- HUMAN LENS CHEMISTRY -

UV FILTERS AND AGE-RELATED NUCLEAR CATARACT

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ABSTRACT

The kynurenine-based UV filters are unstable under physiological conditions and undergo side chain deamination, resulting in α,β-unsaturated carbonyl compounds. These compounds can react with free or protein bound nucleophiles in the lens *via* Michael addition. The key sites of the UV filters kynurenine (Kyn) and 3-hydroxykynurenine (3OHKyn) modification in human lenses include cysteine (Cys), and to a lesser extent, lysine (Lys) and histidine (His) residues. Recent *in vivo* studies have revealed that 3-hydroxykynurenine-*O*-β-D-glucoside (3OHKG) binds to Cys residues of lens crystallins in older normal human lenses. As a result of this binding, human lens proteins become progressively modified by UV filters in an age-dependent manner, contributing to changes that occur with the development of age-related nuclear (ARN) cataract. Upon exposure to UV light, free UV filters are poor photosensitisers, however the role of protein-bound species is less clear. It has been recently demonstrated that Kyn, when bound to lens proteins, becomes more susceptible to photo-oxidation by UV light. Therefore, the investigation of 3OHKG binding to lens proteins, and the effect of UV light on proteins modified with 3OHKG and 3OHKyn, were major aims of this study. As a result of the role of these compounds as UV filters and their possible involvement in ARN cataract formation, it is crucial to understand the nature, concentration and modes of action of the UV filters and their metabolites present in the human lenses. Therefore, an additional aim was to investigate human lenses for the presence of novel kynurenine-based human lens metabolites and examine their reactivity.

As 3OHKG is not commercially available, to conduct protein binding studies, an initial aim of this study was to synthesise 3OHKG (Chapter 2). Through the expansion and optimisation of a literature procedure, 3OHKG was successfully synthesised using commercially available and inexpensive reagents, and applying green chemistry principles, where toxic and corrosive reagents were replaced with benign reagents and solvent-free and microwave chemistry was used. A detailed investigation of different reaction conditions was also conducted, resulting in either the improvement of reaction yields or reaction time compared to the literature method. Applying the same synthetic strategy, and using key precursors from the synthesis of 3OHKG, the UV filters 3OHKyn and 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid-*O*-β-D-glucoside (AHBG), were also successfully synthesised (Chapter 3).

Chapter 4 describes the investigation of both normal and cataractous human lenses in an attempt to identify novel human lens metabolites derived from deaminated Kyn and 3OHKyn

(Chapter 4, Part A). Initially, 4-(2-aminophenyl)-4-oxobutanoic acid (AHA), glutathionyl-kynurenine (GSH-Kyn), kynurenine yellow (Kyn yellow), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid (AHB), glutathionyl-3-hydroxykynurenine (GSH-3OHKyn) and 3-hydroxykynurenine yellow (3OHKyn yellow) were synthesised and human lenses were examined for their presence. AHA and AHB were synthesised from similar precursors to those used in the synthesis of 3OHKG, while the GSH adducts and yellow compounds were synthesised from Kyn and 3OHKyn *via* base induced deamination. Following isolation and structural elucidation, AHA, AHB and GSH-Kyn were confirmed as novel human lens metabolites. They were quantified in low pmol/mg lens (dry mass) levels in normal and cataractous lenses of all ages, while GSH-3OHKyn, Kyn yellow and 3OHKyn yellow were not detected. In contrast to AHA, the lens metabolites AHB, GSH-Kyn and GSH-3OHKyn were found to be unstable at physiological pH. The spectral properties of these compounds suggest that they may act as UV filters.

Chapter 4 (Part B) also describes the identification and characterisation of a novel human lens UV filter, cysteinyl-3-hydroxykynurenine-*O*-β-D-glucoside (Cys-3OHKG). An authentic standard was synthesised *via* Michael addition of cysteine to deaminated 3OHKG. Cys-3OHKG was detected in low pmol/mg lens (dry mass) levels in normal lenses only after the 5^{th} decade of life and was absent in cataractous lenses. Cys-3OHKG showed rapid decomposition at physiological pH.

Chapter 5 describes the identification and quantification of amino acids involved in covalent binding of 3OHKG to lens proteins. Model studies with bovine lens proteins and 3OHKG at pH 7.2 and 9.5 were undertaken. The amino acid adducts were identified *via* total synthesis and spectral analysis, and subsequently quantified upon acid hydrolysis of the modified lens proteins. Under both pH conditions, 3OHKG was found to react with lens proteins predominantly *via* Cys residues with low levels of binding also detected at Lys residues. Comparative studies with Kyn (pH 9.5) and 3OHKyn (pH 7.2 and 9.5) resulted in modified lens proteins at Cys residues, with only minor modification at Lys residues at pH 9.5. The extent of modification was found to be significantly higher at pH 9.5 in all cases. His adducts were not identified. 3OHKG-, Kyn- and 3OHKyn-modified lens proteins were found to be coloured and fluorescent, resembling those of aged and ARN cataractous lenses. In contrast, AHB and AHA, which can not form α,β-unsaturated carbonyl compounds, resulted in non-covalent modification of lens proteins. AHB may contribute to lens colouration and fluorescence as further reactions of this material yielded species that have similar

characteristics to those identified from 3OHKyn modification. These species are postulated to arise *via* auto-oxidation of the *o*-aminophenol moiety present in both 3OHKyn and AHB.

In Chapter 6, the potential roles of 3OHKG and 3OHKyn, and the related species AHA and AHB, in generating reactive oxygen species and protein damage following illumination with UV light was examined. The UV filter compounds were examined in both their free and protein-bound forms. Kyn-modified proteins were used as a positive control. Exposure of these compounds to UV light (λ 305-385 nm) has been shown to generate H_2O_2 and protein-bound peroxides in a time-dependent manner, with shorter wavelengths generating more peroxides. The yields of peroxides were observed to be highly dependent on the nature of the UV filter compound and whether these species were free or protein bound, with much higher levels being detected with the bound species. Thus, protein-bound 3OHKyn yielded higher levels of peroxide than 3OHKG, with these levels, in turn, higher than for the free UV filter compounds. AHB-treated lens proteins resulted in formation of low but statistically significant levels of peroxides, while AHA-treated lens proteins resulted in insignificant peroxide formation. The consequences of these photochemical reactions have been examined by quantifying protein-bound tyrosine oxidation products (3,4-dihydroxyphenylalanine [DOPA], di-tyrosine [di-Tyr]) and protein cross-linking. 3OHKG-modified proteins gave elevated levels of di-Tyr, but not DOPA, whereas 3OHKyn-modified protein gave the inverse. DOPA formation was observed to be independent of illumination and most likely arose *via o*-aminophenol auto-oxidation. AHB- and AHA-treated lens proteins resulted in statistically insignificant di-Tyr formation, while a light independent increase in DOPA was observed for both samples. Both reducible (disulfide) and non-reducible cross-links were detected in modified proteins following illumination. These linkages were present at lower levels in modified, but non-illuminated proteins, and absent from unmodified protein samples.

This work has provided an optimised synthetic procedure for 3OHKG and other lens metabolites (Chapters 2 and 3). Four novel lens metabolites have been identified and quantified in normal and cataractous human lenses (Chapter 4). Subsequent experiments, described in Chapter 5, identified the major covalent binding sites of 3OHKG to lens proteins, while AHA and AHB showed non-covalent binding. Further work described in Chapter 6 showed that protein-bound 3OHKG, Kyn and 3OHKyn were better photosensitisers of oxidative damage than in their unbound state. Together, this research has provided strong evidence that post-translational modifications of lens proteins by kynurenine-based metabolites and their interaction with UV light appear, at least in part, responsible for the age-

dependent colouration of human lenses and an elevated level of oxidative stress in older lenses. These processes may contribute to the progression of ARN cataract.

THESIS DECLARATION

This thesis contains no material that has been accepted for the award of any higher degree or diploma at any University or Institution, and to the best of my knowledge contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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This thesis is dedicated to

my mum and dad

my brother, sister-in-law and nephews

my boyfriend

Love you all forever and thank you

PUBLICATIONS

Sections of the work described in this thesis have been reported or are in the preparation for the following publications:

Peter G. Hains, Jasminka Mizdrak, Isla M. Streete, Joanne F. Jamie, Roger J. W. Truscott, Identification of the new UV filter compound cysteinyl-L-3-hydroxykynurenine-*O*-β-D-glucoside in human lenses. *FEBS letters*, **2006**, 580, 5071-5076.

Jasminka Mizdrak, Peter G. Hains, Danuta Kalinowski, Roger J. W. Truscott, Michael J. Davies, Joanne F. Jamie, Novel human lens metabolites from normal and cataractous human lenses. *Tetrahedron*, **2007**, 63, 4990-4999.

Jasminka Mizdrak, Peter G. Hains, Joanne F. Jamie, Michael J. Davies, Roger J. W. Truscott, Identification of lens protein-bound 3-hydroxykynurenine-*O*-β-D-glucoside. A possible role in Age-Related Nuclear cataract. *FEBS letters*, manuscript in preparation.

Jasminka Mizdrak, Peter G. Hains, Roger J. W. Truscott, Joanne F. Jamie, Michael J. Davies, Tryptophan-derived UV filter compounds covalently bound to lens proteins are photosensitizers of oxidative damage. *Free Radicals in Biology & Medicine*, manuscript in preparation.

Jasminka Mizdrak, Peter G. Hains, Roger J. W. Truscott, Michael J. Davies, Joanne F. Jamie, A convenient synthesis of lenticular UV filters. *Tetrahedron Letters*, manuscript in preparation.

CONFERENCE PRESENTATIONS

The following oral or poster presentations have been made at conferences/symposia on research conducted during this PhD study:

i. The ISMC/RACIOC Organic Chemistry Conference in Cairns (QLD, Australia), 4-8 July 2004. Jasminka Mizdrak, Danuta Kalinowski, Nicole R. Parker, Peter G. Hains, Roger J. W. Truscott, Michael J. Davies, Joanne F. Jamie. Human lens chemistry - UV filters and cataract.

ii. The XVI International Congress of Eye Research in Sydney (NSW, Australia), 29 August-3 September 2004. Jasminka Mizdrak, Danuta Kalinowski, Nicole R. Parker, Peter G. Hains, Roger J. W. Truscott, Michael J. Davies, Joanne F. Jamie. Human lens chemistry - UV filters and cataract.

iii. The 12th Royal Australian Chemical Institute (RACI) Convention "Connect 2005" in Sydney (NSW, Australia), 3-7 July 2005. Jasminka Mizdrak, Peter G. Hains, Nicole R. Parker, Danuta Kalinowski, Roger J. W. Truscott, Michael J. Davies, Joanne F. Jamie. Human Lens Chemistry - Age-Related Nuclear (ARN) cataract.

iv. The Royal Australian Chemical Institute (RACI) Natural Products Group - Annual one day symposium in Sydney (NSW, Australia), 30 September 2005. Jasminka Mizdrak, Peter G. Hains, Danuta Kalinowski, Roger J. W. Truscott, Michael J. Davies, Joanne F. Jamie. Human Lens Natural Products.

v. The 1st European Chemistry Congress in Budapest (Hungary), 28-31 August 2006. Jasminka Mizdrak, Peter G. Hains, Roger J. W. Truscott, Michael J. Davies, Joanne F. Jamie. Human lens chemistry - UV filters and cataract. This international presentation resulted in media coverage, see Appendix C.

vi. One Day Royal Australian Chemical Institute (RACI) Organic Symposium in Canberra (ACT, Australia), 6 November 2006. Jasminka Mizdrak, Peter G. Hains, Roger J. W. Truscott, Michael J. Davies, Joanne F. Jamie. Human lens chemistry - Novel UV filters.

LIST OF ABBREVIATIONS

The following abbreviations are used throughout the text:

ABG	2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide
AcOH	Acetic acid
AGE	Advanced glycation end
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
AHA	4-(2-Aminophenyl)-4-oxobutanoic acid
AHB	4-(2-Amino-3-hydroxyphenyl)-4-oxobutanoic acid
AHBG	4-(2-Amino-3-hydroxyphenyl)-4-oxobutanoic acid-*O*-β-D-glucoside
AHBDG	4-(2-Amino-3-hydroxyphenyl)-4-oxobutanoic acid-*O*-diglucoside
ARN	Age-related nuclear
BAA	β-Benzoylacrylic acid
BLP	Bovine lens proteins
Boc	Butyloxycarbonyl
CH_3CN	Acetonitrile
Cys-BAA	Cysteinyl-β-benzoylbutanoic acid
Cys-3OHKG	Cysteinyl-3-hydroxykynurenine-*O*-β-D-glucoside
DCM	Dichloromethane
DHA	Dehydroascorbic acid
di-Tyr	Di-tyrosine
DOPA	3,4-Dihydrophenyalanine
D_2O	Deuterium oxide
ES-MS	Electrospray mass spectrometry
EtOH	Ethanol
EtOAc	Ethyl acetate
Em	Emission
Ex	Excitation
FOX	Peroxide assay involving the oxidation of the Fe(II)-xylenol orange complex to the Fe(III) species
Gln	Glutamine
Gly	Glycine
GSH	Glutathione
GSH-3OHKG	Glutathionyl-3-hydroxykunurenine-*O*-β-D-glucoside
GSH-Kyn	Glutathionyl-kynurenine
GSH-3OHKyn	Glutathionyl-3-hydroxykynurenine
HCl	Hydrochloric acid
His	Histidine
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
Kyn	Kynurenine
Kyn yellow	Kynurenine yellow
LC-MS	Liquid chromatography-mass spectrometry
Lys	Lysine
MeOH	Methanol
Met	Methionine
MS/MS	Tandem mass spectrometry

MW	Microwave
m/z	Mass to charge ratio
NAD(P)H	Reduced β-nicotinamide dinucleotide (phosphate)
NMR	Nuclear magnetic resonance
δ	Chemical shift
NaOH	Sodium hydroxide
3OHKyn	3-Hydroxykynurenine
$^1O_2$	Singlet molecular oxygen in its $^1\Delta_g$ state
$O_2^{\cdot -}$	Superoxide radical
3OHKG	3 Hydroxykynurenine-*O*-β-D-glucoside
3OHKyn yellow	3-Hydroxykunurenine yellow
3OHKG yellow	3-Hydroxykunurenine-*O*-β-D-glucoside yellow
pet. spirit	Petroleum spirit
Phe	Phenylalanine
Pro	Proline
PSH	Protein sulfhydryl
PTM	Post-translational modification
RP-HPLC	Reversed phase-high performance liquid chromatography
RT	Room temperature
Rt	Retention time
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	Serine
TFA	Trifluoroacetic acid
Thr	Threonine
TLC	Thin layer chromatography
Trp	Tryptophan
Tyr	Tyrosine
UDP	Uridine diphosphate
UV	Ultraviolet