the squares to a new well and then determining the relative number of cells by the Alamar Blue assay. Our data indicate that the numbers of attached and viable cells ranged from 80 to 120% among the different spots compared to an unmodified square (Fig. 4). Statistical comparison showed no significant differences except for the Lys–Lys spot, where only 82% of cells attached ($p < 0.05$). Some glycerinaldeyde-modified MRPs showed significant differences in cell attachment compared to the membrane containing no MRPs (Fig. 4). To take these differences in the degree of attachment in account for all following cytotoxicity experiments, relative cell numbers were always measured on each spot after 2 h. This value was then used as set to 100% and used for normalization, when cell proliferation on this spot was determined.

3.5 Influence of membrane bound MRPs on cell proliferation and survival

To determine the effect of the different MRPs on cell survival and/or growth, squares were cut out from unmodified cellulose membrane, and amino acid and dipeptide spot strips, which had been modified with methylglyoxal, glyceraldehyde or ribose, respectively. Similar to the protocol

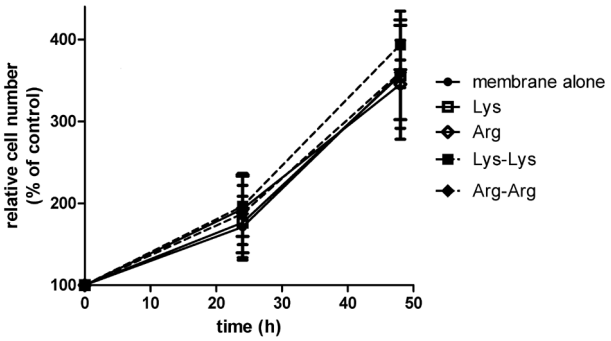

Figure 5. Cell growth of RAW 264.67 macrophages on unmodified cellulose membranes. To establish standard growth parameters of cells on unmodified cellulose membrane, the RAW 264.7 macrophages were seeded on the squares and allowed to attach. After 2 h, the squares were transferred into new wells, and the relative number of membrane attached cells for each individual membrane square was determined with the Alamar Blue assay. After further 24 and 48 h, the relative number of metabolically active cells was determined. Data show the mean ± SD.

above, the RAW 264.7 macrophages were seeded on the squares and allowed to attach. After 2 h, the squares were transferred into new wells (leaving any escapees behind), and the relative number of membrane attached cells for each individual membrane square was determined with the Alamar Blue assay. This value at $t = 0$ was set to 100% for each individual membrane square containing the amino acid or dipeptide spot. After 24 and 48 h, the relative number of metabolically active cells was determined.

As a further controls, the cells were also grown on membrane squares which did not contain dipeptides or amino acids, but had been incubated with all three carbonyl compounds. In general, proliferation of cells on the cellulose alone, the cellulose incubated with the three RCCs (except

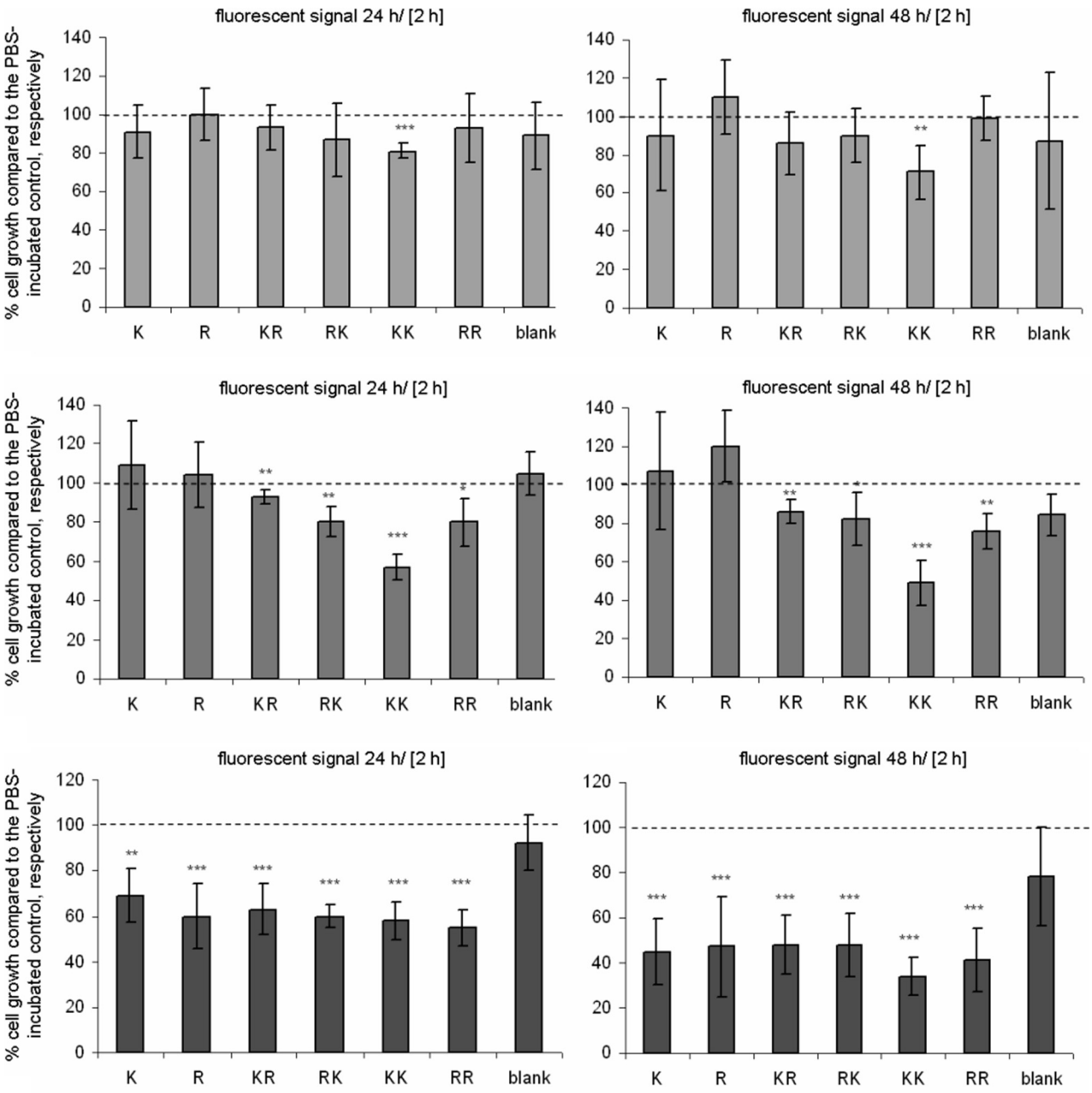

Figure 6. Comparison of inhibition of cell proliferation caused by individual MRPs. RAW 264.7 macrophages (5.5×10^4 cells/well) were seeded on methylglyoxal, glyceraldehyde, ribose modified amino acid (K and R), dipeptide (KR, RK, KK and RR) squares. Proliferation of cells was monitored with the Alamar Blue assay after 24 and 48 h. Cell number on the ribose (C), glyceraldehyde (B) and methylglyoxal (A)-derived MRPs is compared to the same unmodified control spot for each amino acid or dipeptide (which was set to 100%). Data show the mean ± SD ($n = 3$). Significance is indicated by *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$).

for methylglyoxal) and the unmodified amino acid and dipeptide spots were quite similar with cell numbers increasing by ~1.8-fold after 24 h, and 3.6-fold after 48 h (Fig. 5).

When the effect of MRP-modification on cell growth was investigated, some MRPs caused a significant slower proliferation rate (Figs. 6A – C). Among the ribose-derived MRPs, only the Lys – Lys MRPs significantly inhibited cell growth (Fig. 6C). With glycerinaldehyde, all MRP-modified dipeptides (but not the MRP-modified amino acids) inhibited cell proliferation (Fig. 6B). Among the MRP modified dipeptides, the Lys – Lys MRPs had the strongest effect on cell proliferation, with only 49% of the cells growing on the Lys – Lys MRPs after a 48 h (Fig. 6B). The most significant effect on cell growth was observed with methylglyoxal-derived MRPs, which was significant for both MRP-modified amino acids and dipeptides (Fig. 6A). The most toxic methyl-

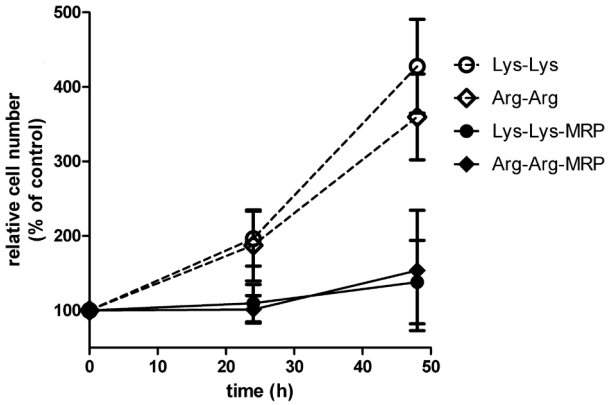

Figure 7. Cytostatic effects of Lys–Lys and Arg–Arg methylglyoxal-derived MRPs. RAW 264.7 macrophages (5.5×10^4 cells/well) were seeded on methylglyoxal-modified dipeptide (KK and RR) squares. The number of cells was monitored with the Alamar Blue assay after 24 and 48 h. Cell number on the ribose (A), glyceraldehyde (B) and methylglyoxal (C)-derived MRPs is expressed as relative cell numbers compared to the start of the experiment. Data show the mean ± SD ($n = 3$).

glyoxal-derived MRP was the Lys–Lys MRP, where cell numbers were reduced by 64% compared to the unmodified Lys–Lys control spot at 48 h (Fig. 6A). When cell numbers at 24 and 48 h were compared to the starting values, the methylglyoxal-derived Lys–Lys MRPs (and also the Arg–Arg MRPs) inhibited cell proliferation almost completely. However, the cell numbers did not fall below the starting value suggesting that the MRPs – at least at this concentration – were rather cytostatic than cytotoxic (Fig. 7).

In summary, our data suggest that the toxicity of MRPs is dependent on the RCC used; it increased in the order of ribose < glycerinaldehyde < methylglyoxal-derived MRPs. Furthermore, the dipeptides were generally more cytotoxic than the amino acids, with Lys–Lys MRPs being the most toxic MRPs in all cases.

3.6 Suitability of the system for larger membrane sheets containing multiple peptide spots

Although the Alamar Blue assay has the advantage of not being destructive and therefore can be repeated many times with the same cell population, it requires that each single peptide spot has to be excised from the membrane and placed in an individual well. This might become quite time consuming with membranes containing multiple peptide spots, as they would be present if peptides containing all 20 amino acids (*e.g.* to include differential activities of their N-termini) would be included. Therefore, a cell viability assay that could be measured on a larger membrane would be highly advantageous. One of the options would be to use a readout, which could be compared among all spots on the membrane (such as the purple formazan crystals formed from MTT, see Fig. 2). These spots could then be photographed and relative cell numbers determined by image analysis.

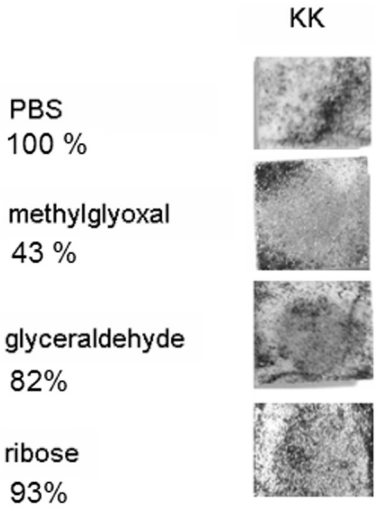

Figure 8. Comparison of cell growth on Lys–Lys-derived MRPs spots using MTT and visualization by image analysis of the blue formazan spots. RAW 264/7 macrophages were incubated with MTT on the MRP-modified Lys–Lys spots after 48 h to allow formazan crystals to form. Cells were photographed, and relative cell numbers on the Lys–Lys-derived MRP spots were detected by densitometric analysis. The colour intensity of the viable blue-stained cells on the spots was quantified using Image MasterTM 2D Platinum software and compared to the unmodified Lys–Lys spot (incubated only with PBS) that was set to 100%.

In a similar approach to the semi-quantitative experiment shown in Fig. 2B, cells were incubated with MTT after a 48 h growth period in the dipeptide spot. Formazan-stained cells on a unmodified Lys–Lys spot (negative control), or three other spots modified with methylglyoxal, glyceraldehyde or ribose-derived MRPs were analysed (Fig. 8). The methylglyoxal-derived Lys–Lys spot showed an obvious low cell density region in the centre of the square. Quantification of the blue staining of this square showed a lower signal ~43%) compared to the unmodified spot. The colour intensities of the glyceraldehyde- and ribose-derived Lys–Lys MRPs spots were determined to be 82 and 93% of control, respectively. However, detailed analysis of the pattern of cell growth showed that formazan producing cells were spread quite irregular across the square. The result indicate that the influence of membrane-bound MRPs on cell proliferation can be generally determined by this technique, but this method needs to be vastly improved (*e.g.* by measuring the number of calls on each spot at the start by a non-destructive method) before it can be used on a large (and expensive) multispot membrane with reliable results.

4 Discussion

The reactivity of amino acids towards MRP formation was determined with a membrane-bound combinatorial amino

acid and dipeptide mini-library. This method enables the screening of amino acids reactivities towards MRP formation under the same experimental setup in a time and reagent saving manner. In previous work, we have shown that dipeptide spot libraries were excellent tools to investigate the reactivity and antigen formation of the proteinogenic amino acids towards Maillard-modification [35, 39]. In the present work, we have established a cell culture model on the cellulose bound amino acid and peptide spots and investigated the influence of the membrane-bound Maillard-modified peptides on cell proliferation. The bioassay was established with a combinatorial dipeptide mini-library, consisting of the single amino acids Lys and Arg, and the dipeptides Lys – Arg, Arg – Lys, Lys – Lys and Arg – Arg, which were MRP-modified using ribose, glyceraldehyde and methylglyoxal. Our results show that none of the MRPs reduced the number of cells below the starting levels, indicating that the spot bound MRPs were rather cytostatic than cytotoxic. However, proliferation of cells exposed to MRP-modified dipeptides was dramatically reduced compared to the non-modified dipeptides. MRPs derived from Lys – Lys dipeptides incubated with methylglyoxal had the strongest cytostatic effect. Similar results were obtained with the glyceraldehyde – modified dipeptides, but the anti-proliferative properties of the MRPS were less intense. Among the glyceraldehyde-modified dipeptides, the MRPs formed with Lys – Lys spots showed the strongest reduction of cell growth (51%). Except of MRPs produced from Lys – Lys dipeptides, the ribose-derived MRPs had no influence on cell proliferation. Among the MRP-modified amino acids, only the methylglyoxal-incubated amino acid spots showed an inhibitory effect on cell proliferation. Independent of the carbonyl compound used for glycation, MRPs derived from Lys – Lys dipeptides exert the strongest inhibitory effect on cell proliferation.

In many other studies, mixtures containing soluble MRPs have often shown to cause cell death. However, in our study, even the most 'toxic' Lys – Lys-derived MRPs did not induce cytotoxicity, but rather inhibited cell proliferation. This effect can be explained by the relatively low concentration of membrane-bound Maillard-modified dipeptides *per* spot (17 nmol/well) compared with the concentration of 'conventional' Maillard reaction mixtures (5 – 25 mM = 0.5 – 2.5 µmol/well). Furthermore, only extracellular AGE effects (e. g. receptor mediated effects) can be assessed with the AGEs covalently attached to the cellulsoe membrane. Effects caused by uptake of AGEs cannot be detected, which may also explain the differences to other reports concerning cytotoxicity. In addition, we tried to maximize cytotoxicity by extending the incubation time to 2 wk at 60°C where the browning appeared to be maximal, taken into account our previous experiment suggesting that toxcity of MRPs/AGEs increases with the degree of glycation [18]. We also used the same buffers and conditions for all spots to ensure that glycation was not influenced by factors such as temperature, impurities (in particular metal ions) or oxygenation, resulting in a comparable pattern of glycation among all spots (Fig. 2). The present work also shows that the anti-proliferative effects of the MRPs were dependent on the RCC. Methylglyoxal-derived MRPs showed the strongest effect on cell proliferation. To a lesser degree glyceraldehyde-derived MRPs also inhibited cell proliferation, whereas only ribose-derived Lys – Lys MRPs reduced cell growth. Interestingly, all MRP-modified dipeptides were more effective than the respective amino acids. It might be speculated whether this might be just an additive effect but it might be possible that the most effective MRPs only form when two amino acids are in close proximity and form a specific 'two amino acid' MRP.

We had originally hoped that a smaller number of MRPs (*e.g.* only Lys, but not Arg-MRPs) would be toxic which would make the search for ‚the most toxic compounds' much easier.

Unfortunately, it appears that a variety of possible toxic MRPs exist which involve both Arg and Lys. However, it might be a good approach for the identification of the most toxic MRPs to start with Arg und Lys dipeptides in the first instance and analyse those mixtures for the most toxic compounds.

The use of a large membrane with multiple spots would be advantageous but the detection by MTT/formazan has serious drawbacks. Firstly, the starting number of cells on each spot can be quite different since seeding of a large membrane appears to be difficult. In addition, only one time point can be used for since the accumulation of formazan in cells is cytotoxic. One option for improvement could be the use of a coloured cell (*e.g.* GFP-transfected) line. Use of such a cell line would allow determination of the number of attached cells on each spot at the start and then at multiple time points to construct a reliable growth curve. In addition, image analysis could be adapted to only include the peptide covered circular spot eliminating the interference from the unmodified areas of the membrane.

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5 References

[1] Maillard, L. C., Action des acides amines sur le sucre: Formation de melanoidines par voie methodique, *C.R. Acad. Sci.* 1912, *154*, 66 – 68.

[2] Monnier, V. M., Sell, D. R., Nagaraj, R. H., Miyata, S., *et al*., Maillard reaction-mediated molecular damage to extracellular matrix and other tissue proteins in diabetes, aging, and uremia, *Diabetes* 1992, *41 (Suppl 2)*, 36 – 41.

[3] Sell, D. R., Lapolla, A., Odetti, P., Fogarty, J., Monnier, V. M., Pentosidine formation in skin correlates with severity of complications in individuals with long-standing IDDM, *Diabetes* 1992, *41*, 1286 – 1292.

[4] Brownlee, M., Advanced protein glycosylation in diabetes and aging, *Annu. Rev. Med.* 1995, *46*, 223 – 234.

[5] Ledesma, M. D., Bonay, P., Colaco, C., Avila, J., Analysis of microtubule-associated protein tau glycation in paired helical filaments, *J. Biol. Chem.* 1994, *269*, 21614 – 21619.

[6] Yan, S. D., Yan, S. F., Chen, X., Fu, J., *et al*., Non-enzymatically glycated tau in Alzheimer's disease induces neuronal oxidant stress resulting in cytokine gene expression and release of amyloid beta-peptide, *Nat. Med.* 1995, *1*, 693 – 699.

[7] Kimura, T., Takamatsu, J., Araki, N., Goto, M., *et al*., Are advanced glycation end-products associated with amyloidosis in Alzheimer's disease? *Neuroreport* 1995, *6*, 866 – 868.

[8] Dickson, D. W., Sinicropi, S., Yen, S. H., Ko, L. W., *et al*., Glycation and microglial reaction in lesions of Alzheimer's disease, *Neurobiol. Aging* 1996, *17*, 733 – 743.

[9] Lüth, H. J., Ogunlade, V., Kuhla, B., Kientsch-Engel, R., *et al*., Age- and stage-dependent accumulation of advanced glycation end products in intracellular deposits in normal and Alzheimer's disease brains, *Cereb. Cortex* 2005, *15*, 211 – 220.

[10] Münch, G., Cunningham, A. M., Riederer, P., Braak, E., Advanced glycation endproducts are associated with Hirano bodies in Alzheimer's disease, *Brain Res.* 1998, *796*, 307 – 310.

[11] Münch, G., Mayer, S., Michaelis, J., Hipkiss, A. R., *et al*., Influence of advanced glycation end-products and AGE-inhibitors on nucleation-dependent polymerization of beta-amyloid peptide, *Biochim. Biophys. Acta* 1997, *1360*, 17 – 29.

[12] Kuhla, B., Haase, C., Flach, K., Lüth, H. J., *et al*., Effect of pseudophosphorylation and cross-linking by lipid peroxidation and advanced glycation end product precursors on tau aggregation and filament formation, *J. Biol. Chem.* 2007, *282*, 6984 – 6991.

[13] Smith, M. A., Monnier, V. M., Sayre, L. M., Perry, G., Amyloidosis, advanced glycation end products and Alzheimer disease, *Neuroreport* 1995, *6*, 1595 – 1596.

[14] Finch, C. E., Cohen, D. M., Aging, metabolism, and Alzheimer disease: Review and hypotheses, *Exp. Neurol.* 1997, *143*, 82 – 102.

[15] Heidland, A., Sebekova, K., Schinzel, R., Advanced glycation end products and the progressive course of renal disease, *Am. J. Kidney Dis.* 2001, *38*, S100 – S106.

[16] Loske, C., Neumann, A., Cunningham, A. M., Nichol, K., *et al*., Cytotoxicity of advanced glycation endproducts is mediated by oxidative stress, *J. Neural Transm.* 1998, *105*, 1005 – 1015.

[17] de Arriba, S. G., Loske, C., Meiners, I., Fleischer, G. *et al*., Advanced glycation endproducts induce changes in glucose consumption, lactate production, and ATP levels in SH-SY5Y neuroblastoma cells by a redox-sensitive mechanism, *J. Cereb Blood Flow Metab.* 2003, *23*, 1307 – 1313.

[18] Gasic-Milenkovic, J., Loske, C., Deuther-Conrad, W., Münch, G., Protein "AGEing" – cytotoxicity of a glycated protein increases with its degree of AGE-modification, *Z. Gerontol. Geriatr.* 2001, *34*, 457 – 460.

[19] Wautier, M. P., Chappey, O., Corda, S., Stern, D. M., *et al*., Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE, *Am. J. Physiol. Endocrinol. Metab.* 2001, *280*, E685 – E694.

[20] Ding, Y., Kantarci, A., Badwey, J. A., Hasturk, H., *et al*., Phosphorylation of pleckstrin increases proinflammatory cytokine secretion by mononuclear phagocytes in diabetes mellitus, *J. Immunol.* 2007, *179*, 647 – 654.

[21] Neumann, A., Schinzel, R., Palm, D., Riederer, P., Münch, G., High molecular weight hyaluronic acid inhibits advanced glycation endproduct-induced NF-kappaB activation and cytokine expression, *FEBS Lett.* 1999, *453*, 283 – 287.

[22] Dukic-Stefanovic, S., Gasic-Milenkovic, J., Deuther-Conrad, W., Münch, G., Signal transduction pathways in mouse microglia N-11 cells activated by advanced glycation endproducts (AGEs), *J. Neurochem.* 2003, *87*, 2609 – 2615.

[23] Berbaum, K., Shanmugam, K., Stuchbury, G., Wiede, F., *et al*., Induction of novel cytokines and chemokines by advanced glycation endproducts determined with a cytometric bead array, *Cytokine* 2008, *41*, 198 – 203.

[24] Krajcovicova-Kudlackova, M., Sebekova, K., Schinzel, R., Klvanova, J., Advanced glycation end products and nutrition, *Physiol. Res.* 2002, *51*, 313 – 316.

[25] Somoza, V., Physiological effects of thermally treated foods, *Mol. Nutr. Food Res.* 2008, *52*, 305 – 306.

[26] Henle, T., A food chemist's view of advanced glycation endproducts, *Perit. Dial. Int.* 2001, *21 (Suppl 3)*, S125 – S130.

[27] Henle, T., Dietary advanced glycation end products – a risk to human health? A call for an interdisciplinary debate, *Mol. Nutr. Food Res.* 2007, *51*, 1075 – 1078.

[28] Kankova, K., Diabetic threesome (hyperglycaemia, renal function and nutrition) and advanced glycation end products: Evidence for the multiple-hit agent? *Proc. Nutr. Soc.* 2008, *67*, 60 – 74.

[29] Uribarri, J., Cai, W., Sandu, O., Peppa, M. *et al*., Diet-derived advanced glycation end products are major contributors to the body's AGE pool and induce inflammation in healthy subjects, *Ann. N. Y. Acad. Sci.* 2005, *1043*, 461 – 466.

[30] Schmidt, A., Kuhla, B., Bigl, K., Münch, G., Arendt, T., Cell cycle related signaling in neuro2a cells proceeds via the receptor for advanced glycation end products, *J. Neural Transm.* 2007, *114*, 1413 – 1424.

[31] Bigl, K., Gaunitz, F., Schmitt, A., Rothemund, S., *et al*., Cytotoxicity of advanced glycation endproducts in human micro- and astroglial cell lines depends on the degree of protein glycation, *J. Neural Transm.* 2008, *115*, 1545 – 1456.

[32] Kuhla, B., Loske, C., Garcia De Arriba, S., Schinzel, R., *et al*., Differential effects of "Advanced glycation endproducts" and beta-amyloid peptide on glucose utilization and ATP levels in the neuronal cell line SH-SY5Y, *J. Neural Transm.* 2004, *111*, 427 – 439.

[33] Frank, R., The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports – principles and applications, *J. Immunol. Methods* 2002, *267*, 13 – 26.

[34] Münch, G., Schicktanz, D., Behme, A., Gerlach, M., *et al*., Amino acid specificity of glycation and protein-AGE crosslinking reactivities determined with a dipeptide SPOT library, *Nat. Biotechnol.* 1999, *17*, 1006 – 1010.

[35] Dukic-Stefanovic, S., Schicktanz, D., Wong, A., Palm, D., *et al*., Characterization of antibody affinities using an AGE-modified dipeptide spot library, *J. Immunol. Methods* 2002, *266*, 45 – 52.

[36] Penndorf, I., Li, C., Schwarzenbolz, U., Henle, T., N-terminal glycation of proteins and peptides in foods and in vivo: Evaluation of N-(2-furoylmethyl)valine in acid hydrolyzates of human hemoglobin, *Ann. N. Y. Acad. Sci.* 2008, *1126*, 118 – 123.

[37] Penndorf, I., Biedermann, D., Maurer, S. V., Henle, T., Studies on N-terminal glycation of peptides in hypoallergenic infant formulas: Quantification of alpha-N-(2-furoylmethyl) amino acids, *J. Agric. Food Chem.* 2007, *55*, 723 – 727.

[38] Sebekova, K., Saavedra, G., Zumpe, C., Somoza, V., *et al*., Plasma concentration and urinary excretion of N epsilon-(carboxymethyl)lysine in breast milk- and formula-fed infants, *Ann. N. Y. Acad. Sci.* 2008, *1126*, 177 – 180.

[39] Richter, T., Münch, G., Lüth, H. J., Arendt, T., *et al*., Immunochemical crossreactivity of antibodies specific for "advanced glycation endproducts" with "advanced lipoxidation endproducts", *Neurobiol. Aging* 2005, *26*, 465 – 474.

