STRUCTURE-ACTIVITY STUDIES ON ANTITUMOUR IMIDAZOTETRAZINONES.

KEITH RONALD HORSPOOL

A thesis submitted for the
degree of Doctor of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

4th May, 1988

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the author's prior, written consent.

The University of Aston in Birmingham

Structure-activity studies on antitumour imidazotetrazinones.

by **Keith Ronald Horspool**

Submitted for the degree of Doctor of Philosophy,
at Aston University, May, 1988.

A review of the chemical and antitumour properties of imidazotetrazinones and in particular for that of mitozolomide, 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (CMCIT) is presented. Previously proposed mechanisms of action for CMCIT are described, with special attention being given to its alkylating properties, conferred by the 3-chloroethyl substituent.

In order to obtain structure-activity data, a series of 8-substituted derivatives was synthesised and the cytotoxicity determined using a murine TLX5 lymphoma cell line. CMCIT and the 8-methylcarbamoyl derivative (MCMCIT) exhibited high cytotoxicity in vitro, which generally paralleled the high antitumour activity observed in vivo. In comparision, the 8-dimethylcarbamoyl derivative (DCMCIT), although equiactive with MCMCIT and CMCIT against the TLX5 lymphoma in the mouse in vivo, was markedly less cytotoxic than MCMCIT or CMCIT when incubated with TLX5 lymphoma cells in vitro. Incubation of DCMCIT with mouse liver microsomes and TLX5 lymphoma cells in vitro produced a significant increase in the cytotoxicity of DCMCIT. The activation was dependent on the presence of an NADPH-generating system. The increased cytotoxicity of DCMCIT when incubated with microsomes was not due to in vitro production of formaldehyde. The stability of CMCIT, MCMCIT and DCMCIT was investigated, but there was no correlation between the rate of ring-opening and cytotoxicity. HPLC analysis of the incubation mixture of DCMCIT with microsomes afforded a peak which co-chromatographed with MCMCIT.

This work implies that the presence of a free -NH group in the 8-carbamoylimidazotetrazinones is required for high antitumour/cytotoxic potential. Proposals have been made concerning the role of the amidic hydrogen, and for possible mechanisms of action of imidazotetrazinones, the most exciting theory envisaging possible targeted alkylation of DNA.

Coplanarity of an oxygen atom in the 8-substituent may also crucially affect the activity of imidazotetrazinones. Maintenance of coplanarity in active derivatives may enable these compounds to fit a theoretical receptor site described for imidazotetrazinones, putatively suggested as DNA.

KEY WORDS: IMIDAZOTETRAZINONES; ANTITUMOUR AGENTS; ALKYLATING AGENTS; STRUCTURE-ACTIVITY RELATIONSHIPS; CYTOTOXICITY.

"Writing is good. thinking is better.
Cleverness is good. patience is better."
Hermann Hesse. Siddhartha.

For Christine. my parents. and for Barry.

Acknowledgements

I would like to thank Professor M.F.G. Stevens and Dr. E. Lunt for their inspiration, guidance and support throughout this project.

I also wish to thank all members of the CRC group in this department for their friendly discussion and advice, including Dr. J.A. Hickman, Dr. C. Quarterman, Dr. J.A. Slack, Dr. M.D. Threadgill, Dr. M.J. Tisdale and especially Dr. A. Gescher for his infectious enthusiasm!

I would also like to acknowledge the technical expertise and assistance of my friends, Mr. C. Bache, Mr. J. Charlton, Mrs. K. Farrow and Dr. P.R. Lowe.

My thanks to all members of the Chemical Research Department at May and Baker, with special thanks to Dr. C.G. Newton for his helpful comments and for proof-reading this document.

I wish to express my gratitude to the Science and Engineering Research Council for provision of a CASE award and to May and Baker Limited, for generously supporting this research programme.

Finally, I would like to thank Ms. S. Swift and Mr. J. Leach for their help in the production of this thesis.

INDEX

Page No

Title Page 1

Summary 2

Dedication 3

Acknowledgements 4

Contents 5

List of Abbreviations 14

List of Figures 16

List of Schemes 18

List of Tables 20

CONTENTS

1. INTRODUCTION 23

1.1 Foreword 23

1.2 Chemistry of imidazotetrazinones 25

1.2.1 Background chemistry to the discovery of imidazotetrazinones 25

1.2.2 Synthesis and chemical reactivity of imidazotetrazinones 27

1.3 Biological and clinical evaluation of imidazotetrazinones 38

1.3.1 Antitumour activity of mitozolomide 38

1.3.2 Clinical investigations with mitozolomide 42

1.3.3 Antitumour activity of substituted imidazotetrazinones 43

1.3.4 Antitumour activity of temozolomide 45

1.4 Mode of action of imidazotetrazinones 45

1.4.1 Carbamoylation reactions 45

1.4.2 Alkylation of DNA 50

INDEX *Page No*

1.4.3 O^6 Alkylation of guanine residues in DNA 51

1.4.4 N^7 alkylation of guanine residues in DNA 57

1.4.5 Studies on the mode of action of mitozolomide 58

1.4.6 Early structure-activity studies on imidazotetrazinones 62

1.5 Current Investigations 67

2 DISCUSSION OF EXPERIMENTAL RESULTS 68

2.1 Chemistry 68

2.1.1 Synthetic routes to imidazotetrazinones 68

2.1.1.1 Optimisation of existing methods 68

2.1.1.2 Preparation of intermediates towards further 8-substituted imidazotetrazinones 76

2.1.1.2.1 Synthesis of 8-azidocarbonyl-3-(2-chloroethyl)imidazo[5.1-d]-1.2.3.5-tetrazin-4(3H)-one 76

2.1.1.2.2 Synthesis of 3-(2-chloroethyl)-8-diazoacetylimidazo[5.1-d]-1.2.3.5-tetrazin-4-(3H)-one monohydrate 78

2.1.1.2.3 Synthesis of 8-aminocarbamoyl-3-(2-chloroethyl)imidazo[5.1-d]-1.2.3.5-tetrazin-4(3H)-one 80

2.1.1.2.4 Synthesis of 8-(N-(ethoxycarbonyl-methyl)-carbamoyl]-3-(2-chloroethyl)-imidazo[5-1-d]-1.2.3.5-tetrazin-4(3H)-one 83

2.1.1.2.5 Synthetic routes towards 8-amino-3-(2-chloroethyl)imidazo[5,1-d]-1.2.3.5–tetrazin-4(3H)-one 84

INDEX *Page No*

2.1.1.2.6 Other reactions involving 3-(2- -chloroethyl)3,4-dihydro- 4-oxoimidazo[5,1-d]-1,2,3,5- tetrazine-8-carbonyl chloride 86

2.1.1.2.7 Preparation of ring-opened triazene derivatives 87

2.1.2 Stability studies on 8-carbamoylimidazo- tetrazinones 90

2.1.2.1 Ultraviolet spectroscopic determination of half-life values for 8-carbamoylimidazo- tetrazinones 90

2.1.2.2 HPLC determination of half-life values for 8-carbamoylimidazotetrazinones 92

2.1.3 X-ray analysis of imidazotetrazinones 97

2.1.3.1 Previous X-ray studies on imidazo- tetrazinones and rationale for current investigations 97

2.1.3.2 Results from present X-ray investigations 103

2.1.4 Conclusions from chemical studies 108

2.2 Biology 109

2.2.1 Cytotoxicity of imidazotetrazinones against the murine TLX5 lymphoma cell line 109

2.2.1.1 Growth characteristics of TLX5 lymphoma cells 109

2.2.1.2 Direct cytotoxicity of imidazotetrazinones 110

2.2.1.3 Cytototoxicity of 8-carbamoylimidazo- tetrazinones in the presence of mouse hepatic microsomes 116

INDEX *Page No*

2.2.1.4 Effect of NADPH and enzyme denaturation upon bioactivation of 8-carbamoylimidazotetrazinones 123

2.2.1.5 Assessment of the role of metabolically generated formaldehyde in the cytotoxicity of 8-dimethylcarbamoyl-3-(2-chloroethyl)-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one 126

2.2.1.6 Discussion of the in vitro cytotoxicity studies involving imidazotetrazinones 128

2.2.2 Metabolism and biological stability studies 132

2.2.2.1 HPLC conditions 132

2.2.2.2 HPLC analysis of the incubate of 8-carbamoylimidazotetrazinones with hepatic microsomes 134

2.2.2.3 Effect of NADPH and enzyme denaturation on metabolite formation 141

2.2.2.4 Discussion of metabolism experiments 149

2.2.2.5 Rates of disappearance and half-life determination 155

2.2.2.6 Discussion of biological stability 159

2.2.3 Conclusions from biological studies 163

2.3 Structure-activity studies 164

2.3.1 Introduction 164

2.3.2 Hansch linear free energy equations 164

2.3.3 Selection of a substituent set for analysis 166

2.3.4 Use of a hydrogen-bonding parameter 168

INDEX *Page No*

2.3.5 Results from structure-activity studies on 8-carbamoylimidazotetrazinones 169

2.3.6 Discussion of structure-activity studies 170

3 CONCLUSIONS: THE ROLE OF THE 8-SUBSTITUENT IN IMIDAZOTETRAZINONES **175**

3.1 Characteristics of hydrogen-bonding 175

3.2 Tautomerism in triazenes 177

3.3 Presence of the carbamoyl moiety in other antitumour agents 182

3.4 Prelude to molecular modelling studies on imidazotetrazinones 189

4 SUMMARY **191**

5 MATERIALS **195**

5.1 Chemicals and investigational compounds 195

5.1.1 Purchased chemicals 195

5.1.2 Gifts 195

5.1.3 Synthesised chemicals 196

5.2 Biological 196

5.2.1 Tissue culture vessels 196

5.2.2 Media 196

5.2.3 Animals 196

6 EXPERIMENTAL DETAILS **197**

6.1 Chemical methods 197

6.1.1 Preparation of 3-(2-chloroethyl)-3.4-dihydro-4-oxoimidazo[5.1-d]-1.2.3.5-tetrazine-8-carboxylic acid 198

INDEX

Page No

6.1.2 Preparation of 3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-d]-1,2,3,5-tetrazine-8-carbonyl chloride 199

6.1.3 Preparation of 8-methylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one monohydrate 199

6.1.4 Preparation of 8-dimethylcarbamoyl-3-(2--chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one 200

6.1.5 Preparation of 8-propylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one 201

6.1.6 Preparation of 8-isopropylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one 201

6.1.7 Preparation of 8-allylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one 202

6.1.8 Preparation of 8-cyclopropylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one monohydrate 203

6.1.9 Preparation of 8-phenylcarbamoyl)-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one 203

6.1.10 Preparation of 8-cyclohexylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one 204

6.1.11 Preparation of 8-neopentylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one 205

6.1.12 Preparation of 8-methoxycarbonyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one 205

6.1.13 Preparation of 8-ethoxycarbonyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one 206

INDEX

Page No

6.1.14 Preparation of 8-propoxycarbonyl-3-(2-chloroethyl)imidazo[5.1-d]-1.2.3.5-tetrazin-4(3H)-one 206

6.1.15 Preparation of 8-isopropoxycarbonyl-3-(2-chloroethyl)imidazo[5.1-d]-1.2.3.5-tetrazin-4(3H)-one 207

6.1.16 Preparation of S.S-dimethyl-N-[3-(2-chloroethyl)[3H]-4-oxoimidazo[5.1-d]-1.2.3.5-tetrazin-8ylcarbonyl]sulphoximide 208

6.1.17 Preparation of 8-azidocarbonyl-3-(2-chloroethyl)imidazo[5.1-d]-1.2.3.5-tetrazin-4(3H)-one 208

6.1.18 Preparation of 3-(2-chloroethyl)-8-diazoacetyl-imidazo[5.1-d]-1.2.3.5-tetrazin-4(3H)-one monohydrate 209

6.1.19 Preparation of 8-phenylaminocarbamoyl-3-(2-chloroethyl)imidazo[5.1-d]-1.2.3.5-tetrazin-4(3H)-one 210

6.1.20 Preparation of 3-(2-chloroethyl)-8-(3.5-difluoro-phenylaminocarbamoyl)imidazo[5.1-d]-1.2.3.5-tetrazin-4(3H)-one 210

6.1.21 Preparation of 1-[3-(2-chloroethyl)-3.4-dihydro-4-oxoimidazo[5.1-d]-1.2.3.5-tetrazin-8-yl-carbonyl]-4-phenylsemicarbazide monohydrate 211

6.1.22 Preparation of 8-methoxycarbonylamino-carbamoyl-3-(2-chloroethyl)imidazo-[5.1-d]-1.2.3.5-tetrazin-4(3H)-one 212

6.1.23 Preparation of 8-[N-(ethoxycarbonylmethyl)-carbamoyl]-3-(2-chloroethyl)imidazo[5.1-d]-1.2.-3.5-tetrazin-4(3H)-one 212

6.1.24 Preparation of 8-ethylthiocarbonyl-3-(2-chloroethyl)imidazo[5.1-d]-1.2.3.5-tetrazin-4(3H)-one 213

INDEX

Page No

6.1.25 Ultra-violet determination of half-life values for decomposition of 8-carbamoylimidazo-tetrazinones in RPMI 1640 media 214

6.1.26 HPLC analysis of imidazotetrazinones 214

6.1.27 Extraction efficiency of the HPLC method 215

6.1.28 Reproducibility of the HPLC method 216

6.1.29 Reproducibility of the instrument used for the HPLC method 216

6.1.30 Infra-red measurement of NH- stretching frequencies in imidazotetrazinones 216

6.2 Biological methods 218

6.2.1 Description and origin of the TLX5S lymphoma cell line 218

6.2.2 Extraction of TLX5 mouse ascites cells 218

6.2.3 Maintenance of TLX5 lymphoma cells 218

6.2.4 In vitro cytotoxicity assays against TLX5 lymphoma cells 219

6.2.5 Preparation of mouse hepatic microsomes 219

6.2.6 Effect of the presence of microsomes upon in vitro cytotoxicity of imidazotetrazinones against TLX5 cells 220

6.2.7 Effect of the presence of NADPH upon metabolic activation of 8-dimethylcarbamoyl-3-(2-chloroethyl)imidazo[5.1-d]-1.2.3.5-tetrazin-4(3H)-one 220

6.2.8 Preincubation with inactivated microsomes and its effect upon metabolic activation of 8-dimethylcarbamoyl-3-(2-chloroethyl)-imidazo[5.1-d]-1.2.3.5-tetrazin-4(3H)-one 221

INDEX *Page No*

6.2.9 Determination of formaldehyde cytotoxicity against TLX5 lymphoma cells 221

7. APPENDICES **222**

8. LIST OF REFERENCES **232**

INDEX

LIST OF ABBREVIATIONS

AIC	5-Aminoimidazole-4-carboxamide
BCNU	1,3-Bis(2-chloroethyl)-nitrosourea
CCNU	1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea
CHO	Chinese hamster ovary
CMEIT	8-Carbamoyl-3-ethylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one
CNU	2-Chloroethylnitrosourea
COSY	Correlated spectroscopy
CYCLO	Cyclophosphamide
DCCI	1,3-Dicyclohexylcarbodiimide
DCMCIT	8-Dimethylcarbamoyl-3-(2-chloroethyl)imidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTIC	5-(3,3-Dimethyltriazen-1-yl)imidazole-4-carboxamide
HMTIC	5-[3-(Hydroxymethyl)-3-methyltriazen-1-yl]imidazole-4-carboxamide
HPLC	High pressure liquid chromatography
HS	Horse serum
ip	Intraperitoneal
ir	Infra-red
iv	Intravenous

INDEX

MCMCIT	8-Methylcarbamoyl-3-(2-chloroethyl)imidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one
MCTIC	5-[3-(2-Chloroethyl)triazen-1-yl]imidazole-4-carboxamide
MNU	N-Methyl-N-nitrosourea
mp	Melting point
MS	Mass spectrum
MTIC	5-(3-Methyltriazen-1-yl)imidazole-4-carboxamide
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
PDMT	3,3-Dimethyl-l-phenyltriazene
PIFA	Phenyl iodosyl bis(trifluoroacetate)
QSAR	Quantitative structure-activity relationship
RPMI	Rosewell Park Memorial Institute
sc	Subcutaneous
TCNU	1-(2-Chloroethyl-3-[2-dimethylaminosulphonyl]-1-ethyl)-1-nitrosourea
TMS	Tetramethylsilane
UV	Ultra-violet

INDEX

Page No

LIST OF FIGURES

1	Synthesis of various 8-substituted imidazotetrazinones	75
2	HPLC trace of authentic imidazotetrazinones	93
3	Kinetics of degradation of CMCIT in RPMI 1640 media at 37°C	94
4	Kinetics of degradation of MCMCIT in RPMI 1640 media at 37°C	95
5	Kinetics of degradation of DCMCIT in RPMI 1640 media at 37°C	96
6	The X-ray structure of CMCIT	98
7	The X-ray structure of DTIC	98
8	The X-ray structure of DCMCIT	100
9	The X-ray structure of 8-cyclohexylcarbamoyl-3-(2-chloroethyl)imidazo[5.1-d]-1.2.3.5-tetrazin-4(3H)-one	101
10	The X-ray structure of 8-ethoxycarbonyl-3-(2-chloroethyl)imidazo[5.1-d]-1.2.3.5-tetrazin-4(3H)-one	102
11	Cytotoxicity of DCMCIT during incubation with and without microsomes	120
12	Cytotoxicity of MCMCIT during incubation with and without microsomes	120
13	Cytotoxicity of CMCIT during incubation with and without microsomes	120
14	Effect of NADPH upon cytotoxicity of DCMCIT when incubated in the presence of microsomes	125
15.16.17	HPLC traces showing metabolism of DCMCIT during incubation with hepatic microsomes	135
18	Degradation of DCMCIT during incubation with microsomes and generation of the metabolite MCMCIT	137

INDEX

Page No

19	HPLC trace of authentic reference compounds for MCMCIT analysis	138
20,21	HPLC traces showing metabolism of MCMCIT during incubation with hepatic microsomes	140
22	Kinetics of decomposition of MCMCIT during incubation with and without microsomes	142
23	Kinetics of decomposition of CMCIT during incubation with and without microsomes	144
24	Kinetics of decomposition of DCMCIT during incubation with microsomes. inactivated microsomes and in the presence of microsomes without NADPH	145
25	Extended kinetic study of CMCIT degradation on incubation in the presence of microsomes	156
26	Extended kinetic study of MCMCIT degradation on incubation in the presence of microsomes	157
27	Extended kinetic study of DCMCIT degradation on incubation in the presence of microsomes	158
28	Extended kinetic study of DCMCIT degradation on incubation in the presence of inactivated microsomes	160
29	Extended kinetic study of MCMCIT degradation on incubation in the presence of inactivated microsomes	161
30	Extended kinetic study of CMCIT degradation on incubation in the presence of inactivated microsomes	162
31	Postulated interaction of CMCIT with DNA	188

INDEX

Page No

LIST OF SCHEMES

1	The Finger reaction	26
2	Theoretical decomposition products of mitozolomide	28
3	Reaction mechanisms for the synthesis of mitozolomide	30
4	Decomposition of mitozolomide under alcoholic conditions	33
5	Decomposition of mitozolomide under aqueous conditions	35
6	Postulated intermediates produced from mitozolomide during reaction with nucleophiles	37
7	Crosslink formation in DNA produced by alkylating agents	54
8	Synthesis of 3-(2-chloroethyl)-3.4-dihydro-4-oxoimidazo[5.1-d]-1.2.3.5-tetrazine-8-carboxylic acid	69
9	Synthesis of 3-(2-chloroethyl)-3.4-dihydro-4-oxoimidazo[5.1-d]-1.2.3.5-tetrazine-8-carbonyl chloride	71
10	Synthesis of 3-(2-chloroethyl)-3.4-dihydro-4-oxoimidazo[5.1-d]-1.2.3.5-tetrazine-8-carbonyl chloride using a dimethylformamide catalyst	72
11	8-Carbamoylimidazotetrazinones synthesised from 3-(2-chloroethyl)-3.4-dihydro-4-oxoimidazo[5.1-d]-1.2.3.5-tetrazine-8-carbonyl chloride	74
12	Synthesis of various 8-substituted imidazotetrazinones	77
13	Synthetic pathway towards 8-amino-3-(2-chloroethyl)-imidazo[5.1-d]-1.2.3.5-tetrazin-4(3H)-one	79

INDEX

Page No

14	Synthetic pathway towards the homologous 8-acetic acid of 3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxylic acid	81
15	Synthetic pathway towards 8-amino-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one using a Hofmann-type reaction	85
16	Degradation of 8-carbamoylimidazotetrazinones in aqueous sodium carbonate	89
17	In vivo metabolism of cyclophosphamide	118
18	Metabolism of DCMCIT in vivo	131
19	Metabolism of 8-piperidinocarbonyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one in vivo	133
20	Metabolism of 3,3-dimethyl-1-phenyltriazene during incubation with hepatic microsomes	151
21	Metabolism of DTIC	153

INDEX

Page No

LIST OF TABLES

1	Activity of mitozolomide and other drugs in the National Cancer Institute murine tumour panel	41
2	Chemical structures and antitumour activity of imidazotetrazinones	44
3	Activity of temozolomide against murine survival-time models, including resistant lines	46
4	Comparison of temozolomide and DTIC against murine tumour systems	47
5	Decomposition of 8-carbamoylimidazotetrazinones in RPMI (pH 7.4) at 37°C as determined by an ultra-violet spectroscopic method	91
6	Kinetics of CMCIT degradation in RPMI 1640 media	94
7	Kinetics of MCMCIT degradation in RPMI 1640 media	95
8	Kinetics of DCMCIT degradation in RPMI 1640 media	96
9	Cell dimensions and space group symmetry for several imidazotetrazinones	104
10	Deviations of non-hydrogen atoms for the least-squares plane through the imidazotetrazinone ring system	105
11	Comparison of torsion angles in various 8-substituted imidazotetrazinones	106
12	Cytotoxicity of 8-substituted imidazotetrazinones against a murine TLX5 lymphoma cell line in vitro	111
13	Cytotoxicity of 8-carbamoylimidazotetrazinones against a murine TLX5 lymphoma cell line in vitro	112

INDEX

Page No

14	Antitumour activity of 8-carbamoylimidazotetrazinones against TLX5 lymphoma in vivo	113
15	In vitro cytotoxicity of cyclophosphamide against the TLX5 cell line during incubations with hepatic microsomes	117
16	Incubation of DCMCIT with hepatic microsomes and the effect on cytotoxicity against TLX5 cells	119
17	Incubation of CMCIT with hepatic microsomes and the effect on cytotoxicity against TLX5 cells	121
18	Incubation of MCMCIT with hepatic microsomes and the effect on cytotoxicity against TLX5 cells	122
19	Effect of NADPH on cytotoxicity of DCMCIT when incubated with hepatic microsomes	124
20	Effect of incubation with inactivated microsomes on cytotoxicity of DCMCIT	127
21	Cytotoxicity of formaldehyde against the TLX5 lymphoma cell line	129
22	Decomposition of DCMCIT in incubations with liver microsomes and generation of the MCMCIT metabolite	136
23	Decomposition of MCMCIT in incubations with and without microsomes	139
24	Decomposition of CMCIT in incubations with and without microsomes	143
25	Results obtained for extraction efficiency of the HPLC method	146
26	Results obtained for reproducibility of the HPLC method	147
27	Results obtained for instrument reproducibility of the HPLC method	148

INDEX

Page No

28	Effect of NADPH and enzyme denaturation on degradation of DCMCIT and metabolite production	150
29	Extended kinetic study of degradation of CMCIT in RPMI 1640 media in the presence of hepatic microsomes	156
30	Extended kinetic study of degradation of MCMCIT in RPMI 1640 media in the presence of hepatic microsomes	157
31	Extended kinetic study of degradation of DCMCIT in RPMI 1640 media in the presence of hepatic microsomes	158
32	Extended kinetic study of degradation of DCMCIT in RPMI 1640 media in the presence of inactivated microsomes	160
33	Extended kinetic study of degradation of MCMCIT in RPMI 1640 media in the presence of inactivated microsomes	161
34	Extended kinetic study of degradation of CMCIT in RPMI 1640 media in the presence of inactivated microsomes	162
35	Rational substituent set for 8-substituted imidazotetrazinones	167

1 INTRODUCTION

1.1 Foreword

The imidazotetrazinones are a novel series of potent antitumour agents which were originally synthesised at Aston University in late 1980. The lead compound, 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one, mitozolomide (CCRG 81010) (1) and the 3-methyl analogue, 8-carbamoyl-3-methylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one, temozolomide (CCRG 81045) (2), were developed to clinical trial by May & Baker Ltd, Dagenham, England in partnership with members of the Pharmaceutical Sciences Institute, Aston University. When tested in animal models and human tumour xenografts, mitozolomide exhibited outstanding antitumour activity. Unfortunately, clinical evaluation of this drug has been extremely disappointing with treatment causing severe unpredictable thrombocytopenia. The clinical failure of mitozolomide has caused scepticism concerning use of the current screening tests for evaluation of new antitumour agents.

The scientific interest generated by the imidazotetrazinones demands further investigations to determine how these compounds exert their antitumour activity. It is known that the 3-substituent is crucial to the

1

2

alkylating properties of these compounds, but interestingly, substitution in the 8-position can cause considerable variation in their antitumour potential. The present study investigates the significance of the 8-substituent for the activity/cytotoxicity of the imidazotetrazinones.

1.2 Chemistry of imidazotetrazinones

1.2.1 Background chemistry to the discovery of the imidazotetrazinones

Mitozolomide exerts its potent antitumour activity probably via hydrolytic ring-opening to give the highly unstable triazene 5-[3-(2-chloroethyl)-triazen-1-yl]imidazole-4-carboxamide (3), MCTIC, NSC 157949. The mechanism for this ring-opening has its origins in the Finger reaction[1]. Finger established that 1,2,3-benzotriazin-4(3H)-one (4) underwent ring-opening in the presence of aqueous alkali to afford anthranilic acid (6) (Scheme 1). Although the 1,2,3-triazinones can decompose to give a variety of products, antitumour activity is not exhibited by these compounds.

Using a reaction based on the interaction of diazoles and isocyanates, Ege & Gilbert[2,3] devised a new general synthesis of azolotetrazinones. Until its synthesis the 1,2,3,5-tetrazine system had been believed to be inherently

$CONH_2$ H N N=N CH_2CH_2Cl N NH

3

N N NH O $\bar{O}H$ → H N N=N H CO_2^-

4

5

$-N_2$ / $+H^+$

NH_2 CO_2H

6

Scheme 1

N=N N N-N N CH_3 O CH_2CH_2Cl

7

unstable[4,5]. Interestingly, the pyrazolo[5,1-d]-1,2,3,5-tetrazinone derivatives synthesised by Ege & Gilbert (e.g. 7) failed to demonstrate any antitumour activity although it is now known that compounds containing this ring system do exhibit potent antitumour activity (See Section 1.2.2).

1.2.2 Synthesis and chemical reactivity of imidazotetrazinones

Previous studies had shown that the 3-substituted-1,2,3-benzotriazin-4(3H)-ones (8) undergo fission at the 1,8a, 2,3 or 3,4 bond[6] (8) and that the sites of these fission reactions are influenced by the nature of the N-3 substituent[7]. In comparison, the 3-substituted-imidazo[5,1-c]-1,2,4-triazin-4(3H)-ones (9) were shown to ring-open in the presence of nucleophiles (e.g. hydrazine) by fission of the 4,5 bond only[8]. Consideration of this data led to a prediction by Stevens that inclusion of an extra heteroatom in the bicyclic 1,2,3,5-tetrazine (1) would induce lability in four different bonds 1,8a, 2,3, 3,4 and 4,5 (10). Synthesis of the imidazo[5,1-d]-1,2,3,5-tetrazinones was attempted in the belief that ring-opening would lead to an array of products, some of which had previously exhibited antitumour activity. For example, it was proposed that mitozolomide might revert to the diazoimidazole (11) and chloroethyl isocyanate (12),

CONH$_2$ N N N N N R O

8 9

10

CONH$_2$ N N N N N CH_2CH_2Cl O

1

CONH$_2$ $\overset{+}{N}\equiv N$ N N^- + $ClCH_2CH_2N{=}C{=}O$ CONH$_2$ H N N N CH_2CH_2Cl N NH

11 12 3

Scheme 2

i.e. by cleavage of the 2,3 and 4,5 bonds, or undergo hydrolytic attack at C-4 with subsequent cleavage of the 3,4 and 4 ,5 bonds to liberate Shealy's MCTIC (3)[9,10,11] (Scheme 2).

The mechanisms proposed for the synthesis of the imidazotetrazinones are shown schematically in Scheme 3. Reaction may occur via an initial [3+2] cycloaddition (i) to form unstable spirobicycles (13) which could rearrange spontaneously by a [1,5] sigmatropic shift to the imidazotetrazinones (1), this pathway being based on previous work which had shown that the reaction of heterocyclic diazo compounds with dipolarophiles proceeded via an initial 1,3-dipolar cycloaddition with subsequent rearrangement[12]. Another possibility is based on the proposal by Ege & Gilbert[2,3] (See Section 1.2.1), of a [7+2] cycloaddition process (iii) (14). Finally, an ionic mechanism (ii) may be involved with initial nucleophilic attack by the imidazole ring nitrogen at the electrophilic carbonyl group of the isocyanate, leading to production of the dipolar intermediate (15) with subsequent ring closure. Stone[10] suggested pathway (ii) to be the most likely candidate for this reaction mechanism.

Imidazotetrazinones produced via this method were cream or pastel coloured powders which were soluble in dimethylformamide (DMF), dimethylsulphoxide (DMSO) and 1-methyl-2-pyrrolidinone, and sparingly

[3+2] (i)

(ii) Ionic

13

1

15

(iii) [7+2]

14

Scheme 3

soluble and unstable in alcohols. Crude products were often contaminated with a maroon pigment which was identified as the imidazolylazo-imidazole[10,11](16) probably formed as a result of a coupling reaction[13] between 5-diazoimidazole-4-carboxamide (Diazo-IC) (11) and some contaminating 5-aminoimidazole-4-carboxamide, AIC (17). Melting points for the imidazotetrazinones ranged between 138° and 210°C and were associated with violent decomposition and effervescence[10]. The imidazotetrazinones showed no detectable deterioration for several months if stored under dry, dark conditions[10].

Decomposition studies employing mitozolomide demonstrated that the route of degradation was extremely sensitive to reaction conditions. In methanol alone conversion to 2-azahypoxanthine (20) and methyl-N-(2-chloroethyl)carbamate (21) was slow; in the presence of ammonia as catalyst, however, the reaction was accelerated. The reaction follows first-order kinetics[10]. The proposed mechanism (Scheme 4, $R=CH_3$) involves initial reversible formation of hemiacetals (18), followed by proton loss from (18) (hence the effect of base), which causes cleavage of the 4,5-bond to produce unstable triazenes (19). Formation of 2-azahypoxanthine (20) and the carbamates (e.g. 21) is probably achieved via intramolecular cyclisation with subsequent expulsion of the terminal N-moiety. The methanolic cleavage is also accelerated by heat. In addition to products

CONH2 HN NH2 N=N N CONH2 N NH

16

CONH2 NH2 N NH

17

$CONH_2$ N N N N N CH_2CH_2Cl R O H O **1**

$CONH_2$ N N N N N CH_2CH_2Cl RO O-H $\bar{O}H$ **18**

NH_2 O N N N CH_2CH_2Cl N NH O OR **19**

$ClCH_2CH_2NHCO_2R$ + O N NH N N H **20**

21

ROH

$R=CH_3$

N CO_2R + N_2 N H NH_2 **22** **23**

Scheme 4

(20) and (21) however, one also obtains secondary methanolic cleavage products of 2-azahypoxanthine (22). Similar degradation reactions are observed using ethanol in place of methanol. It has been suggested that the ester byproducts (22) probably arise by alcohol-initiated ring-opening of 2-azahypoxanthine, since similar cleavage has been observed with 1,2,3-benzotriazin-4(3H)-one[14].

Decomposition of mitozolomide in aqueous conditions has been shown to be pH dependent. Mitozolomide is stable in dilute aqueous acid and in anhydrous strong acids, e.g. in warm (60 —65°C) concentrated sulphuric acid. In contrast, at higher pH values mitozolomide ring-opens; degradation of mitozolomide in 5% aqueous sodium carbonate has been used to prepare MCTIC[11]. Later studies also led to the isolation of AIC (17) and chloroethanol (28) following decomposition of mitozolomide in phosphate buffer. Half-life determinations employing various buffer solutions have established that minor changes in pH can produce a profound effect on the half-lives of imidazotetrazinones[15,16]. The proposed reaction mechanism for decomposition of mitozolomide in aqueous systems is given in Scheme 5[11]. The mechanism involves initial attack at C4 leading to ring-opening to produce unstable carbamic acids ((24) or (25) depending on which bond breaks), with decarboxylation giving MCTIC (26, 27). MCTIC alkylates water via a S_N2-type

$CONH_2$
CH_2CH_2Cl
1

$CONH_2$ CH_2CH_2Cl CO_2H
24

$CONH_2$ HO_2C CH_2CH_2Cl
25

$-CO_2$ $-CO_2$

Azoimidazole tautomer
$CONH_2$ CH_2CH_2Cl
26

Aminoimidazole tautomer
H_2N H_2O: CH_2CH_2Cl
27

AIC
17

N_2
23

$HOCH_2CH_2Cl$
28

Scheme 5

mechanism with subsequent formation of AIC (17), nitrogen (23) and 2-chloroethanol (28). It has also been proposed that the aminoimidazole tautomer (27) might possess an intramolecular hydrogen-bond which leads to activation of the electrophilic α-methylene group of MCTIC to attack by nucleophiles.

Baig & Stevens[17] have recently investigated the discrepancy between the decomposition of mitozolomide and that of other 3-alkylimidazotetrazinones by examining the degradation of different 3-substituted imidazotetrazinones by various nucleophiles. Evidence obtained suggests that mitozolomide cleaves in the presence of oxygen and nitrogen nucleophiles exclusively at the C(4)-N(5) bond, whereas 3-methyl- and 3-ethyl-imidazotetrazinones also fragment at the N(3)-C(4) bond to a minor extent. It has also been suggested that the tetrahedral adduct (29) produced from attack of nucleophiles at C(4) in mitozolomide (Scheme 6) might be aligned favourably for intramolecular cyclisation to form an oxazolidine intermediate (30). Such a species can only be formed from mitozolomide, but although it is theoretically possible that this intermediate might cleave exclusively at the observed bond, it is difficult to imagine why this should occur selectively, since previous evidence has shown that the C-Cl bond remains intact in the presence of nucleophiles[11].

Interest generated by mitozolomide led to synthesis of a large number of

1

29

30

Nuc=Nucleophile

Scheme 6

analogues substituted at various positions in the imidazotetrazinone structure. The scope of the reaction for preparation of these compounds has therefore been extensively studied[10,11,18]. Substitutions at the 3-position of the molecule were performed by reaction with the appropriate isocyanate. Cycloadducts, however, were not formed with cyclohexyl, n-butyl, t-butyl, n-tridecyl and n-pentadecyl isocyanates under the conditions tried. Likewise, phenyl-(p-tolyl)isothiocyanate and NN′-diphenyl-, NN′-di-p-tolyl, and NN′-dicyclohexylcarbodiimides also failed to participate in the reaction[10].

Synthesis of derivatives containing other ring systems in place of the imidazole moiety led to the discovery that, despite previous findings (See Section 1.2.1), the pyrazolotetrazinones if appropriately substituted do display antitumour activity[19,20].

1.3 Biological and clinical evaluation of imidazotetrazinones

1.3.1 Antitumour activity of mitozolomide

Mitozolomide possesses potent antitumour activity against a broad spectrum of murine tumours[21]. In animal screening models, single doses between 20 and 40mg/kg mitozolomide elicited cures against L1210 and P388 leukaemias irrespective of the route of tumour and/or drug

administration. Screening against the TLX5 lymphoma (sc) and B16 melanoma (ip) also demonstrated the potent antitumour activity of mitozolomide. Excellent results were also obtained against various solid tumour models with complete cures of the Lewis lung tumour, colon 38 tumour, M5076 sarcoma & ADJ/PC6A plasmacytoma[22]. These results established mitozolomide to be one of the most potent compounds screened against the National Cancer Institute murine tumour panel. Comparison of the activity of mitozolomide with that of other widely used agents in these animal models demonstrated that it was equiactive with cis-platinum (31), 1,3-bis(2-chloroethyl)-nitrosourea, BCNU (32), adriamycin (33) and cyclophosphamide (34), and superior to 5-(3,3-dimethyltriazen-1-yl)imidazole-4-carboxamide, DTIC (35) (Table 1).

In cross-resistance studies, mitozolomide was inactive against a L1210 leukaemia made resistant to BCNU and against a TLX5 lymphoma resistant to dimethyltriazenes. However, animals bearing the L1210 leukaemia with derived resistance to cyclophosphamide maintained their sensivity towards the effects of mitozolomide, with cures being elicited in these animals.

Fodstad et al[23] examined the antitumour activity of mitozolomide against several xenografted human tumours. Excellent antitumour activity was

$H_3N \rightarrow Pt(Cl)_2 \leftarrow NH_3$

31

$ClCH_2CH_2-NH-C(=O)-N(N=O)-CH_2CH_2Cl$

32

O, OH, O, C–CH_2OH, OH, OCH_3, O, OH, H, O, H_3C, O, H, HO, NH_2

33

H, N, O, P, O, $N(CH_2CH_2Cl)_2$

34

$CONH_2$, N=N–N(CH_3)(CH_3), N, NH

35

Table 1: Activity of mitozolomide and other drugs in the National Cancer Institute murine tumour panel[24]

DRUG	L1210 leukaemia	P388 leukaemia	B16 melanoma	LL carcinoma	Colon 26 tumour	C38 adeno-carcinoma	$CD8F_1$ mammary
Mitozolomide	++	++	++	++	++	++	+
Nitrogen mustard	++	+	++	inactive	++	+	+
BCNU	++	++	++	++	++	+	+
DTIC	++	+	+	++	inactive	+	+
Cisplatin	++	++	++	++	++	+	++
Cyclophosphamide	++	++	++	++	++	++	++
Methotrexate	++	++	inactive	+	inactive	inactive	+
Adriamycin	++	++	++	++	++	inactive	++
% T/C activity criteria (++)	>150	>175	>150	>150	>150	<10	<10
(+)	>125	>120	>125	>140	>130	<42	<42

observed against sarcomas, melanomas and colon cancers; this led to anticipation that mitozolomide might produce clinical responses in sarcoma, melanoma, and possibly colon cancer.

1.3.2 Clinical investigations with mitozolomide

In Phase I clinical trial Newlands et al[25] established that the dose-limiting toxicity of mitozolomide was thrombocytopenia produced at a dose of >115 mg/m^2, with recovery from the thrombocytopenia being delayed for up to eight weeks. Later, Joss et al[26] conducted an abbreviated Phase I clinical trial which resulted in a recommendation that the iv dose of mitozolomide should be 100 mg/m^2 for previously untreated and 90 mg/m^2 for previously treated patients in Phase II trials.

Unfortunately in Phase II clinical trials[27] unpredictable myelosuppression occurred despite dose reduction from 115 mg/m^2 orally to 90 mg/m^2 or 70 mg/m^2. Although some response was seen against small cell lung cancer (response rate 28%) and possibly melanoma (response rate 11%) further clinical investigation was difficult because of the unpredictable and frequently cumulative myelosuppression. Phase II trials conducted by other workers[28] have also shown that orally administered mitozolomide is

active against malignant melanoma. The apparent selective nature of bone marrow toxicity has led to the suggestion[28] that future use of mitozolomide might be in conjunction with bone marrow transplantation, the hypothesis being that following aspiration of the bone marrow, patients may be treated with high doses of drug before reinfusing the bone marrow.

1.3.3 Antitumour activity of substituted imidazotetrazinones

A series of substituted analogues have been tested against murine tumour cell lines (Table 2)[20] with certain derivatives exhibiting outstanding activity, notably the 8-methylsulphonyl and the 8-sulphonamido derivatives. Comprehensive antitumour data for various analogues of mitozolomide has recently been published[19,29]. The search for a less myelosuppressive derivative was unsuccessful with no obvious analogue emerging with a significantly improved therapeutic index in animals. These studies did, however, highlight qualitative differences in the activity of mitozolomide and that of its 3-methyl analogue, temozolomide. These differences were considered important enough to warrant further investigation into the activity of temozolomide.

Table 2: Chemical structures and antitumour activity of imidazotetrazinones

Structure No.	R	R1	R2	Antitumour Activity[a] L1210 leukaemia[b]	Antitumour Activity[a] TLX5 lymphoma[c]
i	$(CH_2)_2Cl$	H	$CONH_2$ (mitozolomide)	+++	+++
ii	CH_3	H	$CONH_2$ (temozolomide)	+[d]	++
iii	C_2H_5	H	$CONH_2$	-	-
iv	$(CH_2)_2Br$	H	$CONH_2$	-	+[e]
v	$(CH_2)_2CH_3$	H	$CONH_2$	NT	-
vi	$(CH_2)_2OCH_3$	H	$CONH_2$	NT	-
vii	$(CH_2)_3Cl$	H	$CONH_2$	-	-
viii	$CH_2CH(Cl)CH_2Cl$	H	$CONH_2$	-	-
ix	$CH_2CH{=}CH_2$	H	$CONH_2$	-	-
x	$CH(CH_3)C_2H_5$	H	$CONH_2$	NT	-
xi	$(CH_2)_5CH_3$	H	$CONH_2$	NT	-
xii	$CH_2C_6H_5$	H	$CONH_2$	NT	-
xiii	$(CH_2)_2Cl$	CH_3	$CONH_2$	++	+++
xiv	$(CH_2)_2Cl$	C_2H_5	$CONH_2$	++	NT
xv	$(CH_2)_2Cl$	C_3H_8	$CONH_2$	+++	NT
xvi	$(CH_2)_2Cl$	$CH(CH_3)_2$	$CONH_2$	+	NT
xvii	$(CH_2)_2Cl$	C_4H_{10}	$CONH_2$	+	NT
xviii	$(CH_2)_2Cl$	$CH_2C_6H_5$	$CONH_2$	+	NT
xix	$(CH_2)_2Cl$	C_6H_{11}	$CONH_2$	-	NT
xx	$(CH_2)_2Cl$	$(CH_2)_2C_6H_5$	$CONH_2$	-	NT
xxi	$(CH_2)_2Cl$	H	$CONHCH_3$	+++	+++
xxii	$(CH_2)_2Cl$	H	$CON(CH_3)_2$	+++	+++
xxiii	$(CH_2)_2Cl$	H	$CONC_5H_{10}$	++	+++
xxiv	$(CH_2)_2Cl$	H	$CONHCH_2C_6H_5$	++	NT
xxv	$(CH_2)_2Cl$	H	$CONHC_6H_5$	NT	+
xxvi	$(CH_2)_2Cl$	H	$CON(CH_3)C_6H_5$	-	NT
xxvii	$(CH_2)_2Cl$	H	$CONHNO_2$	NT	+++
xxviii	$(CH_2)_2Cl$	H	SO_2NH_2	+++	+++
xxix	$(CH_2)_2Cl$	CH_3	SO_2NH_2	+++	+++
xxx	$(CH_2)_2Cl$	H	SO_2NHCH_3	+++	+++
xxxi	$(CH_2)_2Cl$	H	$SO_2N(CH_3)_2$	+++	+++
xxxii	$(CH_2)_2Cl$	CH_3	$SO_2N(CH_3)_2$	+++	+++
xxxiii	$(CH_2)_2Cl$	H	SO_2CH_3	+++	+++
xxxiv	$(CH_2)_2Cl$	CH_3	SO_2CH_3	+++	+++
xxxv	$(CH_2)_2Cl$	CH_3	$SO_2C_2H_5$	+++	+++
xxxvi	$(CH_2)_2Cl$	CH_3	$SO_2C_3H_8$	+++	+++
xxxvii	$(CH_2)_2Cl$	CH_3	$SOCH_3$	+++	NT
xxxviii	$(CH_2)_2Cl$	H	C_6H_5	-	NT
xxxvix	$(CH_2)_2Cl$	H	NO_2	-	NT
xxxx	$(CH_2)_2Cl$	H	CN	NT	-

[a] The antitumour assessment shown represents the optimal result on a single-dose schedule. Activity was rated according to the following scale: +++, T/C >150% with cures at one or more dose levels; ++, T/C >150% with no cures; +, T/C >125%; -, T/C <125%, and NT, not tested.

[b] The murine L1210 leukaemia tests were conducted in accordance with the protocols described by the National Cancer Institute[24].

[c] The TLX5 lymphoma was passaged and used as described previously[24].

[d] On a repeat schedule temozolomide rates ++.

[e] T/C 137% in one test at optimum dose in single-dose schedule.

1.3.4 Antitumour activity of temozolomide

Potent activity was demonstrated by temozolomide against P388 and L1210 leukaemias, the B16 melanoma and M5076 reticulum-cell sarcoma, with curative activity against three solid tumour models i.e. the murine M5076 sarcoma and ADJ/PC6A plasmacytoma and the MX-1 human mammary xenograft[20]. In comparison with mitozolomide, which displayed optimum activity on a single-dose schedule, temozolomide required a divided dose schedule to display maximum activity. Temozolomide has shown cross-resistance to a cell line resistant to mitozolomide and a cell line resistant to DTIC (Table 3), hence temozolomide may be acting by a similar mechanism to DTIC. Comparison with the clinically useful agent DTIC (Table 4) is favourable and this has led to assessment of temozolomide in a disease directed Phase I clinical trial against melanoma.

1.4 Mode of action of imidazotetrazinones

1.4.1 Carbamoylation reactions

Carbamoylation reactions are exhibited by isocyanates produced from decomposition of nitrosoureas, e.g. BCNU (32)[30,31]. The organic isocyanate moieties generated are responsible for carbamoylating intracellular proteins[32,33], chiefly at amino acids at sites of "active"

Table 3: Activity of temozolomide against murine survival-time models, including resistant lines

TUMOUR	Schedule (day of injection)	Optimum dose (mg/kg/day)	Optimal T/C (%)	Assessment
P388 leukaemia	1	200	143	+[c]
	1-4	100	214	++[c]
	1-5	200	>214	+++[c]
P388/mitozolomide	1	200	110	-
	1-4	100	112	-
L1210 leukaemia	1	200	149	+
	1-4	100	200	++
L1210/DTIC	1	200	116	-
	1-4	100	108	-
L1210/BCNU	1	200	148	+
	1-4	100	175	++
B16 melanoma	1-9	100	181	++
M5076 reticulum cell sarcoma	1,5,9,13	200	170	++
TLX5 lymphoma	3	160	151	++
	3,6,9	80	154	++
	3-7	40	181	++

a For reference to protocols, see footnote b. Table 2

b See footnote a. Table 2

c For the P388 tumour, +++. T/C >175% with cures at one or more dose levels; ++. T/C >175% with no cures; +. T/C >120%

Table 4: Comparison of temozolomide and DTIC against murine tumour systems

MODEL	TUMOUR	OPTIMAL T/C% or THERAPEUTIC INDEX	
		Temozolomide	DTIC
MURINE ASCITIC SURVIVAL TIME	TLX5 lymphoma[a]	181	180
	L1210 leukaemia[a]	200	160
	P388 leukaemia[a]	>254	166
	B16 melanoma[a]	181	145[d]
	M5076 reticulum[a] cell sarcoma	200	NT[e]
MURINE SOLID	M5076 reticulum[b] cell sarcoma	6.5	8.1
	ADJ/PC6A plasmacytoma[b]	>8	50
MURINE HUMAN XENOGRAFT	MX-1 mammary[c]	15	37[d]

a Increase in survival time
b Therapeutic index, LD50/ID50
c Tumour volume change
d Date from Goldin et al[24]
e NT, not tested

hydrogen groups, such as Σ-amino groups of lysine or arginine.

A number of workers have suggested a biological role for carbamoylation, and its contribution towards antitumour activity or toxicity. The diversity of biological consequences caused by carbamoylation includes inhibition of DNA polymerase II[34,35] and human leukaemia DNA polymerase II[36], and reduction of nucleolar RNA processing[37], as well as inhibition of glutathione reductase[38], α-chymotrypsin[39,40], and the polymerisation of tubulin[41]. Kann et al[42,43,44] demonstrated that isocyanates can inhibit the repair of X-irradiation damage to DNA, and consequently such inhibition of repair processes could enhance the therapeutic effect of drugs causing alkylation damage, although it may also increase their carcinogenic potential and toxicity in normal tissues.

Studies were intensified following synthesis of chlorozotocin (36)[45] which consisted of a chloroethylnitrosourea moiety from (37) attached to the C-2 position of glucose and which possessed excellent antitumour activity, despite negligible carbamoylation properties. This finding led to the suggestion that carbamoylation is not a major factor in antitumour activity[30,33]. It was also shown that there was no correlation with granulocycte suppression[46], lethal toxicity[47], or myelotoxicity[48,49,50].

36

37

38

In contrast to the above reports, Gibson & Hickman[51] showed that isocyanates generated from BCNU (32) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, CCNU (38), possessed some degree of selective toxicity towards TLX5 tumour cells. They postulated that isocyanate production could be particularly important for the toxicity of nitrosoureas expressed in TLX5 lymphoma cells, and that molecules that release a chloroethyl isocyanate, rather than a haloalkyl species, could be active antitumour agents.

Obviously the role of carbamoylation in cancer chemotherapy has still to be fully explained. However, a recent review[52] relating structure to anticancer activity and toxicity of nitrosoureas has confirmed earlier conclusions that carbamoylation does not seem to be a major factor in the activity or toxicity of these compounds.

1.4.2 Alkylation of DNA

In addition to its carbamoylating properties, mitozolomide was also envisaged as possessing alkylating properties via hydrolytic ring-opening to give MCTIC (3). MCTIC is a chloroethylating species which could alkylate nucleophiles by a S_N2 type mechanism (Scheme 5). In biological macromolecules, alkylation reactions result in addition of alkyl groups to

nitrogen, sulphur or oxygen atoms[53,54,55]. Although a variety of potential alkylation sites exist, for these investigations only O^6 and N^7 lesions of guanine residues of DNA will be considered.

1.4.3 O^6 Alkylation of guanine residues in DNA

Addition of an alkyl group to O^6 of guanine leads to misreading of the genetic code, that is, it is a pro-mutagenic lesion. A GC-AT single point mutation occurs as guanine (39) is now read as adenine (40)[56,57,58]. There is evidence that O^6 alkylation plays a definite role in carcinogenesis and mutagenesis[59,60,61,62] .

In addition to the results outlined above, studies[63,64] involving the chloroethylnitrosoureas have shown that O^6-chloroethyl adducts can undergo a second reaction with elimination of chloride ion. This results in a crosslink consisting of an ethano (CH_2CH_2) bridge between two nucleophilic atoms. Two types of DNA crosslinks have been observed: (i) that formed between two guanine-N^7 positions, (ii) that formed between a guanine-N^1 and a cytosine-N^3 postion[63,64]. Crosslink (i) is almost certainly confined to adjacent guanines in the same DNA strand (intramolecular), and little is known about its biological significance.

39 41

40 42

It is the G-C interaction which is considered to be an interstrand crosslink of major importance. A mechanism for production of this crosslink was postulated by Tong & Ludlum[63] (Scheme 7). It was suggested that initial chloroethylation of the guanine-O^6 position (43) disrupts the hydrogen-bonding to the paired cytosine and makes the guanine-N^1 position accessible. The chloroethyl group at O^6 then reacts intramolecularly with N^1, eliminating chloride and forming an ethano bridge (44). The resulting N, O^6-ethanoguanine moiety (45) is a resonance stabilised cation which is susceptible to nucleophilic attack at the ethano group, the O^6 being the leaving group. If the nucleophile in this reaction is a cytosine-N^3, then the result is thought to be an interstrand cross-link via the ethano-GC moiety (46).

It has been shown that for the chloroethylnitrosoureas cross-linked DNA strands are produced both in reaction with purified DNA[65,66] and in cells[67,68,69]. Experiments designed to assess the extent and significance of cross-link formation have been extremely difficult due to the presence of a repair enzyme, O^6-alkylguanine-DNA-alkyltransferase[70,71,72]. Cells proficient in this type of repair are designated Mer+ or Mex+ while those deficient are Mer- or Mex-. The Mer+ protein appears to possess a broad substrate specificity, being able to remove methyl, ethyl, hydroxyethyl, and, supposedly, chloroethyl adducts[73,74]. The alkyl groups are rapidly removed from the guanine O^6-postion and are transferred to a thiol group

$ClCH_2CH_2-X$

NH_2

41

39

$Cl-CH_2CH_2O$

43

$+ \bar{Cl}$

44

45

dr=deoxyribose

46

Scheme 7

of a protein cysteine moiety[70,75].

Thus if the Mer+ system is able to remove chloroethyl groups from the guanine O^6-position then it is possible that this system can prevent interstrand cross-linking by chloroethylnitrosoureas. Erickson[76] has shown that Mer+ strains in vitro clearly show less interstrand cross-linking than do Mer- strains. There have been many attempts to use this repair system as a device to explain the significance of O^6 alkylation and cross-link formation. Early studies showed that Mer+ character enhances the ability of cells to survive treatment with N-methyl-N-nitrosourea, MNU (47)[77,78] or with chloroethylnitrosoureas[76]. This improved survival to MNU suggests, but does not prove that methylation at guanine-O^6 is an important contributor to cell killing, since the Mer+ phenotype might be able to influence several different repair processes. Similarly, the increased survival of such cells to chloroethylnitrosoureas suggests, but does not prove, that interstrand cross-link formation is a major contributor to cell killing by this drug. Recently a correlation between the ability to repair alkyl lesions at the O^6-postion of guanine and cytotoxicity has been observed with both the nitrosoureas[79] and triazenes[80] and suggests that this base modification is a potentially cytotoxic lesion. The contribution of individual DNA lesions to the mutagenic and cytotoxic effects of alkylating

$$H_2N{-}\overset{\displaystyle O}{\overset{\|}{C}}{-}\underset{\displaystyle N{=}O}{\underset{|}{N}}{-}CH_3$$

47

$$HO{-}N{=}N{-}CH_2CH_2Cl$$

48

agents has also been investigated using cloned genes that code for specific DNA repair functions to complement repair deficient cells[81,82,83,84]. Studies involving Chinese hamster V79 and murine haemopoietic stem cells have provided good evidence to support a major role for O^6-alkylguanine in cellular lethality following alkylating agent treatment.

The bulk of the evidence hence favours alkylation at the O^6-position of guanine as the crucial event in the cytotoxic action of alkylating agents. The role of the subsequent cross-link formation is not clear. However, the majority of evidence suggests that it is probably a less significant event than the initial alkylation step.

1.4.4 N^7 alkylation of guanine residues in DNA

Alkylated purines are lost spontaneously from DNA, producing apurinic sites[85]. This ultimately leads to single strand breaks in the DNA, and is presumed to account for the cytotoxicity of the alkylating agents. Of these alkylated purines, 7-methylguanine has received most attention [86], although there is conflicting evidence concerning the role of N^7 alkylation in the antitumour activity of alkylating agents.

Current investigations examining alkylation of calf thymus DNA by

temozolomide, have shown that the N^7 position of guanine is the main site of alkylation[87,88]. The qualitative differences in antitumour activity between temozolomide and mitozolomide could be explained by their preference for different sites of alkylation. There is no evidence, however, to support this hypothesis.

1.4.5 Studies on the mode of action of mitozolomide

Although 2-chloroethyl isocyanate production is theoretically possible from decomposition of mitozolomide (Section 1.2.2), the initial screening programme suggested that this was unlikely. A comparison between the murine TLX5 lymphoma and L1210 leukaemia screening models had shown that the TLX5 cell line appeared to be sensitive to haloalkyl-nitrosoureas, this being attributed to release of isocyanate moieties from these compounds (See Section 1.4.1). No such sensitivity was observed with the L1210 leukaemia cell line[51]. Screening of mitozolomide showed that it possessed potent and broad spectrum antitumour activity against a wide range of murine tumours, including the L1210 and TLX5 cell lines (See Section 1.3.1). This data, coupled with analysis of breakdown products in vitro, suggested that 2-chloroethyl isocyanate was not, or not alone, the active species involved in the antitumour activity of mitozolomide.

Horgan & Tisdale[89,90] investigated the carbamoylating properties of

mitozolomide by studying its effect on γ-glutamyltranspeptidase, α-chymotrypsin and glutathione reductase. Comparison of mitozolomide with BCNU at equitoxic concentrations showed that whilst BCNU caused inhibition of glutathione reductase in intact TLX5 cells, mitozolomide had no significant effect on enzyme activity. Similarly, BCNU showed inhibition of both α-chymotrypsin and γ-glutamyltranspeptidase activity whereas mitozolomide had no inhibitory effect on enzyme activity. It was suggested that isocyanate generation does not occur during decomposition of mitozolomide, either in the intact cell or in aqueous solution.

Dive et al[91,92] have recently provided data which is contrary to previous findings. Using a flow cytoenzymological method they have demonstrated that mitozolomide may possess some carbamoylating activity. There seems little reason to doubt findings from the enzyme inhibition studies, although results from the flow cytoenzymological assay are intriguing and the dichotomy cannot at present be explained.

The alkylating properties of mitozolomide have also received a great deal of attention. Gibson et al,[93] using L1210 murine leukaemia cells, demonstrated that at equimolar concentrations mitozolomide possessed similar in vitro cytototoxicity to MCTIC (3) and the 2-chloroethyl-nitrosourea, CNU (37); at equitoxic concentrations they produced similar

levels of DNA interstrand cross-linking. The time course for the cross-link formation was found to be somewhat faster for CNU (6 hours) compared with mitozolomide and MCTIC, where the time taken to form cross-links was 9 hours. From these studies it was concluded that mitozolomide probably achieves its cytotoxic action against L1210 cells by ring-opening to give MCTIC (Scheme 5) with subsequent formation of DNA cross-links. The cross-linking ability of mitozolomide was further studied by examining its effect on cell viability and cellular DNA integrity of a Mer proficient cell line, IMR 90 (Mer+) and a repair deficient line VA-13 (Mer-)[94]. Comparison with MCTIC showed that DNA-protein cross-link formation was similar in both cell lines for each drug, suggesting that drug penetration and intracellular drug reactivity were similar. Little or no interstrand cross-link formation was observed in the IMR-90 cells, the VA-13 cells giving interstrand cross-link formation peaking 12 hours after drug removal. The interstrand cross-link formation in VA-13 cells was shown to be concentration dependent and a linear correlation between cross-link formation and log cell kill was obtained. No such correlation was obtained using the IMR-90 cells. By analogy to the chloroethylnitrosoureas it was proposed that mitozolomide may also produce a chloroethyldiazo species (i.e. MCTIC), previously implicated in the formation of chloroethyl-DNA adducts which convert to interstrand cross-links[75] (see section 1.4.3). This led to the hypothesis that DNA interstrand cross-link formation may be a

common mechanism for the in vitro cytotoxicity of mitozolomide and MCTIC.

Following this work, a study investigating the role of possible N^7 alkylations[95] demonstrated that, for mitozolomide, some specific alkylations were obtained in DNA sequences containing runs of three or more guanines. It was postulated, without any substantive evidence, that 2-chloroethyl diazohydroxide (48) was responsible for these alkylations, and that preferential guanine N^7 alkylations at runs of guanines did not contribute to antitumour activity. These studies also implicated reactions at guanine-O^6 in the antitumour activity of mitozolomide.

Indirect evidence for the importance of O^6 alkylations was also provided from work testing a series of alkyltriazenylimidazoles against Mer+ and Mer- cell lines[80]. These monomethylating agents were shown to produce a differential toxicity between the Mer+ and Mer- cell lines which was similar to that of analogous chloroethylating agents. It was suggested that monoalkylation of guanine O^6-positions is sufficient to account for the observed differential cell killing. It is interesting to note that Karran & Williams[96] had previously proposed that adducts at the O^6-position of guanine are not potentially cytotoxic in Raji cells.

More recent attempts to clarify the significance of O^6-guanine lesions have been performed by Margison (see Section 1.4.3). Using an E.coli alkyl-transferase (AT) gene expressed in mammalian cells, increased resistance towards the toxic effects of mitozolomide has been observed. These investigations have indicated that O^6-alkylguanine is responsible for the cytotoxicity of mitozolomide. More detailed investigations are necessary, however, to provide specific information concerning the relevance of DNA alkylation for the biological activity of mitozolomide.

1.4.6 Early structure-activity studies on imidazotetrazinones

Mitozolomide contains a number of structural features which have a long association with antitumour activity. The 'NNN' linkage in either acyclic or cyclic arrangements has been exploited in various ways following the discovery that 3,3-dimethyl-1-phenyltriazene, PDMT (49), was active against mouse sarcoma 180[97]. The most widely used triazene is DTIC (35)[98], which is a useful clinical agent mainly employed in the treatment of malignant melanoma[99,100,101]. Although DTIC was designed as a pro-drug of the highly unstable antitumour agent Diazo-IC (11)[102] it was later shown that the mechanism of action of DTIC had little to do with production of Diazo-IC[103]. Expectations that degradation to AIC (17) would lead to antimetabolite properties[104,105] were also unfounded, since

49

50

inhibition of purine biosynthesis was unrelated to the mechanism of action of DTIC[106]. It was later suggested that monomethyltriazenes such as 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide, MTIC (50), cause methylation of biological nucleophiles such as DNA[107]. Thus to achieve this active structure DTIC requires metabolic N-demethylation[106,108,109]. The requirement for metabolic activation of DTIC to give the active species is one possible reason why DTIC only produces temporary responses in 19% of patients treated[110]. The presence of alkyltransferase repair enzymes may also be a reason for this poor response (see Section 1.4.3). Human beings are rather poor at N-demethylation[111], and thus a considerable escalation in dose is required to produce the necessary concentration of active metabolite in vivo. The metabolic activation also leads to production of formaldehyde which is itself cytotoxic (see Section 2.2.1.5) and may be responsible for some of the side effects of DTIC. In comparison, the imidazotetrazinones were designed to undergo hydrolytic ring-opening to release active triazenes without microsomal metabolism or concomitent release of formaldehyde.

The preferred peripheral substituents of 8-carbamoyl and 3-(2-chloroethyl) in mitozolomide were arrived at by consideration of the structural features of BCNU (32) and DTIC (35). To date the following information is available concerning structural modifications to imidazotetrazinones:

:

(i) Several 3-substituted derivatives of mitozolomide have been prepared but only the 3-methyl derivative (2) displays pronounced antitumour activity (see Section 1.3.3); and (ii) Other 3-alkyl analogues are less active or inactive against the TLX5 tumour on a single dose schedule.

These preliminary findings led to the suggestion that the 2-chloroethyl group is the substituent of choice at the 3-position[20]. It is the 3-substituent which imparts the alkylating and hence antitumour activity to the imidazo-tetrazinones (see Section 1.4.5). However, alteration of the 6- and 8-substituents can also cause perturbation of antitumour activity. Addition of a 6-alkyl group onto the mitozolomide structure gives a series of compounds with graded activity against the L1210 tumour (See Section 1.3.3). This substitution at position 6- of the imidazotetrazinone structure is not deleterious to activity when the group is small or linear, but large, bulky groups (e.g. cyclohexyl and phenethyl) appear to be distherapeutic. Lunt et al[19] have suggested that this possibly reflects:

(i) an increased Π value altering transport characteristics,

(ii) failure of the enlarged molecule to fit a binding site, or

(iii) inhibition of hydrolysis at position 4-, preventing ring-opening to the proposed active triazene.

Screening results obtained from a number of 8-substituted imidazotetrazinones have indicated that the substitution in the 8-position can crucially affect the antitumour activity of these compounds (see Section 1.3.3, Table 2). The preferred substituents in the 8-position of imidazotetrazinones were initially given by Stevens as carboxamido, sulphonamido and alkylsulphonyl[20]. More extensive testing of 8-substituted imidazotetrazinones has enabled the following conclusions to be drawn concerning 8-substitution[19]:

(i) Compounds containing a methyl-substituted 8-carbamoyl moiety are essentially equiactive with mitozolomide but activity decreases as the bulk of the N-alkyl substituent increases;

(ii) The 8-sulphamoyl and 8-methylsulphonyl compounds are extremely potent antitumour agents, with 3-(2-chloroethyl)-8-sulphamoylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (Table 2, xxviii) giving cure rates against the L1210 leukaemia in excess of those generated by mitozolomide;

(iii) It appears that alkyl substitution on 8-sulphamoyl analogues has the same effect as that upon 8-carbamoyl derivatives.

1.5 Current investigations

The present studies aimed to extend the information available concerning the relevance of 8-substitution to the antitumour activity of imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-ones. A number of novel 8-substituted analogues have been synthesised for inclusion in structure-activity studies, to further determine the role of the 8-substituent. A cytotoxicity assay against a murine TLX5 lymphoma cell line has been used to assess the cytotoxicity at a simple in vitro level. The concentration of drug required to produce 50% inhibition of TLX5 cell growth (IC_{50}) has been used in the QSAR studies. It was considered that the metabolic complexities intrinsic to in vivo studies precluded the use of in vivo data in structure-activity studies.

The metabolism of 8-carbamoylimidazotetrazinones has been examined using a bioassay involving incubations of drug with mouse hepatic microsomes. Metabolites produced in these experiments have been analysed using HPLC. Results from these experiments, and data from X-ray determinations, have been used to formulate several hypotheses suggesting why the choice of 8-substituent is crucial to the activity of imidazotetrazinones.

ASTON UNIVERSITY LIBRARY

2 DISCUSSION OF EXPERIMENTAL RESULTS

2.1 Chemistry

2.1.1 Synthetic routes to imidazotetrazinones

2.1.1.1 Optimisation of existing methods

Initial objectives were to improve the existing methods for preparation of 3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxylic acid (53) and 3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo-[5,1-d]–1,2,3,5-tetrazine-8-carbonyl chloride (57), and to extend the range of 8-substituted analogues. Reaction of mitozolomide (CMCIT) with nitrous acid using trifluoroacetic acid as solvent instead of sulphuric acid[29,112] gave increased yields of the acid (53). The mechanism proposed for this reaction (Scheme 8)[112] involves formation of an intermediate N-nitrosoamide (51) which undergoes hydrolysis to give the expected product (53). Present investigations indicate that the volume of water (degree of solvation) is crucial to the success of acid (53) production by the above methods[113]. The acid (53) prepared by this method is a buff coloured powder that has been shown to exist in a number of polymorphic forms[112].

The existing method for preparation of the acid chloride (57) [29,112] involving reaction of the acid (53) with thionyl chloride under reflux

Scheme 8

(Scheme 9), probably proceeds via formation of the mixed anhydride (54), which decomposes by an internal nucleophilic attack by chlorine (55) or by S_N2 displacement (56)[114]. The acid chloride (57) was envisaged as a useful intermediate towards further 8-substituted imidazotetrazinones. Although reactions involving the acid chloride (57) (in situ) were successful, this method was not widely used at May & Baker because the project came to a premature end.

In the present work, attempts were made to isolate the acid chloride (57) by employing triphenylphosphine and carbon tetrachloride in place of thionyl chloride,[115] or using thionyl chloride with a pyridine catalyst[116,117]. These reactions were not successful. Use of the reagent dimethylformamidinium chloride (60a, 60b), prepared by addition of dimethylformamide (58) to thionyl chloride, (59)[114,118] did improve the reaction, enabling isolation of the acid chloride (57) as a red crystalline solid. The postulated reaction mechanism (Scheme 10) involves formation of an intermediate (61) which breaks down to give the acid chloride (57). Preparation of this compound requires extreme care during removal of the toluene solvent. The relatively high boiling point of toluene (109-112°C) and low melting point of the acid chloride (57) (69°C), means that heating for prolonged periods at elevated temperatures to remove solvent can cause degradation of the product, giving a dark red tar.

53 → + $SOCl_2$ → 54 + HCl → 55 / 56 → ($-SO_2$) / ($-\bar{Cl}$, $-SO_2$) → 57

Scheme 9

$HCON(CH_3)_2 + SOCl_2 \rightarrow H\text{-}C(Cl)=\overset{+}{N}(CH_3)_2\ \overline{Cl} \leftrightarrow H\text{-}\overset{+}{C}(Cl)\text{-}N(CH_3)_2\ \overline{Cl}$

58 **59** **60a** **60b**

+ **53**

61

HCl + H–C(=O)–N(CH₃)₂ + COCl ... CH₂CH₂Cl

63 **62** **57**

Scheme 10

The acid chloride (57) synthesised by this method is an extremely reactive compound which reacts vigorously at room temperature with amines and alcohols[119] to give the corresponding 8-carbamoyl derivatives (64a - 64i) (Scheme 11) and 8-esters (65a - 65d) (Fig 1). This route of preparation was shown to have only limited application for synthesis of 8-disubstituted carbamoylimidazotetrazinones. That is, whilst reaction with dimethylamine afforded the 8-dimethylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (DCMCIT) (64b), reactions of the acid chloride (57) with other secondary amines such as diethylamine and dicyclohexylamine were not successful.

The range of 8-substituted derivatives prepared from the acid chloride (57)[120] is shown in Fig 1. Certain analogues were synthesised by a method employing a pyridine catalyst[121].

Previous methods employed to obtain imidazotetrazinones with 8-substituents[19] involved initial preparation of appropriate substituted AIC derivatives followed by formation of the tetrazinone ring. For example, 8-carbamoyl derivatives (64) synthesised in this manner (Scheme 12) were all prepared from 5-nitroimidazole-4-carboxylic acid (73). Reaction of this compound (73) with phosphorus pentachloride afforded the dimeric lactam (74) which in the presence of primary and secondary amines ring-opened to

COCl

HNR_1R_2

$CONR_1R_2$

CH_2CH_2Cl

57

64

a) $R_1{=}H$, $R_2{=}CH_3$
b) $R_1{=}R_2{=}CH_3$
c) $R_1{=}H$, $R_2{=}C_3H_8$
d) $R_1{=}H$, $R_2{=}{-}CH(CH_3)_2$
e) $R_1{=}H$, $R_2{=}{-}CH_2{-}CH{=}CH_2$
f) $R_1{=}H$, $R_2{=}{-}CH(CH_2)_2$
g) $R_1{=}H$, $R_2{=}C_6H_5$
h) $R_1{=}H$, $R_2{=}C_6H_{11}$
i) $R_1{=}H$, $R_2{=}{-}CH_2{-}C(CH_3)_3$

Scheme 11

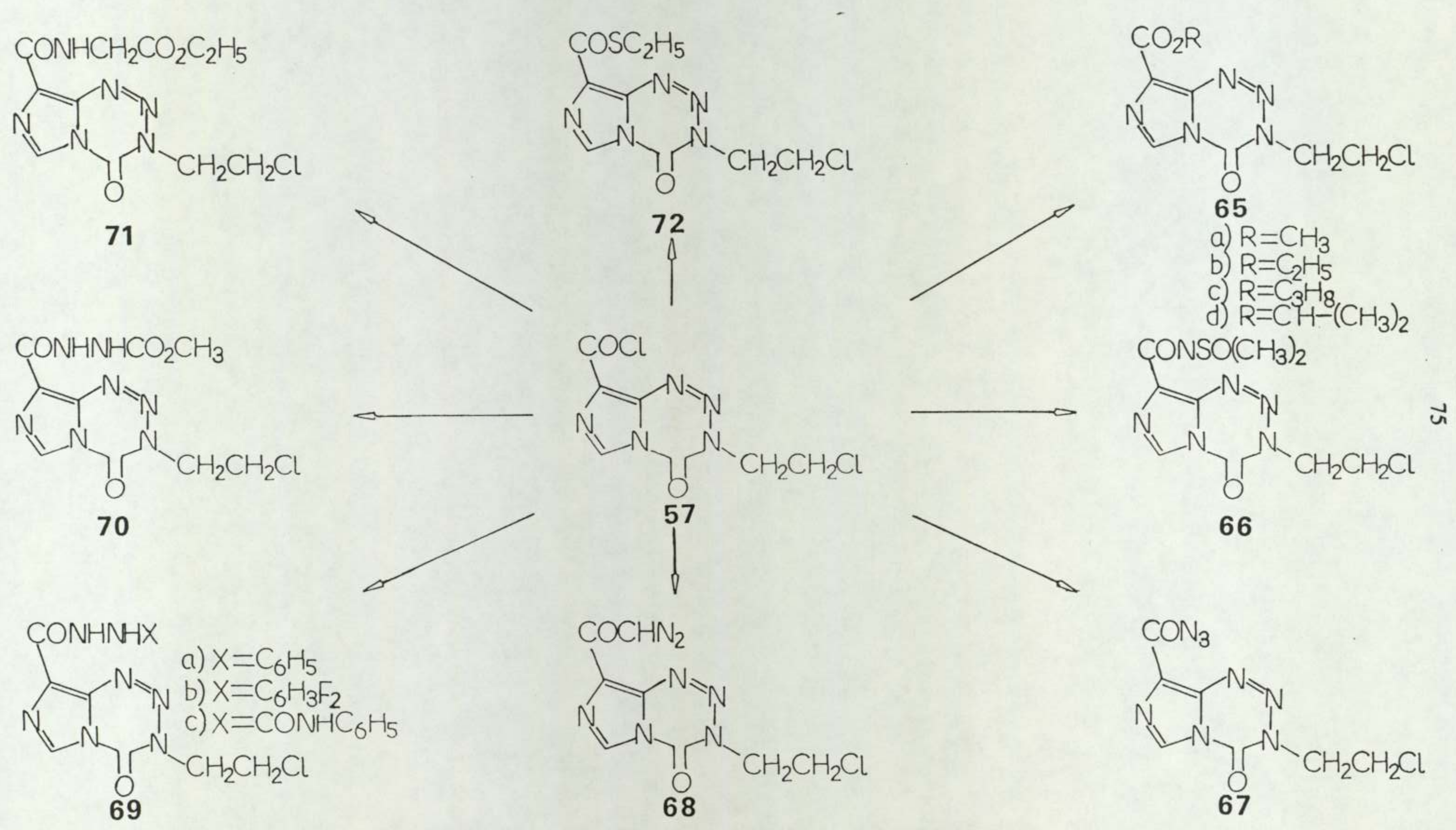


Figure 1 Synthesis of various 8-substituted imidazotetrazinones

give the 5-nitroimidazole-4-carboxamides (75). These derivatives were then reduced catalytically to the corresponding 5-aminoimidazole-4-carboxamides (76), which were diazotised with nitrous acid to give the corresponding diazoimidazoles (77). Reaction of the diazoimidazoles (77) with 2-chloroethyl isocyanate finally afforded the imidazotetrazinones (64). In comparison, the present method for 8-substitution via the acid chloride route is far less laborious and provides an extemely useful alternative for synthesis of 8-carbamoyl substituted imidazotetrazinones.

2.1.1.2 Preparation of intermediates towards further 8-substituted imidazotetrazinones

Several 8-substituted derivatives were prepared with the expectation that they would act as valuable intermediates for extending the range of 8-substituted imidazotetrazinones. The theoretical basis for selection of these intermediates is outlined in the following sections.

2.1.1.2.1 Synthesis of 8-azidocarbonyl-3-(2-chloroethyl)-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

8-Azidocarbonyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (67) was synthesised by two routes, the most effective method involving

CO_2H, NO_2, N, NH — **73**

PCl_5 →

O, NO_2, N, N, N, N, NO_2, O — **74**

R_1R_2NH ↓

$CONR_1R_2$, NO_2, N, NH — **75**

← H_2

$CONR_1R_2$, NH_2, N, NH — **76**

HNO_2 ↓

$CONR_1R_2$, $\overset{+}{N}\equiv N$, N, N^- — **77**

$ClCH_2CH_2NCO$ →

$CONR_1R_2$, N=N, N, N, N–CH_2CH_2Cl, O — **64**

a) R_1, R_2 = $N(CH_2)_4CH_2$ (ring)
b) $R_1 = CH_3$, $R_2 = H$
c) $R_1 = R_2 = CH_3$
d) $R_1 = CH_3$, $R_2 = C_6H_5$
e) $R_1 = CH_2C_6H_4OCH_3$, $R_2 = C_6H_5$
f) $R_1 = CH_2C_6H_4OCH_3$, $R_2 = CH_2C_6H_5$

Scheme 12

reaction of the acid chloride (57) with sodium azide in aqueous acetone. Reaction of the acid chloride (57) with trimethylsilyl azide[122-126] was also successful; however, the sodium azide method was superior. Acid azides can be decomposed to isocyanates and nitrogen, this reaction being known as the Curtius rearrangement[127]. The reaction is a preparative method for isocyanates and for compounds derivable from isocyanates, such as urethans, ureas, amides and amines. It was envisaged that the azide (67) would undergo a similar thermal rearrangement (Scheme 13)[128,129] to yield 8-isocyanato-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (79) and, subsequently, 8-amino-3-(2-chloroethyl)imidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (83). Refluxing the azide (67) in a variety of solvents including toluene, acetone, chloroform and benzene failed to produce the Curtius-type conversion to the isocyanate (79). Thermal rearrangement via direct heating was also unsuccessful, with an explosive degradation reaction occuring at 115°C. Mass spectral analysis of the azide (67) gave a small peak in the spectrum corresponding to the molecular ion for the isocyanate (79) (m/z 240). It is hoped that future experiments utilising flash vacuum thermolysis[130,131] will be effective in converting the azide (67) to the isocyanate (79).

2.1.1.2.2 Synthesis of 3-(2-chloroethyl)-8-diazoacetylimidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one monohydrate

57 + NaN_3/ aqueous acetone → 67 → 78 → 79 → 80 ↔ 81 → 82 → 83 + CO_2

Scheme 13

Direct reaction of the acid chloride (57) with an ethereal solution of diazomethane[132] in the dark afforded 3-(2-chloroethyl)-8-diazoacetyl-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one monohydrate (68). Diazo-ketones can undergo the Wolff rearrangement[133] involving loss of nitrogen followed by rearrangement to give highly reactive ketenes. The Wolff rearrangement constitutes part of the Arndt-Eistert procedure[134] by which an acid is converted to its homologue. Homologisation of the the acid (53) (Scheme 14) was of interest to determine what effect separation of the 8-substituent from the imidazole ring by a methylene link had upon antitumour activity. Unfortunately efforts to effect Wolff-type degradation of the imidazotetrazinone (68) failed to produce the homologous 8-acetic acid (88), the dark brown residue produced being indicative of chemical breakdown during these reactions.

2.1.1.2.3 Synthesis of 8-aminocarbamoyl-3-(2-chloroethyl)-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

On the basis of results from biological experiments suggesting that the carbamoyl -NH group was important for optimal activity in 8-carbamoyl-imidazotetrazinones (Section 2.3.6), it was interesting to consider what effect addition of another -NH group to the 8-carbamoyl moiety had upon the activity of these compounds. Unfortunately, preparation of 8-amino-carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one by

Ag_2O

$-N_2$

68 84 85 86 87 88

Scheme 14

reaction of the acid chloride (57) with hydrazine hydrate. was extremely difficult with degradation occuring, presumably due to nucleophilic attack at C-4 and ring-opening in an analogous manner to that reported by Baig[112].

A route towards 8-aminocarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one was designed involving initial reaction of the acid chloride (57) with t-butylcarbazate followed by cleavage of the t-butyl group with trifluoroacetic acid. to give 8-aminocarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one. Reaction of the acid chloride (57) with methylcarbazate did afford 3-(2-chloroethyl)-8-methoxycarbonylaminocarbamoylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (70) (Fig 1). However. reaction with the more bulky t-butylcarbazate gave a product for which spectroscopic evidence was encouraging. but satisfactory elemental analysis was never obtained. Tosyl hydrazine also failed to participate in these reactions.

Latter reactions (Fig 1) were effective in the synthesis of 3-(2-chloroethyl)-8-(N-phenylaminocarbamoyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (69a) and 3-(2-chloroethyl)-8-(2,4-difluorophenylaminocarbamoyl) imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (69b).

2.1.1.2.4 Preparation of 8-[N-(ethoxycarbonylmethyl)carbamoyl]-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

There are many reports of antitumour agents linked to peptide moieties[135] and monoclonal antibodies[136,137]. As a prelude to investigating such possibilities with CMCIT, the acid chloride (57) was reacted with a selection of amino-acids and derivatives including glycine ethyl ester hydrochloride, glycine, serine benzyl ester hydrochloride, phenylalanine hydrochloride and glycinamide. The only successful reaction was that involving reaction with glycine ethyl ester hydrochloride to give 8-[N-(ethoxycarbonylmethyl)carbamoyl]-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (71).

Methods employing reaction of the acid chloride (57) in the presence of diphenylphosphoryl azide (DPPA)[138] with various amino-acid derivatives such as phenylalanine ethyl ester, glycine ethyl ester and serine benzyl ester were also unrewarding. However, a recent method for conversion of the acid (53) to the glycinamide (71) utilising the peptide linking agent 1,3-dicyclohexylcarbodiimide, DCCI, has been reported as successful[139].

2.1.1.2.5 Synthetic routes towards 8-amino-3-(2-chloroethyl)-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one.

8-Amino-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (83) was considered a key intermediate in reactions towards further 8-substituted imidazotetrazinones. A major part of the synthetic programme involved efforts to prepare this compound, (83) mainly via the azide/isocyanate route (Scheme 13) (see section 2.1.1.2.1). The exquisite sensitivity of CMCIT towards basic conditions precluded the use of the classical method for conversion of an amide to an amine of one less carbon atom, i.e. the Hofmann reaction, since this method requires the presence of alkali[140]. However, a method has recently been published which employs the Hofmann reaction under acidic conditions[141]. The method involves use of the reagent, phenyl iodosyl bis(trifluoroacetate), PIFA (89)[142,143]. During reaction PIFA is converted into its dimer (90) which is presumed to be the reactive species. The reaction mechanism for reaction of PIFA with CMCIT (Scheme 15) would presumably involve formation of an intermediate (91) which might then rearrange to the isocyanate (79) with subsequent hydrolysis to (83). Preparation of PIFA, followed by reaction with CMCIT, failed to produce the expected product (83). Further investigations are planned with PIFA, based on a method supplied by other workers[144]. Schmidt-type reactions[145] of CITCA with polyphosporic acid

$CF_3-C(=O)-O-I(Ph)-O-C(=O)-CF_3$ (89) → $CF_3COO-I(Ph)-O-I(Ph)-OCOCF_3$ (90)

+ 1

H_2O: ... $C(=O)-NH-I(Ph)-O-I(Ph)-OCOCF_3$ ... N N N N N N CH_2CH_2Cl O

91

N=C=O ... N N N N N N CH_2CH_2Cl O 79

+ H_2O

NH_2 ... N N N N N N CH_2CH_2Cl O + CO_2

83

Scheme 15

and nitromethane[146] were not effective in producing the expected imidazotetrazinone (83) and did not warrant further investigation.

2.1.1.2.6 Other reactions involving 3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-d]-1,2,3,5-tetrazine-8-carbonyl chloride

In an attempt to substitute a halogen atom in the 8-position of imidazotetrazinones the acid chloride (57) was reacted with chlorotris(triphenyl) rhodium (1) catalyst[147,148]. This decarbonylation method failed to afford the desired product. Methods towards 8-halogen compounds involving the imidazotetrazinone (83) were not possible due to the extreme difficulties experienced in synthesising this compound (See Section 2.1.1.2.5).

It had been suggested earlier[20] that the presence of an 8-carbamoyl or 8-sulphonamide group was important to the activity of imidazotetrazinones. The activity of compounds containing a mixture of these substituents was considered worthy of investigation. Synthesis of an 8-tosylcarbamoyl derivative by reaction of the acid chloride (57) with toluene-p-sulphonamide was attempted in addition to attempts to synthesise a 8-tosylaminocarbamoyl derivative (Section 2.1.1.2.3). Although spectroscopic evidence

was encouraging, results from elemental analysis of these compounds were never satisfactory.

Finally, during disposal of the acid chloride (57) by treatment with ammonia and methanol, small amounts of a precipitate were obtained; treatment of the acid chloride (57) with ammonia alone producing appreciable amounts of this light brown solid. The compound was isolated and subsequent ^1H nmr analysis gave a spectrum exhibiting two triplet signals at δ4.0 (CH_2) and 4.6 ppm (CH_2), a singlet at 8.3 ppm (imidazole CH) and a broad band at 7-8 ppm (NH_4^+). This suggests that the acid chloride (57) can undergo hydrolysis to the acid (53) in aqueous ammonia followed by conversion to the ammonium salt.

2.1.1.2.7 Preparation of ring-opened triazene derivatives

Information from the present structure-activity studies (Section 3) suggesting that differences in tautomerism in the ring-opened triazenes (116a, 116b, 118) may be of importance for the activity of the 8-carbamoylimidazotetrazinones CMCIT, 8-methylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (MCMCIT) and DCMCIT, led to attempts to prepare the triazenes using a method based on that of Lunt[149]. Experiments were conducted involving reaction of the 8-carbamoyl compound with aqueous sodium carbonate at various concentrations (5-20%) at room temperature, for varying lengths of time

(1-4 hours). Reaction with CMCIT afforded the corresponding triazene MCTIC (3), as reported earlier[19], although similar reactions with MCMCIT and DCMCIT failed to produce the expected products. This method was also used in attempts to synthesise the triazene corresponding to 8-ethoxycarbonyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (65b). Successful reaction has been reported for synthesis of the above triazene (65b) via this route[112], although subsequent attempts to repeat the experiment were unsuccessful. The present attempts to prepare the triazene derivative from the ethyl ester (65b) also met with disappointment.

The successful preparation of MCTIC from CMCIT by reaction with aqueous sodium carbonate may be attributed to the fact that MCTIC precipitates out of solution during the conversion, whereas the other derivatives do not. When in solution MCMCIT, DCMCIT and ECNCIT presumably undergo a base-catalysed degradation reaction, producing deep maroon coloured liquids, with isolation of the products seemingly impossible. The presence of maroon pigments in imidazotetrazinones has been attributed to production of imidazolylazoimidazole (16). This product is thought to be produced by a coupling interaction between diazo-IC (11) and AIC (17)[11]. By analogy, the pigmentation produced in ring-opening of MCMCIT, DCMCIT suggests breakdown to the corresponding AIC

$CONNR_1R_2$ … $N-CH_2CH_2Cl$ — **64** $\xrightarrow{Na_2CO_3}$ **92a**

Na_2CO_3

77b,c + **76b,c**

93b,c

a) $R_1=R_2=H$
b) $R_1=H, R_2=CH_3$
c) $R_1=R_2=CH_3$

Scheme 16

(76b, 76c) and diazo-IC (77b, 77c) derivatives, followed by coupling of the degradation products (Scheme16).

2.1.2 Stability studies on 8-carbamoylimidazotetrazinones

2.1.2.1 Ultra-violet spectroscopic determination of half-life values for 8-carbamoylimidazotetrazinones

Stability studies on CMCIT, MCMCIT and DCMCIT were important to investigate whether a difference in chemical stability could explain differences in the cytotoxicity of these compounds (See Section 2.2.1.2 , Table 13).

Previous workers[15,16] have reported half-life values for CMCIT and several analogues at various pH values determined in phosphate buffers. In the present studies, an ultra-violet method was devised to provide half-life determinations for CMCIT, MCMCIT and DCMCIT in cell culture medium, RPMI 1640 containing 17% horse serum (HS), at 37°C. Use of this buffer caused considerable difficulties since it gave a strong absorbance peak at 302nm which increased in magnitude with time until it completely overwhelmed the 8-carbamoylimidazotetrazinone peak (λmax 325nm). This problem was partly resolved by use of a RPMI 1640+HS blank, but the high background absorbance still led to quite large errors. Results obtained are shown in Table 5. The large standard errors inherent in this

Table 5: Decomposition of 8-carbamoylimidazotetrazinones in RPMI 1640 (pH 7.4) at 37°C as determined by an ultra-violet spectroscopic method

	HALF LIFE (T1/2) MINUTES		
EXPERIMENT	CMCIT	MCMCIT	DCMCIT
1	-	80.8	67.5
2	70.8	65.8	75.4
3	55.8	56.7	50.8
4	57.9	57.5	40.8
$\bar{x}$	61.5	65.2	58.6
s.d.	± 8.12	± 11.18	± 15.69

method led to the decision to conduct a HPLC determination of half-life values for these compounds.

2.1.2.2 HPLC determination of half-life values for 8-carbamoyl-imidazotetrazinones

The method was based on that of Slack et al[150,151]. Separation of CMCIT, MCMCIT and DCMCIT from each other was achieved using a 250 Lichrosorb RP select B column and a methanol/acetic acid (0.5%) in water (35:65) mobile phase. 8-Carbamoyl-3-ethylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (ethazolastone, CMEIT) was found to be a suitable internal standard. The peaks in mixtures of authentic reference compounds (Fig 2) were assigned as: CMEIT (i), CMCIT (ii), MCMCIT (iii) and DCMCIT (iv), the compounds eluting in decreasing order of polarity. Half-life values obtained for the decomposition of CMCIT, MCMCIT and DCMCIT in RPMI 1640 + HS at 37°C are given in Tables 6, 7 and 8. The decomposition was shown to follow first-order kinetics for all three compounds (Tables 6, 7 and 8, Figs 3, 4 and 5). The decomposition of CMCIT in RPMI 1640 at 37°C ($T_{1/2}$ =33.9 minutes) was accelerated compared with its decomposition in phosphate buffer at 37°C, previously determined as $T_{1/2}$ = 55.2 minutes[151].

The small differences in half-life values between CMCIT, MCMCIT and DCMCIT indicate that differences in stability of the tetrazinone ring are

Figure 2 HPLC trace of authentic imidazotetrazinones

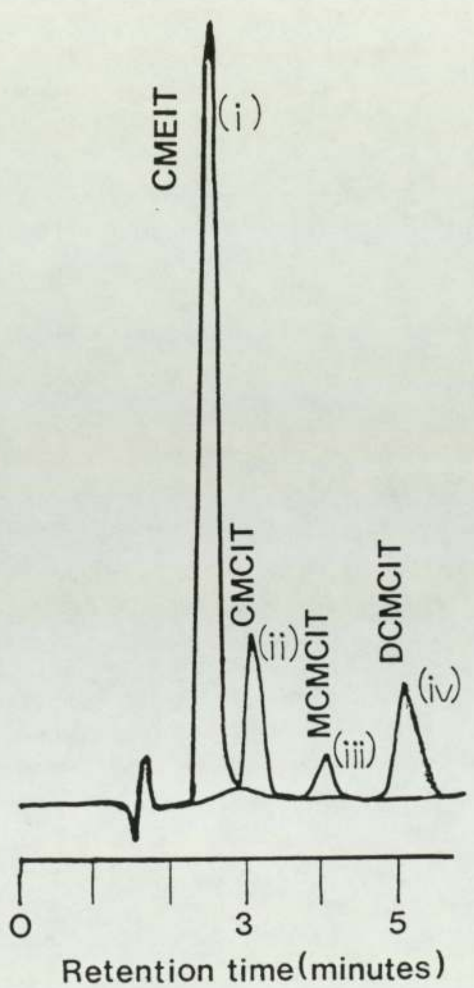


Figure 3 Kinetics of degradation of CMCIT in RPMI 1640 media at 37°C

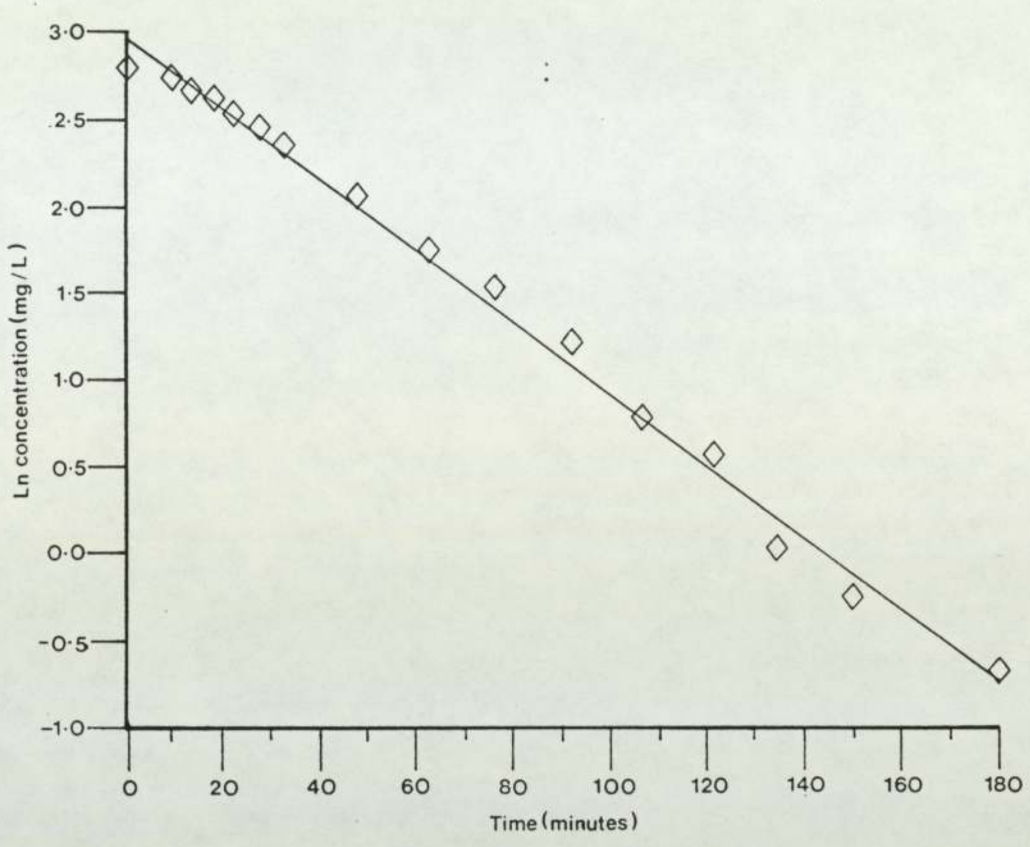


Table 6: Kinetics of CMCIT degradation in RPMI 1640 medium

TIME (min)	CONC (mg/L)	LN CONC (mg/L)
0.000	16.367	2.795
9.000	15.428	2.736
13.000	14.238	2.656
18.000	13.677	2.616
22.000	12.461	2.523
27.000	11.488	2.441
32.000	10.417	2.343
47.000	7.769	2.050
62.000	5.731	1.746
76.000	4.574	1.520
92.000	3.316	1.199
106.000	2.165	0.772
121.000	1.752	0.561
135.000	1.040	0.039
150.000	0.784	-0.243
180.000	0.511	-0.671

ELIMINATION HALF-LIFE (min) = 33.9040

ELIMINATION RATE CONSTANT (1/min) = -0.0204

R = -0.997

Figure 4 Kinetics of degradation of MCMCIT in RPMI 1640 media at 37°C

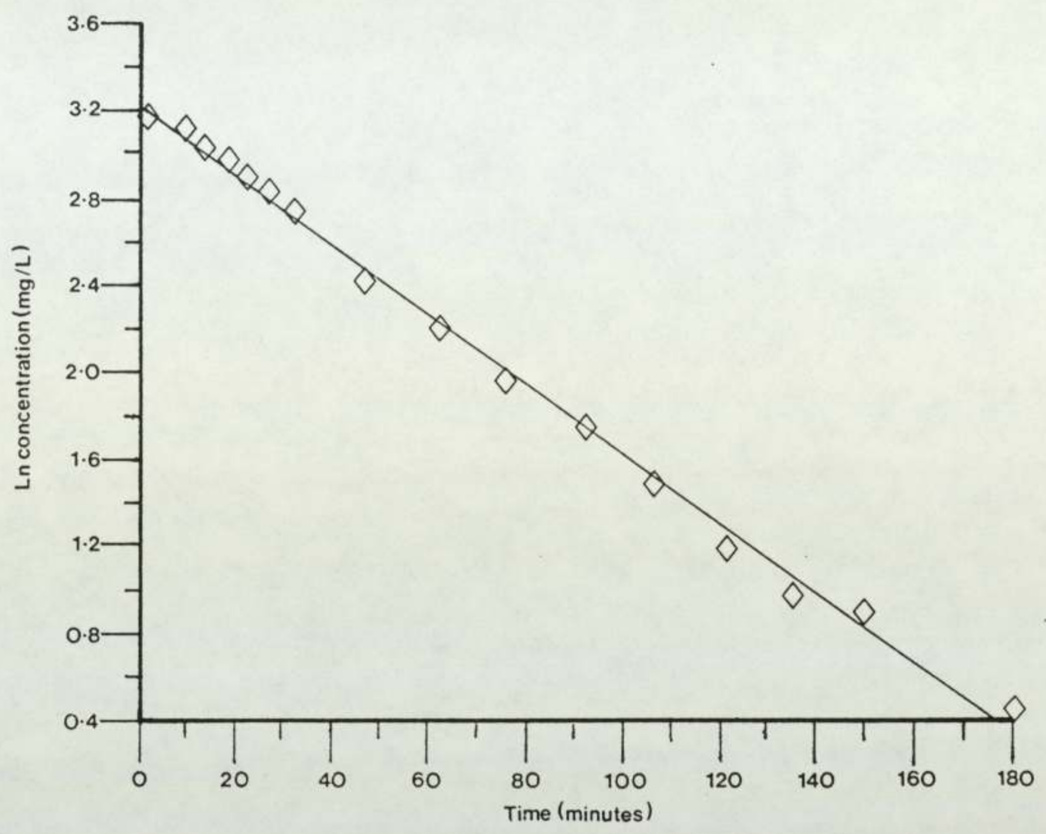


Table 7: Kinetics of MCMCIT degradation in RPMI 1640 medium

TIME (min)	CONC (mg/L)	LN CONC (mg/L)
1.000	23.881	3.173
9.000	22.403	3.109
13.000	20.657	3.028
18.000	19.504	2.971
22.000	18.071	2.894
26.000	16.845	2.824
32.000	15.407	2.735
46.000	11.231	2.419
62.000	9.025	2.200
76.000	7.020	1.949
92.000	5.707	1.742
106.000	4.401	1.482
121.000	3.274	1.186
135.000	2.672	0.983
150.000	2.462	0.901
180.000	1.585	0.461

ELIMINATION HALF-LIFE (min) = 43.5100

ELIMINATION RATE CONSTANT (1/min) = -0.0159

R = -0.9979

Figure 5 Kinetics of degradation of DCMCIT in RPMI 1640 media at 37°C

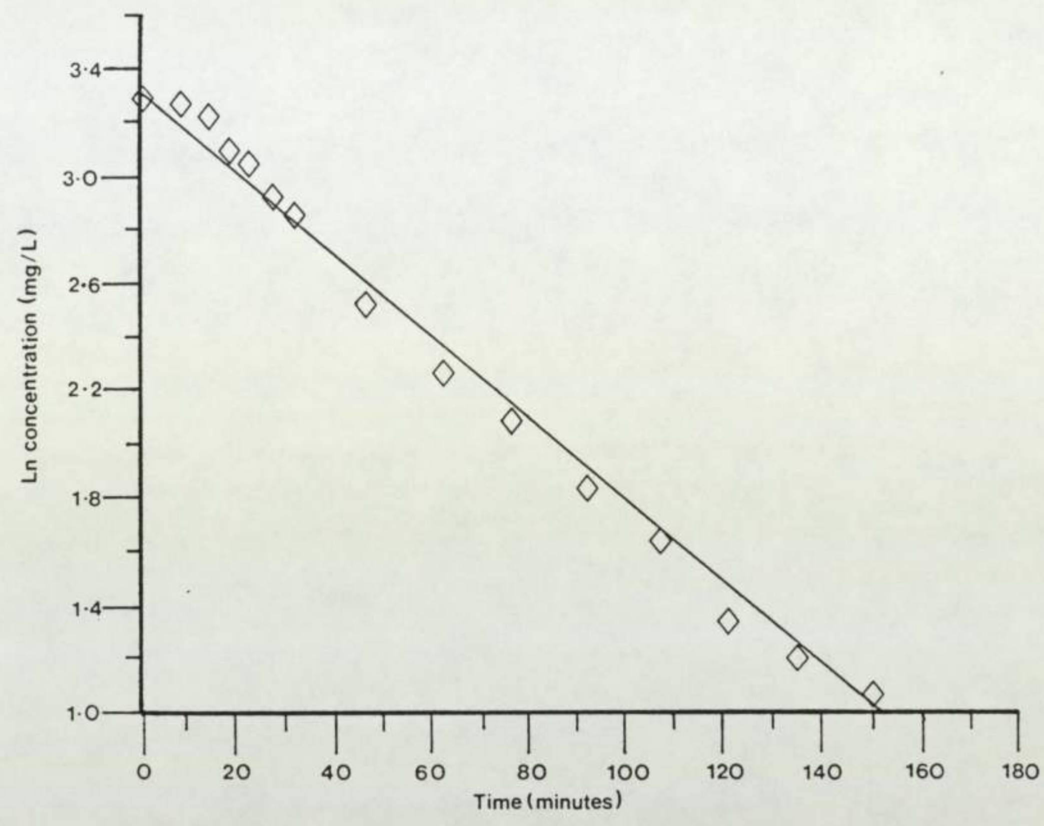


Table 8: Kinetics of DCMCIT degradation in RPMI 1640 medium

TIME (min)	CONC (mg/L)	LN CONC (mg/L)
0.000	26.826	3.289
8.000	26.092	3.262
14.000	24.908	3.215
18.000	21.955	3.089
22.000	20.980	3.044
27.000	18.463	2.916
31.000	17.307	2.851
46.000	12.417	2.519
62.000	9.586	2.260
76.000	8.020	2.082
92.000	6.258	1.834
107.000	5.155	1.640
121.000	3.821	1.341
135.000	3.345	1.207
150.000	2.906	1.067
180.000	2.294	0.830

ELIMINATION HALF-LIFE (min) = 45.8244

ELIMINATION RATE CONSTANT (1/min) = -0.0151

R = -0.9934

not significant enough to account for the difference in cytotoxicity (Table 13, Section 2.2.1.2) observed between these compounds.

2.1.3 X-ray analysis of imidazotetrazinones

2.1.3.1 Previous X-ray studies on imidazotetrazinones and rationale for current investigations

X-ray analysis of CMCIT (Figs 6a, 6b)[152] showed that there were four molecules per unit cell and two independent molecules per asymmetric unit. These two conformationally distinct molecules are rotamers about the C(8)-C(81) ring to carboxamide bond, the one amide being intramolecularly hydrogen-bonded to N(1) of the tetrazinone ring (Fig. 6a, unprimed structure), the other not (Fig. 6b, primed structure). It was also reported that with the exception of the chloroethyl sidechain, both molecules (a), (b) were approximately planar with intermolecular hydrogen-bonds holding the four molecules together around the centre of symmetry.

The X-ray structure of CMCIT had structural analogies with that of DTIC (Fig 7)[153]and its monohydrate hydrochloride[154] with the carbamoyl group nearly coplanar with the imidazole ring, bent in-plane at the point of attachment to the ring, and the angle NCC < OCC. In the unprimed

Figure 6 The X-ray structure of CMCIT

Figure 7 The X-ray structure of DTIC

rotamer of CMCIT (Fig. 6a) the coplanarity of the 8-carbamoyl group with the ring system was maintained by an intramolecular hydrogen-bond analogous to that observed in DTIC and its monohydrate hydrochloride. It was suggested that the coplanarity observed in the CMCIT structure possibly affords conjugation between the 8-carbamoyl group and the imidazole ring[152]. Structures of other imidazotetrazinones[155] have also shown the imidazotetrazinone ring system to be substantially planar.

Consideration of X-ray data from CMCIT together with biological results suggesting a requirement for a -NH moiety for optimum activity in 8-carbamoylimidazotetrazinones, led to the hypothesis that coplanarity of the 8-substituent to the ring system was important to the activity of these compounds. It was also suggested that coplanarity in CMCIT and mono-substituted carbamoyl derivatives such as MCMCIT, was facilitated by intramolecular hydrogen-bonding, giving increased activity relative to compounds unable to hydrogen-bond, e.g. DCMCIT. To test this hypothesis, attempts were made to obtain X-ray data for MCMCIT, DCMCIT, 8-cyclohexylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (64h) and the ethyl ester (65b), for comparison with previously determined structures. Whilst the structures of DCMCIT (Fig. 8), the cyclohexylamide (64h) (Fig. 9) and the ethyl ester (65b) (Fig. 10) were determined, the X-ray structure of MCMCIT remains unsolved, crystallisation affording material which was unsuitable for X-ray studies.

Figure 8 The X-ray structure of DCMCIT

Figure 9 The X-ray structure of 8-cyclohexylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

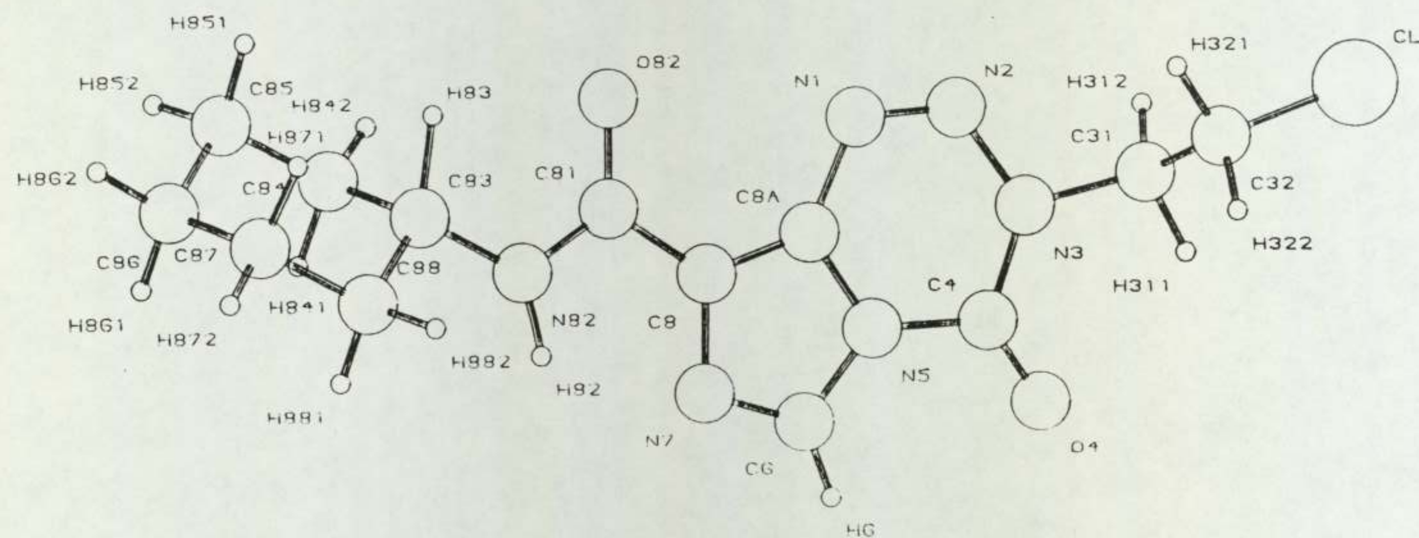
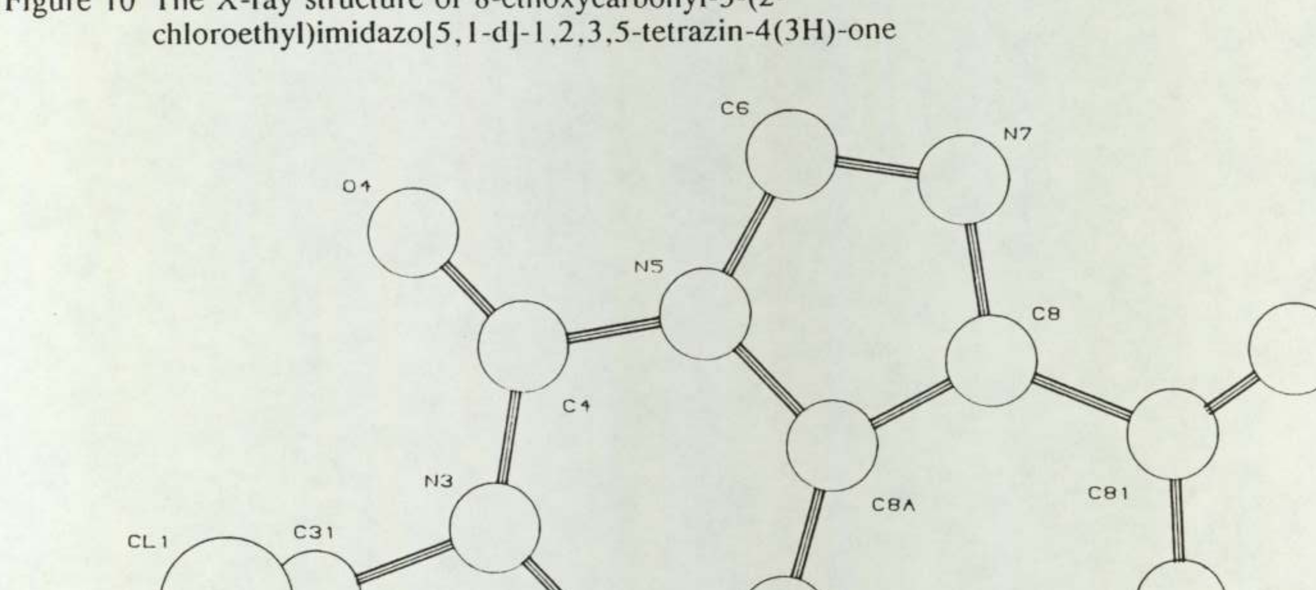


Figure 10 The X-ray structure of 8-ethoxycarbonyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one



The orientation of the 8-substituent to the bicyclic ring system was examined to determine whether the position of the 8-substituent was different in poorly active compounds such as DCMCIT and the ethyl ester (65b), compared with the position of the 8-group in structures of active compounds. In addition, interference with hydrogen-bond formation and coplanarity of the 8-substituent by the bulky non-planar N-substituent in the cyclohexylamide (64h) was also considered.

2.1.3.2 Results from present X-ray investigations

The data reported here were supplied by Dr. P.R. Lowe, this department, and Dr. C. Whiston, Department of Physical Sciences, Wolverhampton Polytechnic. The reported structures were determined by Dr. P.R. Lowe using an Enraf Nonius CAD 4 four circle diffractometer using graphite monochromated Mo-Kα radiation.

For these investigations only limited data is provided for previously unpublished compounds DCMCIT, 64h, 65b, (Table 9).
More important to this project are values for deviations in Å from the least-squares plane through the nine atoms of the bicyclic system (Table 10), and the value of the torsion angle associated with the 8-substituent (Table 11).

Table 9: Cell dimensions and space group symmetry for several imadazotetrazinones

	$R = CON(CH_3)_2$	$R = CONHC_6H_{11}$	$R = COOC_2H_5$
Space Group	$P2_1$	Pbca	Pbca
z	2	8	8
U($Å^3$)	616 (1)	3070 (1)	2423
a(Å)	9.078 (2)	9.485	9.587
b(Å)	7.518 (5)	9.123 (1)	8.223
c(Å)	9.780 (2)	35.488 (4)	28.646
α(°)	90	90	90
β(°)	112.71 (1)	90	90
δ(°)	90	90	0.097
R	0.04	0.096	

Table 10: Deviations of non-hydrogen atoms for the least-squares plane through the imidazotetrazinone ring system. (Atoms used in the plane calculation are marked with an asterisk.)

ATOM	R = $CONH_2$ unprimed	R = $CONH_2$ primed	R = $CONHC_6H_{11}$	R = $CON(CH_3)_2$
	Å	Å	Å	Å
* N (1)	-0.004	-0.017	0.0382	0.0044
* N (2)	0.002	-0.002	-0.0611	0.0041
* N (3)	0.007	0.035	0.0176	-0.0055
* C (4)	0.003	-0.004	0.0216	-0.0250
* N (5)	-0.010	-0.029	0.0105	0.0275
* C (6)	-0.003	-0.009	-0.0119	0.0152
* N (7)	0.005	0.017	-0.0157	-0.0111
* C (8)	0.008	0.019	0.0007	-0.0185
* C (8a)	-0.007	-0.010	-0.0579	0.0089
C (31)	0.011	0.142	-	0.0161
C (32)	-1.346	1.547	-	1.3304
Cl	-2.355	2.646	-	2.6380
O (4)	0.006	-0.006	-	-0.0735
C (81)	0.030	0.080	0.0665	-0.2222
O (82)	-0.126	0.220	-0.1992	-1.1211
N (82)	0.188	0.019	-	0.5475
C (83a)	-	-	-	0.2521
C (83b)	-	-	-	1.7350

Table 11: Comparison of torsion angles in various 8-substituted imidazotetrazinones

R Substituent	Torsion Angle(°) C8A, C8, C81, 082	R Substituent	Torsion Angle(°) C8A, C8, S1, O81
NH_2	- 6.0	$NHCH_3$	- 17.3
NHC_6H_{11}	8.1	$N(CH_3)_2$	- 18.4
OH	6.7	CH_3	- 23.4
OC_2H_5	16.1	-	-
$N(CH_3)_2$	45.5	-	-

The X-ray structure for the cyclohexylamide (64h) (Fig 9) showed that even in the presence of a bulky, non-planar group attached to the 8-carbamoyl moiety, hydrogen-bonding via the carbamoyl -NH group was still possible. The possibility that involvement of the -NH moiety in intramolecular or intermolecular hydrogen-bonding is important for optimum activity will be discussed in Sections 2.3 and 3.3.

It was realised that the 'hydrogen-bonding theory' postulated for 8-carbamoylimidazotetrazinones (Section 2.3) could not explain the excellent antitumour activity of the dimethylsulphamoyl (Table 12, iv) and alkylsulphonyl (Table 12, ix) derivatives. However, comparison of coplanarity of an oxygen atom in the 8-position in structures of active compounds, with coplanarity in structures of inactive compounds, may explain how 8-substitution can affect activity in these compounds. It was shown that in structures of active imidazotetrazinones CMCIT and the cyclohexylamide (64h) an oxygen atom of the 8-substituent was coplanar with the bicyclic ring system, as determined from least-squares plane measurement (Table 10).

However, the structure of the relatively non-cytotoxic (Section 2.2.1.2) derivative DCMCIT was significantly different from other structures (Table 10). In the DCMCIT structure the oxygen atom of the carbamoyl group showed a large deviation from coplanarity, the deviation from the least-

square plane giving a value of -1.211 Å. Comparison of the torsion angle C8A, C8, C81, O82 (Table 11) also indicated a large deviation from coplanarity, the oxygen atom lying 45.5° out of plane. The torsion angles about the 8-position in CMCIT, the cyclohexylamide (64h), the 8-methylsulphone (Table 12, ix) and the highly active sulphonamoyl derivatives (Table 12, iv, x) all gave evidence of an oxygen atom coplanar to the ring nitrogen (N^1).

From these findings it was hypothesised that deviation of coplanarity of an oxygen atom in the 8-substituent might crucially affect the activity of imidazotetrazinones.

2.1.4 Conclusions from chemical studies

The extreme synthetic difficulties intrinsic to imidazotetrazinones has made synthesis of further 8-substituted derivatives very limited. Ring-opening degradation reactions have prohibited the use of base-catalysed reactions, and attempts to decarbonylate 8-substituents are also restricted due to effects on the ring carbonyl group. A new method of synthesis, via a modified acid chloride route, has been used to extend the range of available 8-substituted imidazotetrazinones. However, these reactions remain both difficult and hazardous.

Chemical stability studies on CMCIT, MCMIT and DCMCIT have

indicated that differences in the rate of ring-opening cannot be responsible for the biological differences of DCMCIT relative to CMCIT and MCMCIT.

X-ray analysis has shown, that, contrary to findings for highly cytotoxic (in vitro) compounds e.g. CMCIT and the cyclohexylamide (64h), the 8-carbamoyl oxygen atom is not coplanar with the imidazotetrazinone ring system for the relatively inactive derivative DCMCIT. If one considers the presence of a 'receptor' for imidazotetrazinones then such pertubation of planarity could result in failure of DCMCIT to fit such a receptor, with a reduction in activity. This possibility will be discussed later (See Section 3.3).

2.2 Biology

2.2.1 Cytotoxicity of imidazotetrazinones against the murine TLX5 lymphoma cell line

2.2.1.1 Growth characteristics of murine TLX5 lymphoma cells

The decision to use the TLX5 cell line for the study of the cytotoxicity of imidazotetrazinones in vitro was taken on the basis of the fact that the properties of these cells have been investigated in depth in the CRC laboratories at Aston University, and that they have been shown to be sensitive to imidazotetrazinones (Section 1.3.3). A study was undertaken

to determine the characteristics of the cellular growth curve. Under the culture conditions employed the doubling time for the TLX5 cell line was 17 hours. For the cytotoxicity assays an initial cell concentration of $2x10^4$ cells/ml was selected, and cell numbers were counted 72 hours after commencement of the culture to ensure that the cells were still in the linear phase of their growth.

2.2.1.2 Direct cytotoxicity of imidazotetrazinones

Results obtained from the cytotoxicity assays are given in Tables 12 and 13. For CMCIT, MCMCIT and the 8-sulphamoyl derivatives the high cytotoxicity in vitro paralleled the antitumour activity observed in vivo (Tables 2, 12,14). In Section 3 some structural requirements necessary for optimal antitumour activity/cytotoxicity of imidazotetrazinones are discussed. In view of this discussion, the acid (53) should theoretically possess good antitumour activity and high cytotoxicity, as it possesses an 8-carbonyl group coplanar to the tetrazinone ring and a hydroxyl group which can participate in hydrogen-bonding. But the antitumour activity of this compound is weak and its cytotoxicity is relatively low. This unexpected result might have a physicochemical explanation. The acid (53) was thought to be ionised at physiological pH, a hypothesis substantiated by pKa determination[156,157] which gave a value of 3.08 ± 0.1.

Table 12: Cytotoxicity of 8-substituted imidazotetrazinones against a murine TLX5 lymphoma cell line

R Substituent	IC_{50} (µM)	R Substituent	IC_{50} (µM)
(i) $COCHN_2^{(2)}$	0.75	(xv) $CO_2C_2H_5^{(1)}$	3.50
(ii) $CON_3^{(2)}$	1.49	(xvi) $CONHC_3H_8^{(1)}$	3.71
(iii) $CONHNHC_6H_5^{(3)}$	1.74	(xvii) $CONHCH(CH_2)_2^{(3)}$	3.83
(iv) $SO_2N(CH_3)_2^{(3)}$	1.89	(xviii) $CO_2CH_3^{(2)}$	4.18
(v) $CONHCH(CH_3)_2^{(3)}$	1.93	(xix) $CONHC_6H_{11}^{(3)}$	4.47
(vi) $CONHCH_2CHCH_2^{(1)}$	1.95	(xx) $CONHOCH_2C_6H_5^{(3)}$	5.67
(vii) $CONHCH_2CH_2Cl^{(1)}$	1.97	(xxi) $CONHCH_2C(CH_3)_3^{(1)}$	7.37
(viii) $CONHC(CH_3)_3^{(1)}$	2.01	(xxii) $CONHCH_2CO_2C_2H_5^{(1)}$	7.92
(ix) $SO_2CH_3^{(3)}$	2.31	(xxiii) $CONHNO_2^{(1)}$	8.71
(x) $SO_2NHCH_3^{(3)}$	2.38	(xxiv) $CONHOH^{(1)}$	12.0
(xi) $SO_2NH_2^{(3)}$	2.48	(xxv) $CON(CH_3)_2^{(3)}$	14.55
(xii) $CONSO(CH_3)_2^{(2)}$	2.51	(xxvi) $CO_2H^{(4)}$	15.8
(xiii) $CONHOCH_3^{(2)}$	3.20	(xxvii) $CONC_5H_{10}^{(3)}$	19.0
(xiv) $CONHC_6H_5^{(4)}$	3.33	(xxviii) $CON(CH_3)C_6H_5^{(3)}$	20.01

( ) No. in brackets represents number of determinations made

Table 13: Cytotoxicity of 8-carbamoylimidazotetrazinones against a murine TLX5 lymphoma cell line

R-Substituent	IC_{50}(μM*)
$CONH_2$ (CMCIT)	2.27 ± 0.29
$CONHCH_3$ (MCMCIT)	2.97 ± 0.44
$CON(CH_3)_2$ (DCMCIT)	14.55 ± 1.1

* average of 3 assays

Table 14: **Antitumour activity of 8-carbamoylimidazotetrazinones against TLX5 lymphoma *in vivo***

R / N=N / N–CH2CH2Cl / O

R-Substituent	Dose mg/kg	T/Cx100%	Long term survivors
$CONH_2$ (CMCIT)	10.0	120	-
	20.0	145	-
	40.0	>458	4/5
	80.0	67	-
$CONHCH_3$ (MCMCIT)	10.0	133	-
	20.0	>256	1/5
	40.0	>550	5/5
	80.0	72	-
$CON(CH_3)_2$ (DCMCIT)	20.0	161	-
	40.0	>508	5/5
	80.0	>361	3/5
	160.0	68	-

T/C = treated versus control.

Therefore in its carboxylate anion form the acid (53) would not be able to penetrate cells, and this would account for its poor activity, assuming an intracellular target for the initiation of cytotoxicity.

In comparison the ethyl ester (65b), which is unionised at physiological pH, should be able to pass through the cell membrane and this would explain its increased cytotoxic potential relative to the acid (53) (Table 12). The cytotoxicity of the ethyl ester (65b) is interesting since this compound does not fit the criteria for a highly cytotoxic derivative as suggested in Section 3.3. However, again assuming an intracellular target for cytotoxicity, it is conceivable that the ethyl ester (65b) acts as a transport form of the acid (53). That is, the ethyl ester (65b) should pass through the cell membrane and then might undergo intracellular hydrolysis to give the acid (53) which then might be responsible for the antitumour/cytotoxic effects. This theory would also explain the cytotoxicity of the 8-methyl ester derivative (65a). Whether hydrolysis in the target cell is required for the cytotoxicity of such esters is still unclear. To test this conjecture, attempts were made to synthesise a derivative with a bulky, non-hydrolysable ester moiety which could then be tested for cytotoxic potential. Unfortunately reactions to prepare the 8-t-butyl ester or the 8-neopentyl ester did not

succeed, and further work is required to elucidate the biological fates of the methyl ester (65a), the ethyl ester (65b) and the acid (53). The finding that the esters are no more active than the acid (53) in vivo was somewhat surprising. This presumably indicates rapid hydrolysis of the esters to the acid (53) in vivo, with poor absorption of the acid metabolite.

Interestingly, the 8-dimethylcarbamoyl analogue (DCMCIT) displayed relatively low cytotoxicity in vitro (Table 13) in spite of its good anti-tumour activity in vivo (Table 14). To explain this discrepancy, experiments were designed to test the hypothesis that DCMCIT undergoes metabolism to a more cytotoxic species.

2.2.1.3 Cytototoxicity of 8-carbamoylimidazotetrazinones in the presence of mouse hepatic microsomes

A cytotoxicity assay involving pre-incubation of the test compound with hepatic microsomes, essentially as described by Dolfini et al[158], was used to test the hypothesis that DCMCIT was being metabolically activated in vivo. Microsomes were obtained from CBA/CA mice, the host of the TLX5 lymphoma in vivo. Preliminary experiments showed that a concentration of microsomes equivalent to 50mg of liver/ml was optimal and this concentration was used in the experiments described here. In the experiments, cyclophosphamide (34) (25 μg/ml) (Table 15) was used as a control substance to indicate whether the microsomes prepared were functionally viable. Cyclophosphamide is known to undergo metabolic activation catalysed by hepatic oxidising enzymes (Scheme 17)[159,160], and in the past metabolic activation of cyclophosphamide has been demonstrated frequently in in vitro experiments[161].

The results in Table 16 show that DCMCIT indeed undergoes metabolism to a more toxic species. Graphical representation of this data (Fig 11) demonstrates a significant increase in the cytotoxicity of this derivative when incubated with microsomes, as compared to its cytotoxicity in the absence of microsomes. Similar experiments were performed with CMCIT

Table 15: In vitro cytotoxicity of cyclophosphamide (CYCLO) against the TXL5 cell line during incubations with hepatic microsomes

CONDITIONS	% Inhibition of TXL5 Cell Growth			$\bar{x}$	S.D. %
	EXPT. 1	EXPT. 2	EXPT. 3		
100mg/L CYCLO+microsomes	99.43	-	-	99.43	-
100mg/L CYCLO	0.48				
Difference	98.95				
50mg/L CYCLO+microsomes	97.84	99.09	96.71	97.88	±1.19
50mg/L CYCLO	0	15.11	0	5.04	±8.72
Difference	97.84	83.98	96.71	92.84	
25mg/L CYCLO+microsomes	95.14	95.99	100.07	97.07	±2.64
25mg/L CYCLO	2.43	9.80	8.72	6.98	±3.98
Difference	92.71	86.19	91.35	90.08	
12.5mg/L CYCLO+microsomes	-	73.99	-		
12.5mg/L CYCLO	-	17.59			
Difference	-	56.40			

34 —Metabolism→ 94 → 95 ⇌ 96 → 97 + 98 ($CH_2{=}CH{-}CHO$) → 99 + 100

Scheme 17

Table 16: Incubation of DCMCIT with hepatic microsomes and the effect on cytotoxicity against TLX5 cells

CONDITIONS	% Inhibition of TLX5 Cell Growth					$\bar{x}$	S.D. %
	EXPT. 1	EXPT. 2	EXPT. 3	EXPT. 4	EXPT. 5		
20 mg/L DCMCIT + microsomes	-	97.34	87.19	94.48	-	93.00	± 5.23
20 mg/L DCMCIT	-	77.39	77.07	54.59	-	69.68	± 13.07
Difference	-	19.95	10.12	39.89	-	23.32	
10 mg/L DCMCIT + microsomes	86.12	95.66	89.96	81.51	80.29	86.71	± 6.31
10 mg/L DCMCIT	39.15	45.86	31.58	16.36	23.88	31.37	± 11.75
Difference	46.97	49.80	58.38	65.15	56.41	55.34	
5 mg/L DCMCIT + microsomes	49.11	76.74	56.84	51.12	35.26	53.81	± 15.07
5 mg/L DCMCIT	17.66	27.88	15.95	35.53	14.50	22.30	± 9.07
Difference	31.45	48.86	40.89	15.59	20.76	31.51	
2.5 mg/L DCMCIT + microsomes	30.32	60.15				45.23	
2.5 mg/L DCMCIT	15.94	13.67				14.81	
Difference	14.38	46.48				30.43	

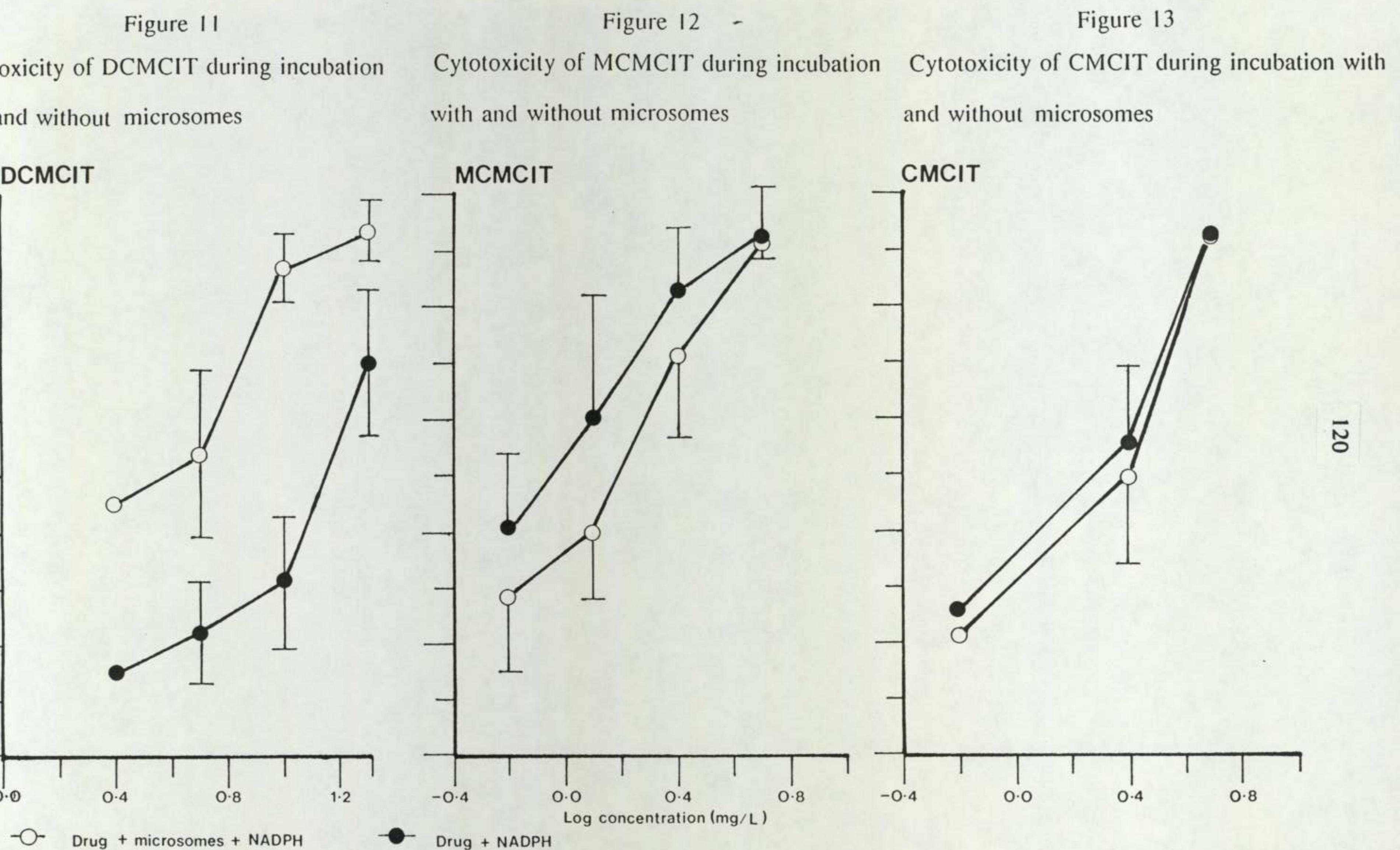


Figure 11
Cytotoxicity of DCMCIT during incubation with and without microsomes

Figure 12
Cytotoxicity of MCMCIT during incubation with and without microsomes

Figure 13
Cytotoxicity of CMCIT during incubation with and without microsomes

Table 17: Incubation of CMCIT with hepatic microsomes and the effect on cytotoxicity against TLX5 cells

CONDITIONS	% Inhibition of TLX5 Cell Growth			$\bar{x}$	S.D. %
	EXPT. 1	EXPT. 2	EXPT. 3		
2.5 mg/L CMCIT + microsomes	-	-	93.36	-	-
2.5 mg/L CMCIT	-	-	93.28	-	-
Difference	-	-	+ 0.08	-	-
1.25 mg/L CMCIT + microsomes	49.42	34.61	64.93	49.65	± 15.16
1.25 mg/L CMCIT	53.48	43.13	69.42	55.34	± 13.24
Difference	- 4.06	- 8.52	- 4.49	- 5.69	± 2.46
0.6 mg/L CMCIT + microsomes	27.72	15.47	-	21.60	-
0.6 mg/L CMCIT	31.02	21.14	-	26.08	-
Difference	- 3.30	- 5.67	-	- 4.49	-

Table 18: Incubation of MCMCIT with hepatic microsomes and the effect on cytotoxicity against TLX5 cells

CONDITIONS	% Inhibition of TLX5 cells				$\bar{x}$	S.D. %
	EXPT. 1	EXPT. 2	EXPT. 3	EXPT. 4		
5.0 mg/L MCMCIT + microsomes	93.64	-	89.16	89.59	90.80	±2.47
5.0 mg/L MCMCIT	99.72	-	93.94	83.86	92.51	±8.03
Difference	-6.08	-	-4.78	+ 5.73	-1.71	±6.48
2.5 mg/L MCMCIT + microsomes	80.20	83.70	51.41	69.98	71.32	±14.50
2.5 mg/L MCMCIT	95.30	88.85	71.92	73.79	82.47	±11.43
Difference	-15.10	-5.15	-20.51	- 3.81	-11.14	±8.02
1.25 mg/L MCMCIT + microsomes	42.25	28.06	34.25	55.12	39.92	±11.68
1.25 mg/L MCMCIT	79.13	28.69	66.60	65.33	59.94	±21.74
Difference	-33.88	-0.63	-32.35	-10.21	-19.27	±16.47
0.6 mg/L MCMCIT + microsomes	36.01	2.98	-	47.89	28.96	±23.27
0.6 mg/L MCMCIT	53.83	26.38	-	41.15	40.45	±13.74
Difference	-17.82	-23.40	-	+ 6.74	-11.49	±16.04

(Table 17, Fig 13) and MCMCIT (Table 18, Fig 12). Neither of these two compounds were more cytotoxic in the presence of microsomes than in their absence.

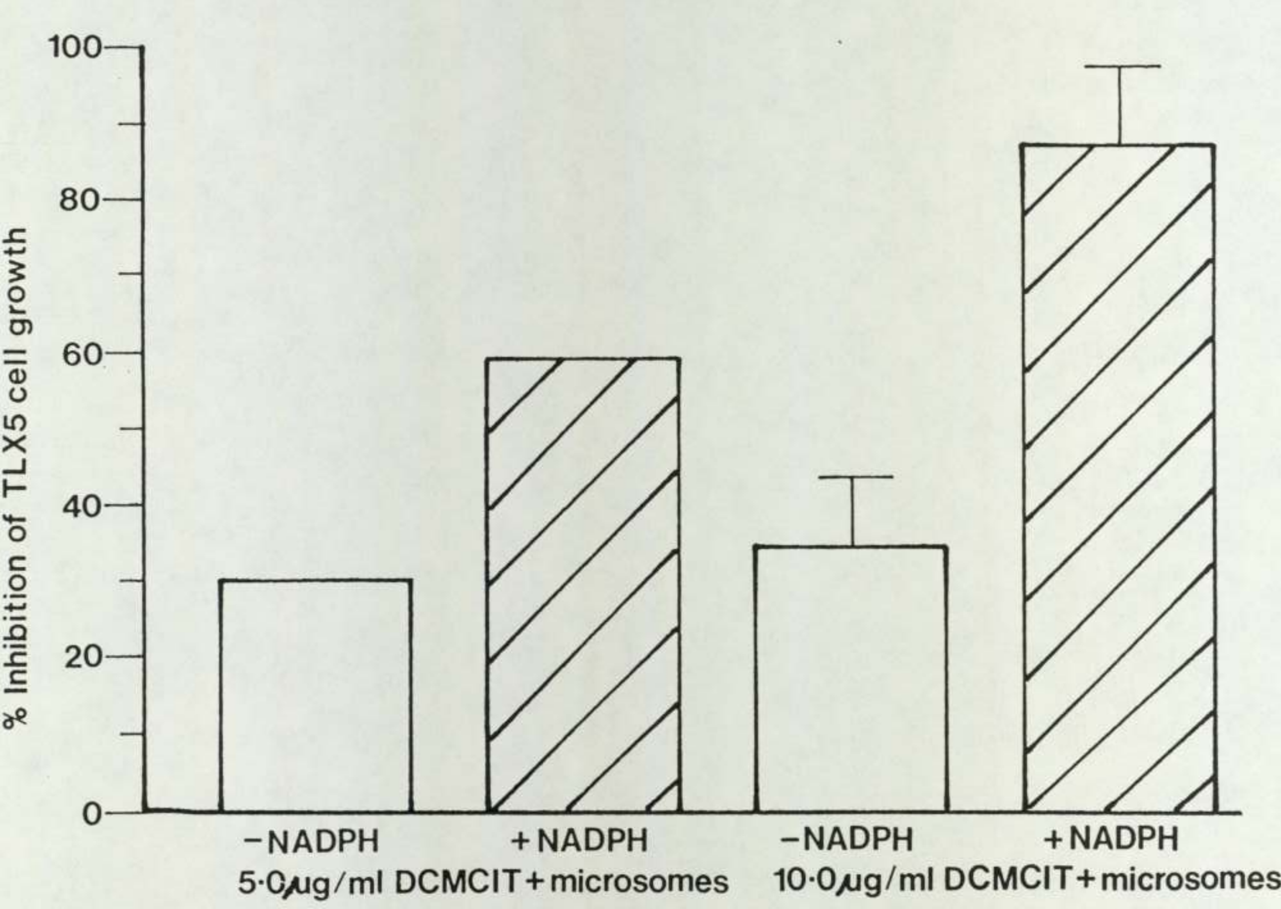
2.2.1.4 Effect of NADPH and enzyme denaturation upon bioactivation of 8-carbamoylimidazotetrazinones

Metabolic oxidation by hepatic microsomal enzymes requires reducing equivalents, and most of the enzymes work only in the presence of a NADPH generating system. To ensure that the observed microsomal activation (Section 2.2.1.3.) was NADPH-dependent, DCMCIT was incubated with microsomes in the presence and absence of NADPH. The results (Table 19, Fig 14) indicate that NADPH is required for the activation. That is, the cytotoxicity of DCMCIT was considerably increased when incubated with microsomes together with NADPH compared with the cytotoxicity of DCMCIT on incubation with microsomes without NADPH. This result suggests strongly that the microsomal cytochrome P450 enzyme system is responsible for the bioactivation observed here, even though more experiments would be required in order to substantiate this hypothesis. The drug metabolising ability of hepatic microsomes can be eradicated by denaturation of the enzymes via heating. Experiments were performed using heat-inactivated microsomes to determine the effect upon the bioactivation of DCMCIT. Not surprisingly, use of heat-inactivated microsomes did not give rise to a consistent increase in the cytotoxicity of DCMCIT compared with

Table 19: Effect of NADPH on cytotoxicity of DCMCIT against TLX5 cells when incubated with hepatic microsomes

CONDITIONS	% Inhibition of TLX5 Cell Growth			$\bar{x}$	S.D. %
	EXPT. 1	EXPT. 2	EXPT. 3		
10 mg/L DCMCIT + microsomes + NADPH	80.29	98.77	81.51	86.86	±10.34
10 mg/L DCMCIT+ microsomes	23.88	41.53	37.14	34.18	±9.19
Difference	56.41	57.24	44.37	52.67	±7.21
5 mg/L DCMCIT + microsomes + NADPH	35.26	83.12	-	59.19	
5 mg/L DCMCIT + microsomes	14.50	45.10	-	29.80	
Difference	20.76	38.02	-	±29.39	

Figure 14 Effect of NADPH upon cytotoxicity of DCMCIT when incubated in the presence of microsomes



its cytotoxicity in the absence of microsomes (Table 20). The interpretation of the results obtained in these experiments is not without difficulties, due to the problems encountered in including heat-inactivated microsomes in cell cultures. The use of such preparations was problematical due to the continued appearance of precipitated microsomal protein in the culture dish, which affected the counting of the cell cultures. This problem was only partially overcome by homogenising the deactivated microsomes, under aseptic conditions, prior to addition to the cell cultures. Other ways of microsomal inactivation, e.g. by incubation at 37°C for three days, did not improve the method, since it was almost impossible to maintain the sterility of such inactivated microsomal preparations.

2.2.1.5 Assessment of the role of metabolically generated formaldehyde in the cytotoxicity of 8-dimethylcarbamoyl-3-(2-chloroethyl)-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

The oxidative metabolism of N-methyl compounds leads ultimately to production of formaldehyde (102), a cytotoxic agent. The metabolically-generated release of formaldehyde has been considered to contribute to the antitumour activity of certain N-methyl containing agents[162,163]. It was therefore thought possible that the increase in cytotoxicity of DCMCIT

Table 20: Effect of incubation with inactivated microsomes on cytotoxicity of DCMCIT against TLX5 cells

CONDITIONS	% inhibition of TLX5 cells		$\bar{x}$
	EXPT. 1	EXPT. 2	
10 mg/L DCMCIT + microsomes + NADPH	92.02	97.62	94.82
10 mg/L DCMCIT + inactivated microsomes + NADPH	77.02	21.95	49.49
10 mg/L DCMCIT	67.58	13.23	40.40
5 mg/L DCMCIT + microsomes + NADPH	-	67.31	67.31
5 mg/L DCMCIT + inactivated microsomes + NADPH	-	19.30	19.30
5 mg/L DCMCIT	-	6.34	6.34

seen in the presence of microsomes may at least partially be due to the formaldehyde generated as the ultimate oxidation product of the dimethylcarbamoyl N-methyl moiety[164]. To test this conjecture, the cytotoxicity of formaldehyde against TLX5 cells was measured (Table 21). Formaldehyde was generated by treatment of paraformaldehyde with sodium hydroxide. The concentration of formaldehyde present was determined using a spectroscopic method based on that by Nash[165]. The growth of the cells was not affected by exposure for 90 minutes to up to 10 nmoles/ml formaldehyde, the maximum concentration theoretically achievable by metabolic oxidation of one of the two N-methyl groups in DCMCIT (10 mg/L) (Section 2.2.2.2). These results suggest that formaldehyde does not cause the increased cytotoxicity of DCMCIT when incubated with microsomes.

2.2.1.6 Discussion of the in vitro cytotoxicity studies involving imidazotetrazinones

Cytotoxicity testing against the TLX5 lymphoma cell line has indicated that this in vitro model provides useful information regarding possible in vivo activity of imidazotetrazinones. However, the use of such simplistic in vitro studies is open to criticism, since there are often complex metabolic processes in vivo which are not apparent in vitro. This has been clearly demonstrated by cytotoxicity testing of DCMCIT. Although highly active

Table 21: Cytotoxicity of formaldehyde against the TLX5 cell line

Formaldehyde Concentration nmoles/ml	% Inhibition of TLX5 cell growth	
	Experiment	
	1	2
20	28.43	21.00
10	12.60	8.00
5	7.80	5.00

in vivo, this compound exhibited relatively low in vitro cytotoxicity. Subsequent bioactivation studies have shown the that DCMCIT can be metabolically activated to a more cytotoxic species in vitro. It is logical to postulate that such a metabolic toxification occurs also in vivo, which would explain the dichotomy between the poor in vitro cytotoxic potential but good antitumour activity in vivo observed for this compound. Studies have also shown that the increased cytotoxicity of DCMCIT, when incubated with hepatic microsomes, cannot be accounted for by the production of formaldehyde.

On the basis of the results described above it can be hypothesised that production in vivo of MCMCIT and/or CMCIT by oxidative N-demethylation (with subsequent ring-opening) might be responsible for, or greatly contribute to, the antitumour activity of DCMCIT (Scheme 18).

Among the compounds investigated in this project, derivatives other than DCMCIT might also be metabolically activated. For example, 8-piperidinocarbonyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (104), like DCMCIT, showed pronounced antitumour activity in vivo (Table 2), with relatively low cytotoxicity in vitro (Table 12). By analogy to the postulated oxidative N-metabolism of cyclophosphamide (Scheme 17), (104) might undergo enzymatic hydoxylation at the piperidine α carbon, followed by opening of the piperidine ring by an

CH_3
$CON\text{–}CH_3$
CH_2CH_2Cl
64c

[O] →

CH_3
$CON\text{–}CH_2OH$
CH_2CH_2Cl
101

↓ − HCHO **102**

CH_3
$CON\text{–}H$
CH_2CH_2Cl
64b

[O] ←

CH_2OH
$CON\text{–}H$
CH_2CH_2Cl
103

↓ − HCHO

$CONH_2$
CH_2CH_2Cl
1

→ Ring-opening

Scheme 18

elimination reaction, to give the corresponding -NH containing species (107) (Scheme 19). Another N-disubstituted derivative, 8-(N-methyl)phenylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one was tested, and unlike DCMCIT or (104), it exhibited weak cytotoxicity in vitro (xxviii, Table 12) together with poor antitumour activity in vivo (Table 2). One might speculate that in this derivative metabolic activation by N-demethylation to 8-phenylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one does not occur. This could be easily explained by steric hindrance due to the bulky phenyl substituent which might prevent easy access of the drug metabolising enzymes to the N-methyl moiety.

2.2.2 Metabolism and biological stability studies

2.2.2.1 HPLC conditions

In this part of the project the hypothesis was tested, by HPLC studies, that the metabolite(s) which are responsible for the antitumour activity of DCMCIT is (are) N-demethylated species. The HPLC conditions and a description of a chromatogram for a mixture of authentic reference compounds of CMCIT, MCMCIT and DCMCIT are given in Section 2.1.2.2.

Scheme 19

2.2.2.2 HPLC analysis of the incubate of 8-carbamoylimidazotetrazinones with hepatic microsomes

Analysis of microsomal incubation mixtures of DCMCIT afforded three peaks (Figs 15,16,17), one of which coeluted with unchanged DCMCIT (iii). Another peak coeluted with MCMCIT (ii). This species appeared almost immediately after addition of DCMCIT to the microsomes, and its maximal concentration, corresponding to 28% of the initial concentration of DCMCIT, was observed after an incubation time of 45 minutes (Table 22, Fig 18). In samples of the microsomal suspension obtained within 30 minutes of the incubation period, appreciable amounts of another metabolite (identity unknown) were eluted from the column (x) (Fig 16). Collection and concentration of the eluant corresponding to peaks (x) and (ii) failed to yield sufficient material for mass spectroscopic characterisation[166].

Incubation of MCMCIT gave chromatograms (Table 23, Figs. 20, 21) which exhibited a (unknown) metabolite peak (y) (at t = 30 minutes, Fig 21) which did not correspond to any peak observed in mixtures of authentic reference compounds (Fig 19) (methanol/acetic acid (0.5%) in water (30:70) mobile phase), or in the extracts of microsomal incubations with DCMCIT. The unknown metabolites (x and y) are thought to be hydroxymethyl derivatives, the reason for this suggestion will be discussed

HPLC traces showing metabolism of DCMCIT during incubation with hepatic microsomes

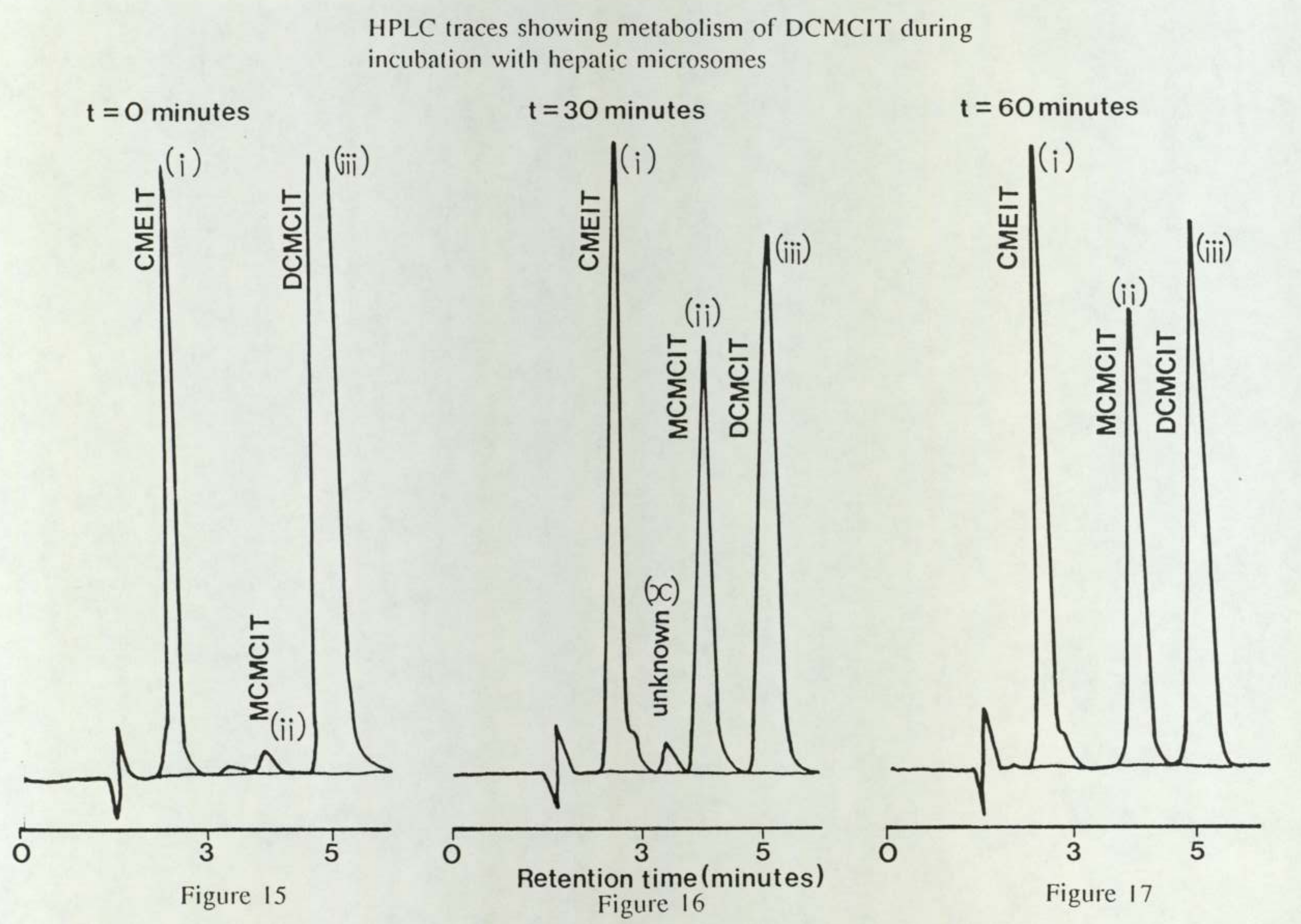


Figure 15

Figure 16

Figure 17

Table 22: Decomposition of DCMCIT in incubations with liver microsomes and generation of the MCMCIT metabolite

Time (Minutes)	DCMCIT Concn. (mg/L) Experiment 1	2	3	$\bar{x}$	S.D. mg/L	MCMCIT metabolite Concn. (mg/L) Experiment 1	2	3	$\bar{x}$	S.D. mg/L
0	23.57	20.71	19.65	21.31	±2.03	0.268	0.076	0.214	0.186	±0.099
15	14.52	13.71	10.83	13.02	±1.94	6.604	3.380	4.622	4.869	±1.626
30	8.27	9.66	7.03	8.32	±1.32	6.098	5.508	5.780	5.795	±0.295
45	8.63	7.18	6.03	7.28	±1.30	6.590	5.812	5.582	5.995	±0.528
60	8.86	6.39	5.89	7.05	±1.59	6.682	5.562	5.476	5.907	±0.673
75	8.12	6.27	5.22	6.54	±1.47	6.002	5.538	4.782	5.441	±0.616
90	6.44	5.97	-	6.21	-	4.608	5.226	-	4.917	-

Figure 18 Degradation of DCMCIT during incubation with microsomes and generation of the metabolite MCMCIT

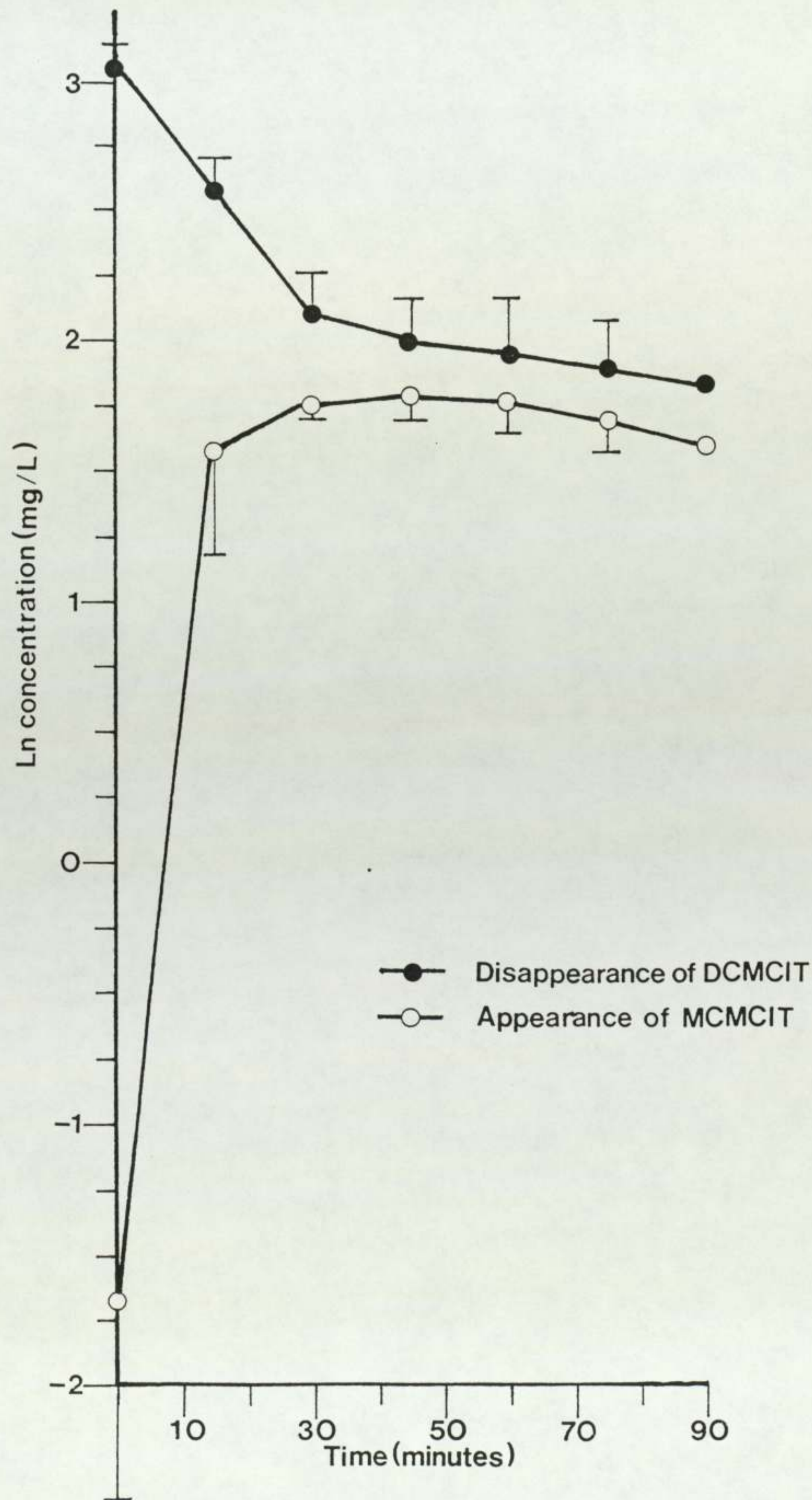


Figure 19 HPLC trace of authentic reference compounds for MCMCIT analysis

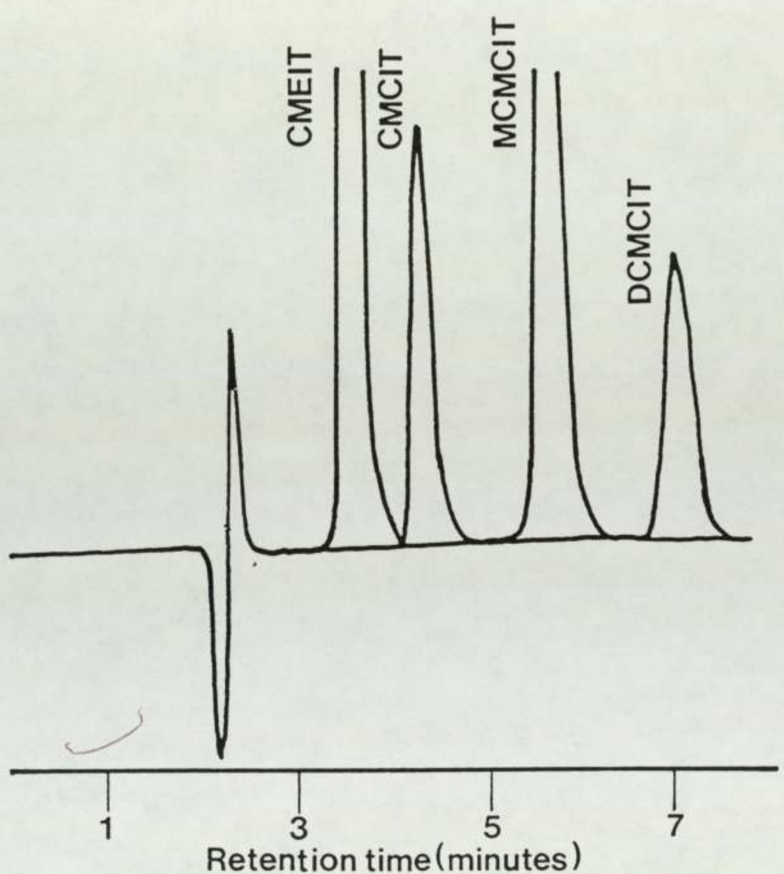


Table 23: Decomposition of MCMCIT in incubations with and without microsomes

Time (minutes)	MCMCIT Concn. (mg/L) Experiment 1	2	3	$\bar{x}$	S.D. mg/L
0	24.198	20.358	20.210	21.589	± 2.261
15	16.468	16.722	18.210	17.133	± 0.941
30	15.758	14.880	13.050	14.563	± 1.382
45	12.664	12.390	11.762	12.272	± 0.462
60	9.824	10.692	9.686	10.067	± 0.545
75	7.834	9.580	9.018	8.811	± 0.891
In the absence of microsomes					
0	-	21.722	23.704	22.713	-
75	-	10.476	12.470	11.473	-

HPLC traces showing metabolism of MCMCIT during incubation with hepatic microsomes

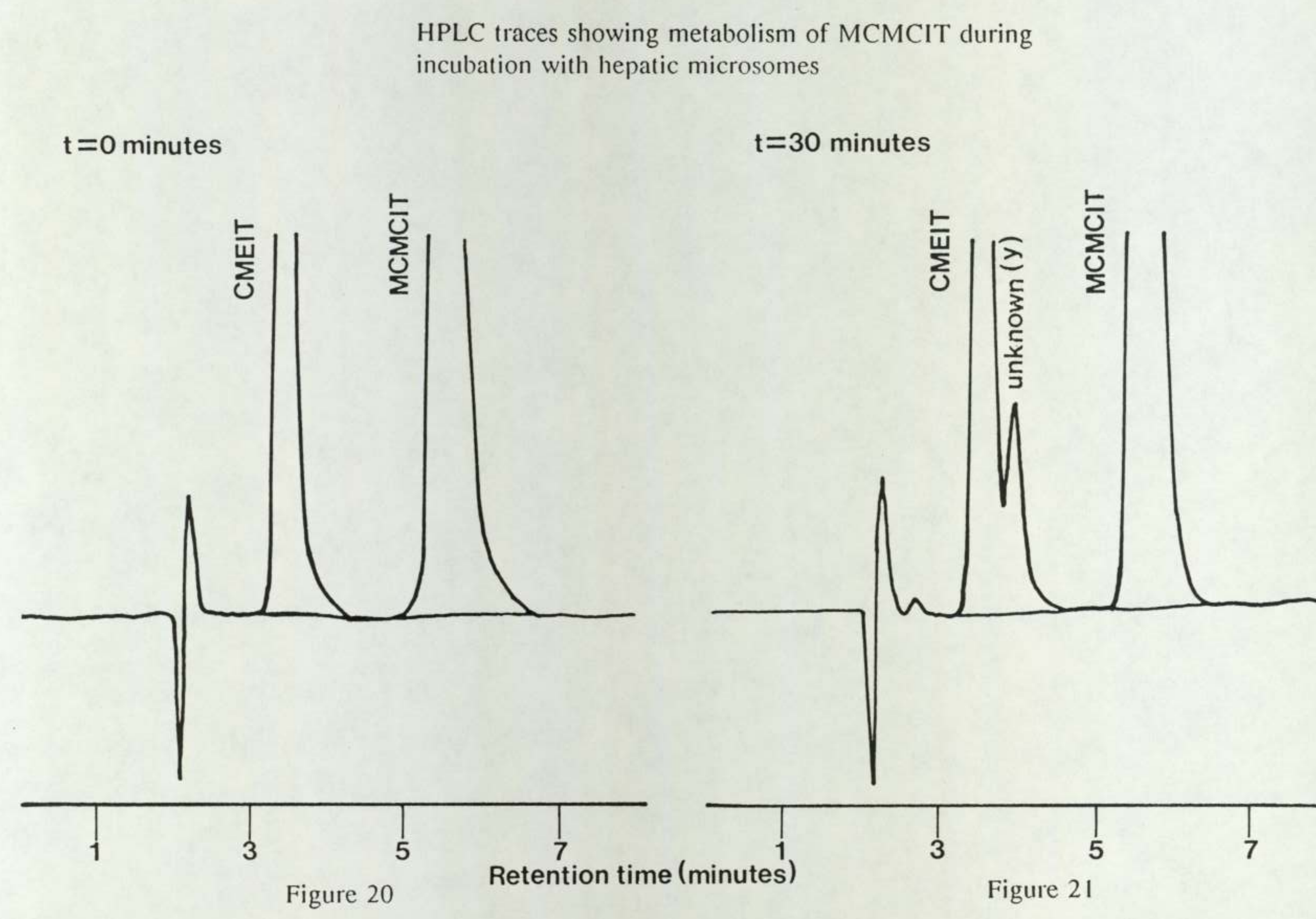


Figure 20

Figure 21

in more detail in Section 2.2.2.4.

In these experiments it was also demonstrated that the kinetics of drug disappearance (due to decomposition and/or metabolism) during incubation with hepatic microsomes was a first-order process for MCMCIT (Table 23, Fig 22) and CMCIT (Table 24, Fig 23). Values obtained for the disappearance of DCMCIT at time points beyond 45 minutes incubation suggest first-order kinetics (Table 22, Fig 24). However, considerable deviation from the first-order plot was observed for data obtained within the initial 45 minutes of the incubation. This deviation is presumably due to the rapid enzymatic conversion of DCMCIT to MCMCIT, which occured in addition to the chemical degradation of the tetrazinone moiety.

Experiments conducted to verify the HPLC method gave satisfactory values for extraction efficiency (Table 25), reproducibility of the assay (Table 26) and the instrument (Table 27). The linearity of the method was satisfactory, calibration curves using at least six evenly distributed points when analysed by least-squares regression all gave values > 0.98 and in many cases > 0.99.

2.2.2.3 Effect of NADPH and enzyme denaturation on metabolite formation

When suspensions of microsomes were incubated with DCMCIT in the

Figure 22 Kinetics of decomposition of MCMCIT during incubation with and without microsomes

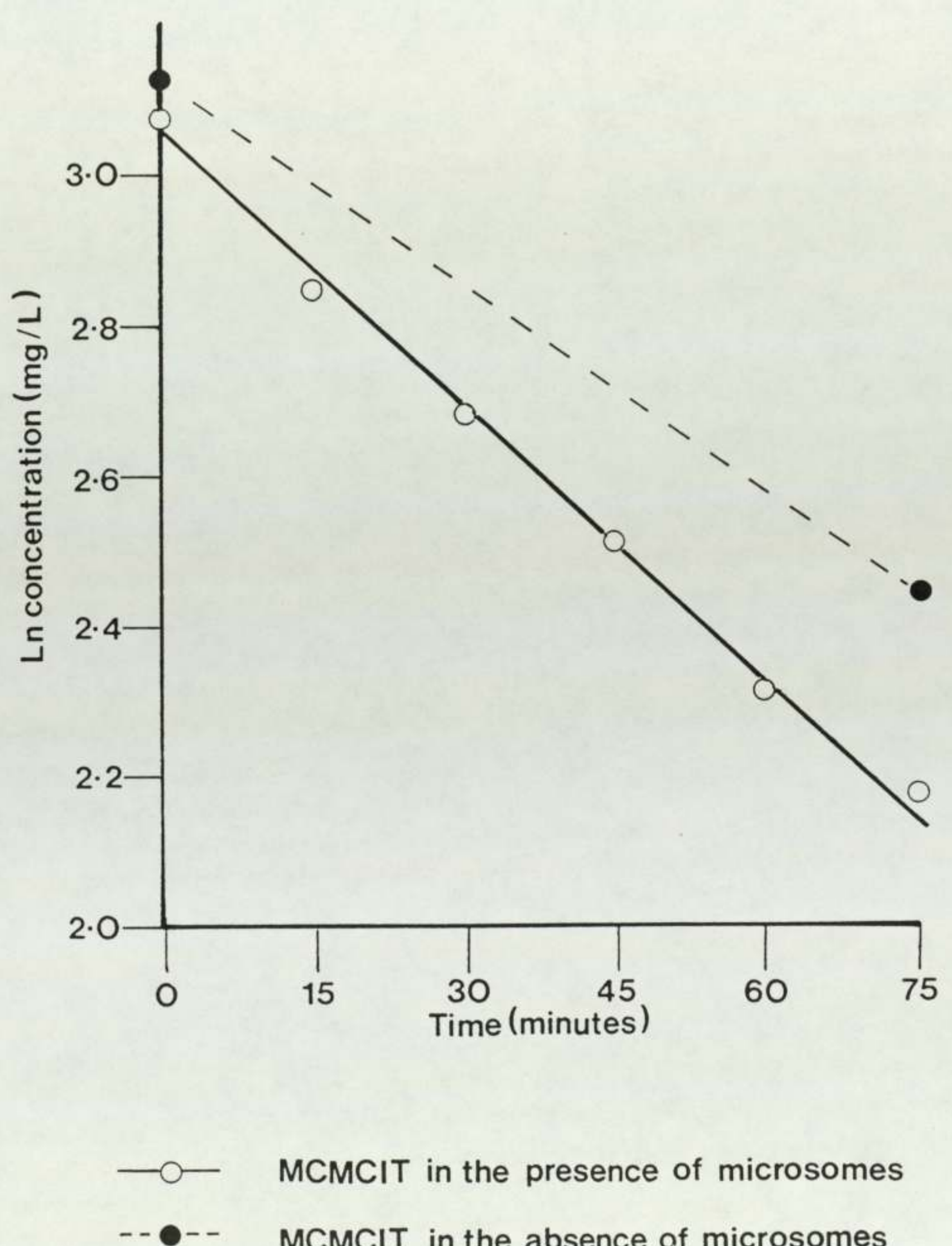


Table 24: Decomposition of CMCIT in incubations with and without microsomes

Time (minutes)	MCMCIT Concn. (mg/L) Experiment 1	2	$\bar{x}$
0	21.332	20.034	20.683
15	15.632	16.428	16.030
30	14.812	13.662	14.237
45	11.496	13.018	12.257
60	9.176	10.976	10.076
75	8.364	7.884	8.124
In the absence of microsomes			
0	21.286	21.540	21.413
75	8.110	7.876	7.993

Figure 23 Kinetics of decomposition of CMCIT during incubation with and without microsomes

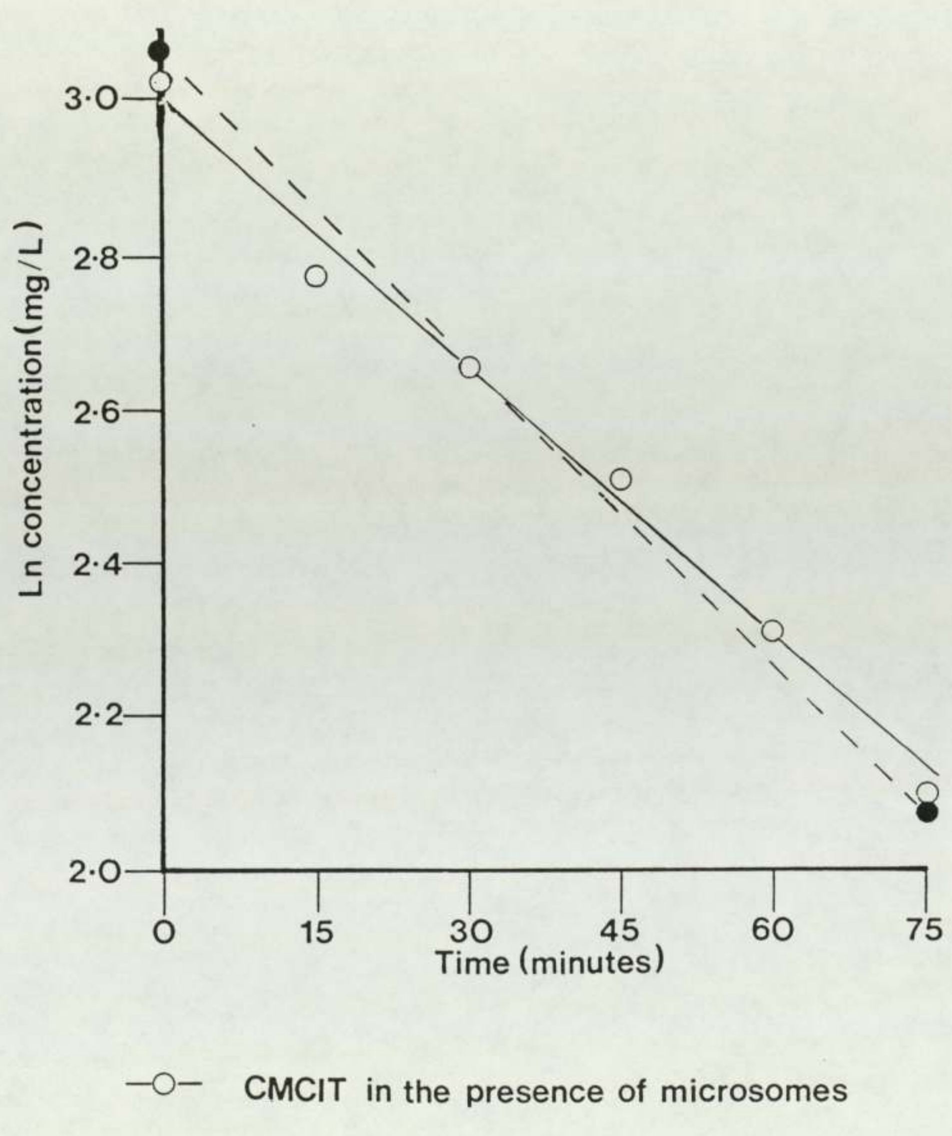


Figure 24 Kinetics of decomposition of DCMCIT during incubation with microsomes, inactivated microsomes and in the presence of microsomes without NADPH

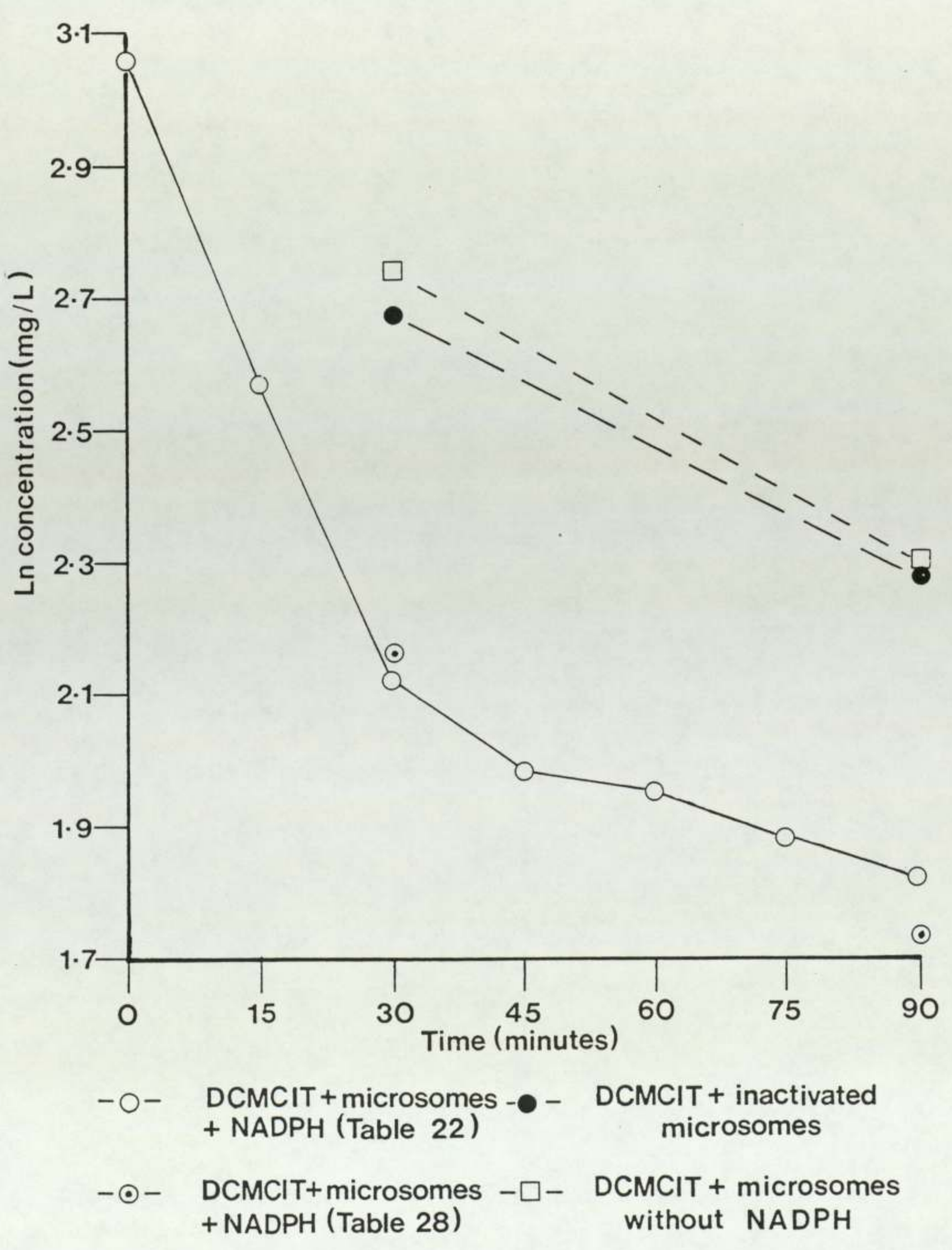


Table 25: Results obtained for extraction efficiency of the HPLC method

Amount of drug recovered as % of initial concentration			
CMCIT	MCMCIT	DCMCIT	CMEIT
73.25	83.34	93.06	49.61
69.49	84.36	88.66	46.20
65.63	83.62	85.95	44.70
67.73	76.81	86.61	44.14
64.64	76.32	81.78	43.99
$n = 5$	$n = 5$	$n = 5$	$n = 5$
$\bar{x} = 68.15$	$\bar{x} = 80.89$	$\bar{x} = 87.21$	$\bar{x} = 45.73$
S.D. = $\pm$ 3.41	S.D. = $\pm$ 3.97	S.D. = $\pm$ 4.11	S.D. = $\pm$ 2.34

Table 26: Results obtained for reproducibility of the HPLC method

Actual concentration mg/L	Concentration from assay mg/L		
	CMCIT	MCMCIT	DCMCIT
12.5	13.674	12.210	11.858
	13.943	11.991	12.136
	14.100	12.121	12.552
	14.080	11.942	12.305
		12.485	
		12.262	
Mean mg/L	13.949	12.168	12.213
Standard deviation mg/L	0.196	0.198	0.292
Coefficient of variation %	1.405	1.627	2.391
0.25	0.253	0.257	0.270
	0.271	0.284	0.261
	0.246	0.291	0.245
	0.250	0.270	0.257
	0.277		0.280
	0.267		0.263
Mean mg/L	0.261	0.276	0.263
Standard deviation mg/L	0.013	0.015	0.012
Coefficient of variation %	4.981	5.435	4.563

Table 27: Results obtained for instrument reproducibility of the HPLC method

Actual concentration mg/L	Concentration from assay mg/L		
	CMCIT	MCMCIT	DCMCIT
25	16.117	18.243	20.560
	15.541	18.343	19.764
	15.763	18.422	20.116
	15.688	18.357	20.148
	15.814	18.329	20.163
	15.792	18.298	20.141
Mean mg/L	15.786	18.332	20.149
Standard deviation mg/L	0.190	0.060	0.252
Coefficient of variation %	1.204	0.327	1.251
0.5	0.325	0.447	0.461
	0.322	0.504	0.445
	0.319	0.466	0.452
	0.314	0.494	0.447
	0.319	0.500	0.446
	0.318	0.469	0.441
Mean mg/L	0.319	0.480	0.449
Standard deviation mg/L	0.004	0.023	0.007
Coefficient of variation %	1.254	4.792	1.559

absence of NADPH, chromatograms obtained on HPLC analysis failed to show the presence of metabolites (x) and (ii) (Table 28). Similarly, metabolites (x) and (ii) could not be detected in the HPLC chromatograms of suspensions containing heat-inactivated microsomes with DCMCIT (Table 28). Again, as in the case of results of the microsomal bioassay (Section 2.2.1.4), these findings suggest the involvement of an enzyme, and more specifically, cytochrome P450.

2.2.2.4 Discussion of metabolism experiments

During the development of antitumour triazenes a plethora of theories have been proposed to explain how the dialkyltriazenes exert their activity. Preusmann et al[167] originally demonstrated that 3,3-dimethyl-1-phenyltriazene (49), when incubated with hepatic microsomes and NADPH, produced formaldehyde (102) as a metabolite, together with another oxidation product postulated as the 3-methyl-1-phenyltriazene (108) (Scheme 20). Conclusions from these and other studies led to the supposition that an oxidative metabolic pathway was responsible for the carcinogenicity, and possibly the antitumour activity, of aryldialkyltriazenes. Later investigations utilising in vitro bioactivation studies[168] were successful in providing further evidence for metabolic activation of dialkyltriazenes to monoalkyltriazenes.

Table 28: Effect of NADPH + enzyme denaturation upon degradation of DCMCIT and metabolite production

		DCMCIT Concn.(mg/L)						MCMCIT Concn.(mg/L)					
		Experiment						Experiment					
Time (Minutes)	Conditions	1	2	3	$\bar{x}$	Ln Concn.	S.D.	1	2	3	$\bar{x}$	Ln Concn.	S.D.
30	With microsomes	8.82	9.02	8.32	8.72	2.17	±0.360	4.36	5.58	5.78	5.24	1.66	±0.768
30	With inactivated microsomes	14.48	-	-	14.48	2.67	-	-	-	-	-	-	-
30	Without NADPH microsomes	15.52	15.34	-	15.43	2.74	-	-	-	-	-	-	-
90	With microsomes	5.04	5.70	6.20	5.64	1.73	±0.582	3.48	3.64	4.92	4.013	1.39	±0.789
90	With inactivated microsomes	10.02	9.58	-	9.80	2.28	-	-	-	-	-	-	-
90	Without NADPH	9.44	9.98	10.48	9.967	2.30	±0.520						

$$\text{PhN=N-N(CH}_3)_2 \; (\mathbf{49}) \longrightarrow \text{PhN=N-NH(CH}_3) \; (\mathbf{108}) + \text{HCHO} \; (\mathbf{102})$$

$$\mathbf{49} \longrightarrow \text{PhN=N-N(CH}_2\text{OH)(CH}_3) \; (\mathbf{109}) \longrightarrow \mathbf{108}$$

Scheme 20

$$\text{2,4,6-Cl}_3\text{C}_6\text{H}_2\text{N=N-N(CH}_3)_2 \; (\mathbf{110})$$

Gescher et al[169] challenged the assumption that monoalkyltriazenes were responsible for the antitumour activity of dimethyltriazenes. Studies involving 1-aryl-3,3-dimethyltriazenes (49) demonstrated that the monoalkyltriazene (108) was non-selectively toxic to sensitive and resistant TLX5 lymphoma cells, whereas incubation of the dimethyltriazene (49) with a liver homogenate gave cytotoxic metabolites which were more selectively toxic than the monomethyltriazene (108). Isolation and characterisation of these metabolites was unsuccessful. However synthesis and testing of the immediate metabolic precursors of 1-aryl-3-methyl-triazenes, the 3-(hydroxymethyl)-3-methyltriazenes (109), showed that these compounds were equally cytotoxic as their decomposition product, the monomethyltriazenes[170]. Such a carbinolamine has been identified unambiguously as a metabolite of 3,3-dimethyl-1-(2,4,6- trichloro-phenyl)triazene (110)[171]. Investigations with DTIC have shown that it too requires metabolic activation for optimum antitumour activity. The metabolic precursor of MTIC (3), 5-[3-(hydroxymethyl)-3-methyltriazen-1-yl]imidazole-4-carboxamide, HMTIC (111) was characterised as a urinary metabolite of DTIC in rats[171]. HMTIC was reported to be more stable than MTIC in polar solvents. This observation led to the proposal that HMTIC may act as a transport form of MTIC which was presumed to be the ultimate antitumour species derived from DTIC (Scheme 21), acting via its alkylation of biological nucleophiles such

35

111

50

Scheme 21

112

113

as DNA[172].

The dispute continues concerning the active species produced on metabolism of DTIC, with a recent report[173]suggesting that the enzymatic hydroxylation of DTIC has little, if any, role in the action of this agent toward cultured CHO cells. In another study[174] the metabolic generation of the monomethyltriazene has been shown to correlate with the antitumour activity of 1-(4-acetylphenyl)-3,3-dimethyltriazene (112). However, the toxicity caused by this compound was speculatively attributed to the production of one or more metabolites of the monomethyltriazene.

Present studies with DCMCIT, outlined in Section 2.2.2.2, have shown that incubation with hepatic microsomes leads to the generation of MCMCIT. By analogy to work conducted on N-methylbenzamides (113)[175,176] it could be speculated that the unidentified metabolite (x) was the N-methyl-N-hydroxymethyl intermediate (101) (Scheme 18). The rapid appearance and disappearance of this metabolite is consistent with the suggestion that it is a semi-stable hydroxymethyl species which can only be seen whilst the microsomal enzymes are oxidising the methyl group at a fast rate, i.e. during the initial 30-45 minutes of the incubation, but then disappears rapidly as the enzyme activity is reduced. The unidentified metabolite (y) found in microsomal incubations with MCMCIT might have

been the corresponding N-hydroxymethyl compound (103) (Scheme 18). Absolute proof of the structure of these metabolites was attempted but could not be obtained.

2.2.2.5 Rates of disappearance and half-life determination

Previous studies (See Section 2.2.2.2) had indicated that disappearance in the presence of microsomes followed first-order kinetics for CMCIT, for MCMCIT, and for DCMCIT beyond 45 minutes incubation. This conclusion was based on data obtained from incubations for a period of only 90 minutes, which was considered insufficient in the case of compounds which have half-lives in the order of the observation period. Further investigations in which disappearance was examined over three hours were conducted in collaboration with Dr. C. Quarterman. These experiments showed that, in accordance with the earlier findings, the disappearance of CMCIT (Table 29, Fig 25) and MCMCIT (Table 30, Fig 26), in the presence of microsomes over a period of three hours, can be described by first-order kinetics. The results also demonstrated that disappearance of DCMCIT (Table 31, Fig. 27) in the presence of microsomes was a first-order process, the previously observed deviation from first-order kinetics for DCMCIT (See Fig 24) not being seen in this experiment. The reason for this discrepancy is presumably the relatively low metabolic conversion of DCMCIT to MCMCIT in this particular

Figure 25 Extended kinetic study of CMCIT degradation on incubation in the presence of microsomes

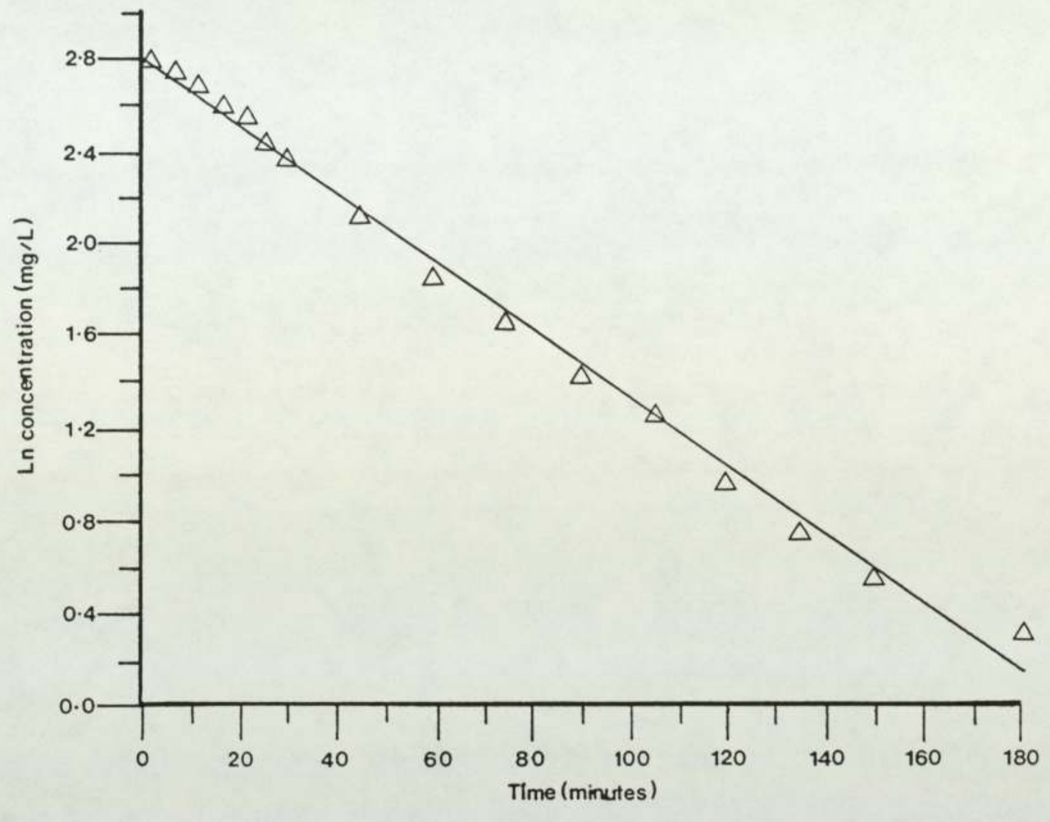


Table 29: Kinetics of degradation of CMCIT in RPMI 1640 media in the presence of hepatic microsomes

TIME (min)	CONC (mg/L)	LN CONC (mg/L)
2.000	16.177	2.784
7.000	15.409	2.735
12.000	14.494	2.674
17.000	13.211	2.581
22.000	12.606	2.534
26.000	11.218	2.418
30.000	10.511	2.352
45.000	8.095	2.091
60.000	6.246	1.832
75.000	5.081	1.626
90.000	4.041	1.396
105.000	3.376	1.217
120.000	2.552	0.937
135.000	2.055	0.720
150.000	1.686	0.522
180.000	1.325	0.281

ELIMINATION HALF-LIFE (min) = 46.1412

ELIMINATION RATE CONSTANT (1/min) = -0.0150

R = -0.9972

Figure 26 Extended kinetic study of MCMCIT degradation on incubation in the presence of microsomes

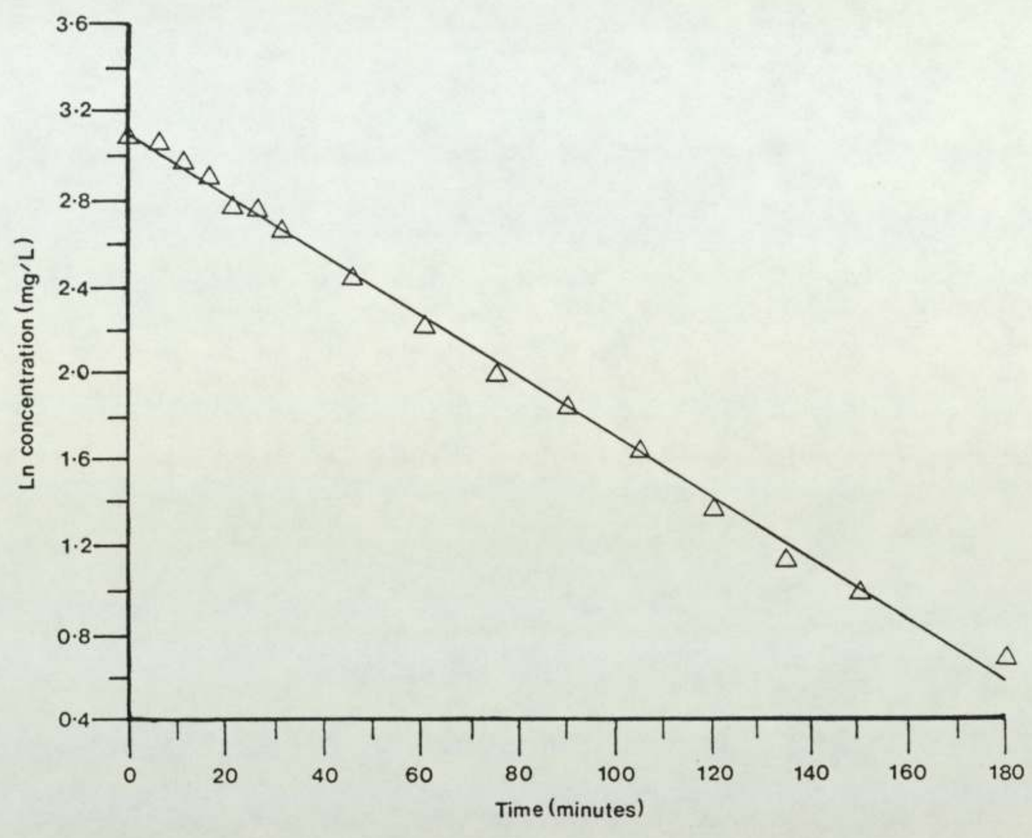


Table 30: Kinetics of degradation of MCMCIT in RPMI 1640 media in the presence of hepatic microsomes

TIME (min)	CONC (mg/L)	LN CONC (mg/L)
0.000	21.594	3.072
6.000	21.036	3.046
11.000	19.293	2.960
16.000	18.081	2.895
21.000	15.679	2.752
26.000	15.335	2.730
31.000	14.128	2.648
45.000	11.175	2.414
60.000	8.964	2.193
75.000	7.209	1.975
90.000	6.115	1.811
105.000	5.123	1.634
120.000	3.887	1.358
135.000	3.096	1.130
150.000	2.668	0.981
180.000	1.984	0.685

ELIMINATION HALF-LIFE (min) = 49.7755

ELIMINATION RATE CONSTANT (1/min) = -0.0139

R = -0.9982

Figure 27 Extended kinetic study of DCMCIT degradation on incubation in the presence of microsomes

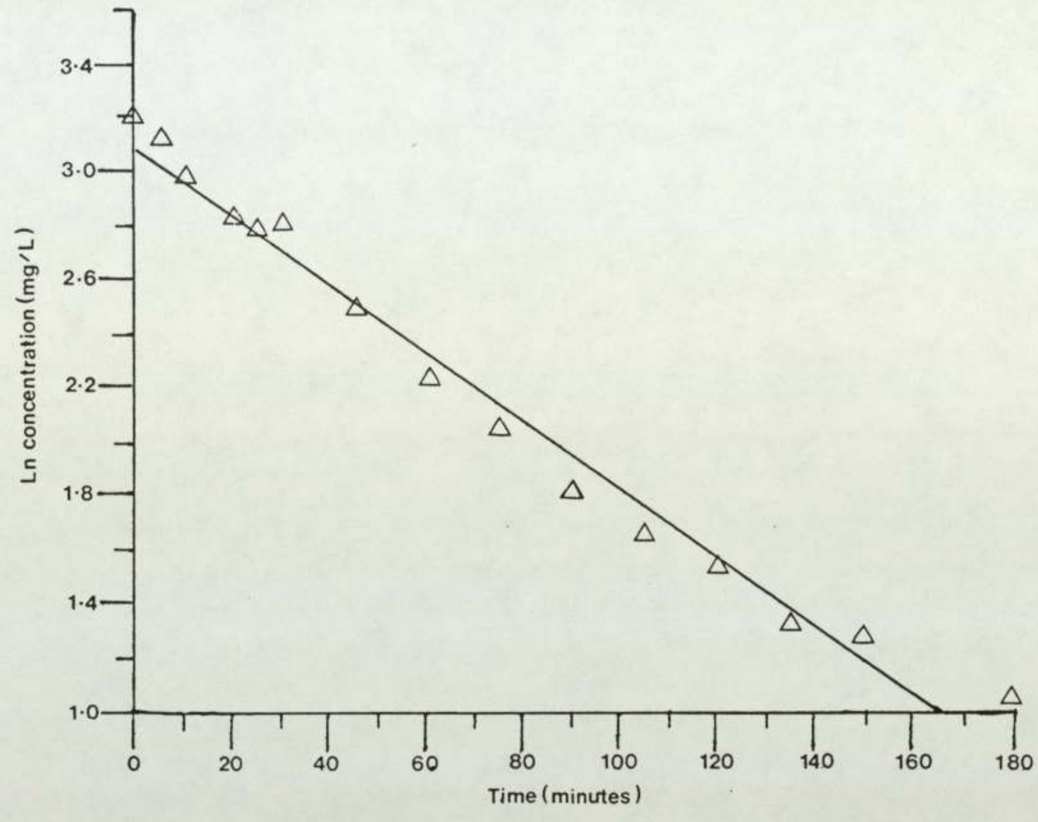


Table 31: Kinetics of degradation of DCMCIT in RPMI 1640 media in the presence of hepatic microsomes

TIME (min)	CONC (mg/L)	LN CONC (mg/L)
0.000	24.425	3.196
5.000	22.786	3.126
10.000	19.753	2.983
20.000	17.029	2.835
25.000	16.251	2.788
30.000	16.583	2.808
45.000	12.140	2.497
60.000	9.381	2.239
75.000	7.743	2.047
90.000	6.158	1.818
105.000	5.280	1.664
120.000	4.655	1.538
135.000	3.813	1.338
150.000	3.604	1.282
180.000	2.859	1.050

ELIMINATION HALF-LIFE (min) = 55.1761

ELIMINATION RATE CONSTANT (1/min) = -0.0126

R = -0.9904

experiment. In addition, these studies demonstrated that disappearance of drug in mixtures containing heat-inactivated microsomes also followed first-order kinetics for DCMCIT (Table 32, Fig 28), MCMCIT (Table 33, Fig 29), and CMCIT (Table 34, Fig 30), in a similar manner to their decomposition in RPMI 1640 medium alone (Section 2.1.2.2). Exposure to the air/CO_2 mixture during these incubations had little effect on the pH of the mixtures, values of pH varying between 7.33-7.77.

The half-life values were determined and are shown in Tables 29, 30, 31. The results from these biological stability studies, coupled with earlier findings from chemical stability studies (Section 2.1.2.2), support the contention that the rate of decomposition due to hydrolytic ring-opening is very similar for DCMCIT, MCMCIT and CMCIT. Therefore differential stability is unlikely to explain the differences observed in the cytotoxicity between DCMCIT and the other two 8-carbamoyl derivatives MCMCIT, CMCIT (Table 13).

2.2.2.6 Discussion of biological stability

Although the exact timing of hydrolytic ring-opening and oxidative N-demethylation is still not known, it would appear that enzymatic conversion of DCMCIT to MCMCIT occurs faster than hydrolytic ring-opening of DCMCIT in the biological environment. Comparison of disappearance rates of DCMCIT in the presence or absence of microsomes has shown a faster

Figure 28 Extended kinetic study of DCMCIT degradation on incubation in the presence of inactivated microsomes

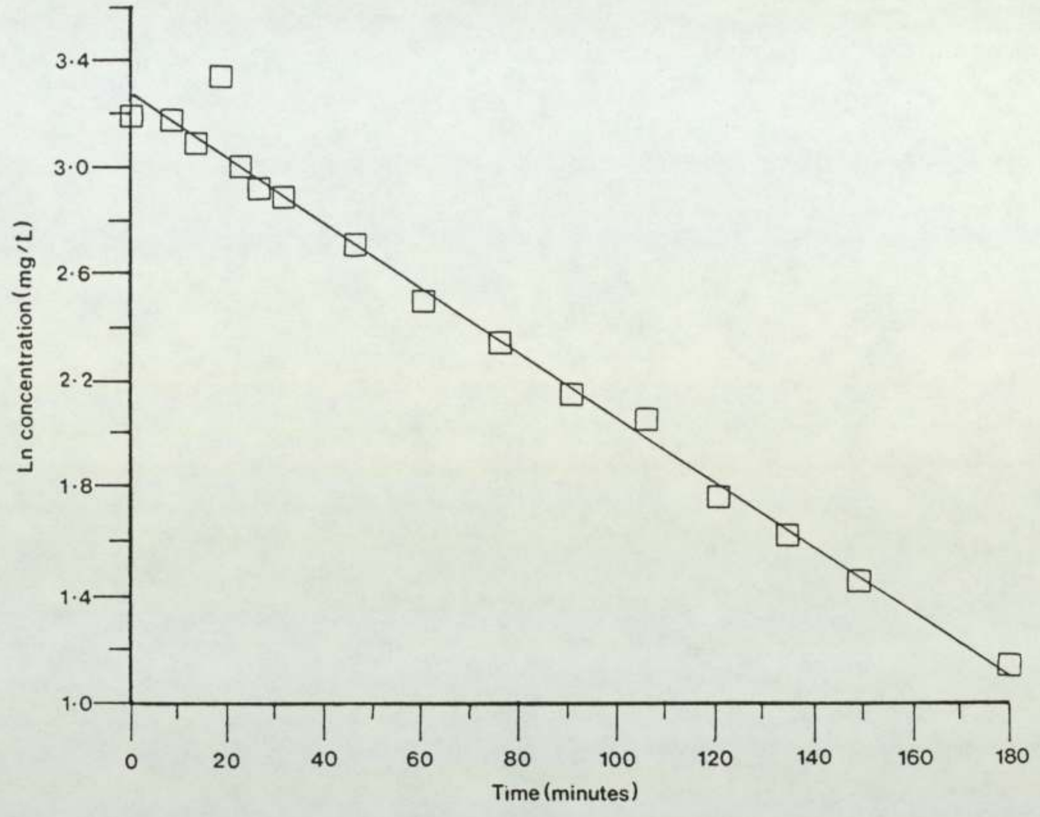


Table 32: Kinetics of degradation of DCMCIT in RPMI 1640 media in the presence of inactivated hepatic microsomes

TIME (min)	CONC (mg/L)	LN CONC (mg/L)
0.000	24.121	3.183
8.000	23.858	3.172
13.000	21.823	3.083
18.000	28.045	3.334
22.000	19.870	2.989
26.000	18.481	2.917
31.000	17.929	2.886
45.000	14.958	2.705
60.000	12.100	2.493
76.000	10.267	2.329
91.000	8.540	2.145
106.000	7.735	2.046
121.000	5.833	1.764
135.000	5.057	1.621
150.000	4.243	1.445
180.000	3.143	1.145

ELIMINATION HALF-LIFE (min) = 57.3092

ELIMINATION RATE CONSTANT (1/min) = -0.0121

R = -0.9928

Figure 29 Extended kinetic study of MCMCIT degradation on incubation in the presence of inactivated microsomes

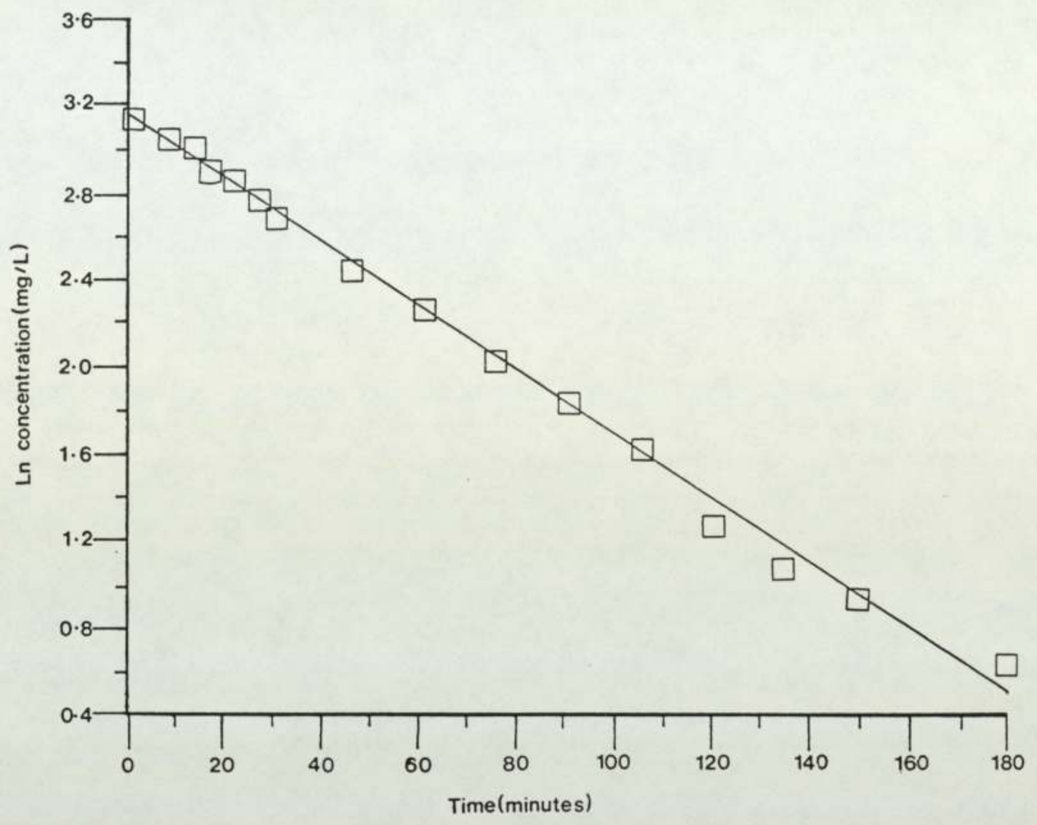


Table 33: Kinetics of degradation of MCMCIT in RPMI 1640 media in the presence of inactivated hepatic microsomes

TIME (min)	CONC (mg/L)	LN CONC (mg/L)
1.000	22.913	3.132
8.000	20.852	3.037
14.000	20.081	3.000
17.000	18.047	2.893
22.000	17.190	2.844
27.000	15.737	2.756
31.000	14.558	2.678
46.000	11.386	2.432
61.000	9.487	2.250
76.000	7.456	2.009
91.000	6.183	1.822
106.000	5.016	1.613
121.000	3.541	1.264
135.000	2.898	1.064
150.000	2.506	0.919
180.000	1.856	0.618

ELIMINATION HALF-LIFE (min) = 46.9361

ELIMINATION RATE CONSTANT (1/min) = -0.0148

R = -0.9980

Figure 30 Extended kinetic study of CMCIT degradation on incubation in the presence of inactivated microsomes

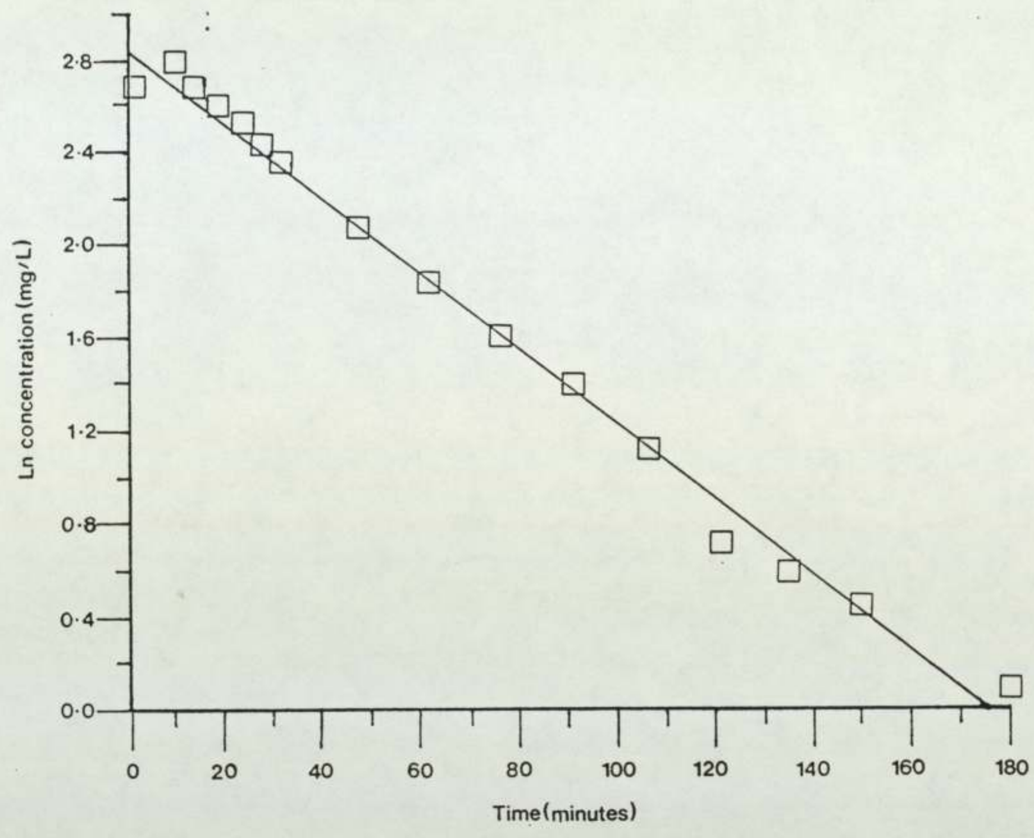


Table 34: Kinetics of degradation of CMCIT in RPMI 1640 media in the presence of inactivated hepatic microsomes

TIME (min)	CONC (mg/L)	LN CONC (mg/L)
1.000	14.583	2.680
9.000	16.219	2.786
13.000	14.505	2.674
18.000	13.360	2.592
23.000	12.439	2.521
27.000	11.214	2.417
31.000	10.467	2.348
46.000	7.909	2.068
61.000	6.248	1.832
76.000	4.982	1.606
91.000	4.048	1.398
106.000	3.065	1.120
121.000	2.057	0.721
135.000	1.811	0.594
150.000	1.572	0.452
180.000	1.091	0.087

ELIMINATION HALF-LIFE (min) = 43.1617

ELIMINATION RATE CONSTANT (1/MIN) = -0.0161

R = -0.9962

rate in the presence of microsomes. The increased rate of disappearance occured only during the first 45 minutes of incubation. Beyond this time the drug disappeared at a rate indistinguishable from that in buffer alone, i.e. at a rate similar to that due to hydrolytic ring-opening.

Further evidence to substantiate the hypothesis that DCMCIT is metabolised *in vivo* to MCMCIT, is provided by the fact that current *in vivo* studies in CBA/CA mice, conducted in The Pharmaceutical Sciences Institute, Aston University, have shown a rapid appearance of MCMCIT, as a metabolite in the blood, following administration of DCMCIT[177].

2.2.3 Conclusions from biological studies

The results here summarised support the hypothesis that there is a requirement for the presence of a -NH moiety in the 8-carbamoyl group in 8-carbamoylimidazotetrazinones for optimal antitumour activity and cytotoxicity.

a) CMCIT and MCMCIT exhibit good antitumour activity *in vivo* and high cytotoxicity *in vitro*, whereas DCMCIT exhibits good antitumour activity *in vivo* but relatively low cytotoxicity *in vitro*.

b) The cytotoxicity of DCMCIT *in vitro* is increased by metabolic activation with mouse hepatic microsomes.

c) HPLC results indicate that the major species produced on metabolic activation of DCMCIT is the monomethyl derivative MCMCIT. The production of MCMCIT could account for the increased cytoxicity of DCMCIT in the presence of microsomes.

d) Similar metabolic activation of DCMCIT is likely to occur in vivo, and preliminary in vivo results confirm rapid production of MCMCIT after administration of DCMCIT.

2.3 Structure-activity studies

2.3.1 Introduction

It has now been more than 100 years since Crum-Brown and Fraser proposed that the physiological action of a molecule was a function of its chemical constitution[178,179]. The term "quantitative structure-activity relationships" was eventually used to describe attempts to relate the activity of a group of drugs to their structural or physico-chemical characteristics, and there have been many reviews written on this subject[180,181,182].

2.3.2 Hansch Linear Free Energy Equations

The most widely adopted method in structure-activity studies is that of Hansch and Fujita[183,184,185], and it is this method that has been used in

work with the imidazotetrazinones.

The Hansch approach involves application of the equations similar to the following:

$$\log 1/C = a + b\pi + c\pi^2 + dEs + eMR + \delta f$$

Equation 1

log l/C represents the relative potency of the analogue, in which C is the concentration required to produce some standard biological response, in the present case, the IC_{50}.

π is a measure of the effect of the substituent on the logarithm of the octanol-water partition coefficient P;

δ, the Hammett value of a substituent, is the logarithm of the effect of that substituent on the acid dissociation constant of benzoic acid.

Es, the Taft constant, is experimentally derived from the relative rates of hydrolysis of esters, but for spherically symmetric substituents it is proportional to the radius of the substituent.

MR, the molar refractivity, is a steric parameter derived from the equation:

$$MR = (n - 1/n + 2)\ MW/d,$$

Equation 2

where MW is molecular weight, n is refractive index and d is density of compound.

2.3.3 Selection of a substituent set for analysis

In quantitative structure-activity relationships one aims to select a set of substituents with independence among several parameters, this requirement first being emphasised by Craig[186]. In addition, the use of the Hansch approach necessitates the inclusion of a wide range of substituents in the analysis if one is to be confident that the equations derived are meaningful. It was therefore decided that a rational substituent set for the imidazotetrazinones would be that given in Table 35[187,188].

However, it has already been mentioned that the chemistry of imidazotetrazinones is complex and engenders unique synthetic problems (see section 1.2.2). To achieve a reasonable spread of substituents without introducing major synthetic complications, the derivatives prepared were all 8-(substituted carbamoyl) derivatives (substituent sets 1 and 2, Appendices 1 and 2). There are very few carbamoyl substituents for which theoretical values of π, π^2, MR are available[189]. In the present work, values for π, π^2 and MR for the substituted carbamoyl groups were estimated using the Pomona College SMILES and CLOGP computer programs by calculating

Table 35: Rational substituent set for 8-substituted imidazotetrazinones

X substituent	Parameter Values for X substituent				
	Π	Es	MR	F	R
H	0.000	1.240	1.030	0.000	0.000
$(CH_2)_5CH_3$	3.050	-0.400	28.870	-0.060	-0.090
SCH_3	0.610	0.170	13.820	0.200	-0.180
OC_6H_{14}	2.550	0.020	30.900	0.250	-0.550
CN	-0.570	0.730	6.330	0.510	0.190
Br	0.860	0.080	8.880	0.440	-0.170
OH	-0.670	0.690	2.850	0.290	-0.640
$C(CH_3)_3$	1.830	-1.540	19.620	-0.070	-0.130
$NHOCH_3$	-0.970	-1.740	14.930	0.280	-0.260
$N(CH_3)_2$	0.180	-1.600	15.500	0.100	-0.920
SO_2CH_3	-1.630	-1.390	13.490	0.540	0.220
CF_3	0.880	-1.160	5.020	0.380	0.190
$N(CH_3H_8)_2$	2.180	-1.600	34.030	0.100	-0.920
NH_2	-1.230	0.630	5.420	0.020	-0.680
SO_2NH_2	-1.820	-1.380	12.280	0.410	0.190

values for substituted benzamides followed by subtraction of the value for the phenyl group.

2.3.4 Use of a hydrogen-bonding parameter

Consideration of results from the cytotoxicity studies led to the decision that it was imperative to include a hydrogen-bonding term into the equation. Hansch and Leo have suggested that since a set of hydrogen-bonding parameters is not available, a "dummy" value can be substituted into the analysis[189]. This approach was used for analysis of substituent set 1 (Appendix 1) which includes CMCIT, monosubstituted and disubstituted 8-carbamoyl derivatives. The disubstituted carbamoyl compounds cannot form a hydrogen-bond since they do not possess an amidic hydrogen; they are therefore assigned the value 0. CMCIT and its monosubstituted carbamoyl derivatives are capable of hydrogen-bonding, and so are given the value of 1 for the hydrogen-bonding term DV.

For substituent set 2 the -NH stretching frequency in the infra-red spectrum was determined for each substituent. The shift in this frequency relative to the averaged value for the hydrogen atoms attached to the carbamoyl nitrogen in CMCIT was used in the analysis as a measure of hydrogen-bonding capacity (H2)[190,191,192,193].

2.3.5 Results from structure-activity studies on 8-carbamoyl-imidazotetrazinones

Structure-activity data were generated using the MIXAR computer program developed by A. Loveless, May & Baker Ltd., Dagenham.

Use of the MR term in these investigations failed to demonstrate any dependence on this parameter. Using substituent set 1, a reasonable equation was obtained involving the hydrogen-bonding parameter (DV) alone (equation 3, Appendix 1, b):

$$\log 1/C = -1.2567 + 0.7400\ DV$$

Equation 3

(N = 18 S = 0.228 F = 26.226 R = 0.78809 P = 0.00012)

Inclusion of the parameters π & π^2 did improve the relationship obtained, although the contribution of these parameters to the overall equation was relatively minor (equation 4, Appendix 1, f):

$$\log 1/C = -1.1756 - 0.2120\pi - 0.2298\pi^2 + 0.7869\ DV$$

Equation 4

(N = 18 S = 0.166 F = 21.965 R = 0.90817 P = 0.00002)

A reasonable equation was obtained incorporating π & π^2 parameters from substituent set 2 (equation 5, Appendix 2, c):

$$\log 1/C = -0.3700 - 0.2064\pi - 0.2493\pi^2$$

Equation 5

($N = 15$ $S = 0.167$ $F = 8.909$ $R = 0.77302$ $P = 0.00428$)

An improvement in the equation was achieved by inclusion of the hydrogen-bonding term (H2) (equation 6, Appendix 2, g):

$$\log 1/C = -0.3363 - 0.2234\pi - 0.2513\pi^2 + 0.0048\ H2$$

Equation 6

($N = 15$ $S = 0.161$ $F = 7.024$ $R = 0.81057$ $P = 0.00667$)

These results suggest that there is a requirement for the presence of a carbamoyl -NH group for optimum cytotoxicity/antitumour activity in 8-carbamoylimidazotetrazinones. Once this group is present, alteration of π can also affect the cytotoxicity/antitumour activity of these compounds.

2.3.6 Discussion of structure-activity studies

Triazene chemistry provided an abundance of information for structure-activity scientists and this inevitably led to a great variety of studies by numerous workers. Most of these studies were performed using phenyltriazenes, on the premise that these compounds were just as active as DTIC and, moreover, it was easier to study systematic structure-activity relationships with the phenyltriazenes than with heterocyclic triazenes. Connors and co-workers[194] conducted experiments which involved testing

ring-substituted phenyltriazenes for antitumour activity against a TLX5 lymphoma cell line. All of these compounds exhibited antitumour activity irrespective of whether the aromatic substituent was electron-donating, such as p-methoxy, or electron-withdrawing, such as p-trifluoromethyl or p-methanesulphonyl, or whether the substituent was hydrophilic or hydrophobic.

The crucial requirement for in vivo activity against TLX5 lymphoma was attributed to the presence of a N-methyl group in the aryldialkyltriazenes. This conclusion had been reached by earlier workers[195,196]. Linn and Loo[197], in testing ring-substituted alkyltriazenes against mouse leukaemia L1210 tumours, established that the carbamoyl substituent in phenyltriazenes could be replaced by a methyl ester group with no adverse effect on antitumour activity. Further, variation of the position of these substituents relative to the dimethyltriazenyl sidechain was not crucial to activity, since all three derivatives (ortho, meta and para) gave comparable antileukaemic activity. Consideration of these results, together with those from other studies[198,199], led to the premise that the imidazole ring, the phenyl ring and the pyrazole ring were all therapeutically equivalent in triazenes. In agreement with these suggestions is the fact that potent antitumour activity is also seen with pyrazolotetrazinones as well as with the imidazotetrazinones (see section 1.2.2).

Extensive studies by Hansch et al[200,201] substantiated the findings of Linn and Loo following the assessment of the antitumour activity of DTIC analogues against the L1210 leukaemia. It was found that the imidazole grouping conferred no special potency; it could be replaced by other π excessive or π deficient heterocyclic rings, or by an aryl moiety, with no deleterious effect. Derivatives having logP values of 1.1 were the compounds exhibiting optimum potency. However, drug modification studies led to the recommendation that there was virtually nothing to be gained therapeutically by manipulation of logP for these compounds.

The structure-activity studies on triazene compounds conducted by Hansch culminated in two provocative statements[200,201]:

> **"Since we have not been able to separate the structural features for toxicity from those for efficacy, there is little support to encourage further work on triazenes as antitumour agents";**

and

> **"Unless one had new biochemical or molecular biological information suggesting that a new triazene might be more effective in some specific way, we would not recommend the synthesis and testing of new cogeners".**

Present results from structure-activity studies on imidazotetrazinones, involving correlation of in vitro cytotoxicity with physical properties, may challenge the above statements. Although it is not possible to draw firm conclusions from the data or provide physical values for observations and inferences from cytotoxicity assays and metabolism studies, it is tentatively suggested that there is a possible dependence on hydrogen-bonding capacity and π for optimal antitumour activity in the 8-carbamoyl-imidazotetrazinones. The capacity to hydrogen-bond via a carbamoyl -NH appears to be quite crucial to activity, along with some minor contribution from π. However, once an amidic -NH is present, the actual strength of the hydrogen-bond is not a major determinant in the activity; it is the hydrophobicity, incorporated in π and π^2, which is more important. Predictions from this data suggest that the ideal carbamoyl substituent should possess an amidic hydrogen and have a negative π value, e.g. $CONH_2$, $CONHCH_3$, $CONHOCH_3$, $CONHCH_2CH_2Cl$. Derivatives based on the above criteria should possess high activity, but they are also likely to produce considerable host toxicity. Although previous attempts to discover imidazotetrazinones with improved therapeutic index were unsuccessful (section 1.3.3), the findings from present QSAR and biological studies suggest that manipulation of the 8-carbamoyl substituent may provide a method for increasing the therapeutic index of these compounds.

For example, peptide pro-drugs have previously been considered in the design of antitumour agents[202-206]. The rationale for the design of these agents was that the peptide moiety would be removed by an enzyme specific to the tumour site, giving localised release of the anticancer species. Little success has been achieved using these methods; however, future studies on the molecular biology of cancer may discover further tumour-specific enzymes which may be exploited in the above manner. Use of a blocking peptide moiety in the 8-position of 8-carbamoyl-imidazotetrazinones, with release of the active mono- or un-substituted carbamoyl species at the tumour site, may provide a means to increasing the therapeutic index of these compounds.

The findings from structure-activity studies with imidazotetrazinones also suggest that if earlier workers had employed in vitro cytotoxicity methods in addition to in vivo studies, then the carbamoyl substituent may have been found to be implicated in the activity/cytotoxicity of phenyltriazenes, DTIC and its derivatives, and pyrazolotriazenes.

3 CONCLUSIONS: THE ROLE OF THE 8-SUBSTITUENT IN IMIDAZOTETRAZINONES

In this section it will be argued that a hydrogen-bonding interaction involving the carbamoyl -NH moiety may be involved in the activity of 8-carbamoylimidazotetrazinones. Following a brief general description of hydrogen-bonding, tautomerism and intramolecular hydrogen-bonding in triazene compounds will be discussed and related to possible intramolecular hydrogen-bonding in imidazotetrazinones. The relevance of the carbamoyl -NH moiety for possible intermolecular hydrogen-bonding interactions with imidazotetrazinones will be considered by analogy to DNA binding drugs, and a receptor site for imidazotetrazinones will be postulated. In addition, the hypothesis that steric requirements at the receptor site may play an important role in the mode of action of imidazotetrazinones will also be mentioned.

3.1 Characteristics of hydrogen-bonding

The distinguishing feature of hydrogen-bonding is the involvement of a specific hydrogen atom of a proton donor group (A-H) with a localised site of high electron density (B), where A is an electronegative atom, e.g. O, N, S, X, (F, Br, I, Cl) or C, and B is a lone electron pair of an electronegative atom, or a Π electron orbital of a multiple bond (unsaturated) system. This interaction can involve two functional groups in the same or different molecules. These last two situations correspond to

the formation of intramolecular and intermolecular H bonds respectively.

Hydrogen-bonding is a distinctly directional and specific interaction and is more localised than any other type of weak intermolecular interaction. The total bond length R(A....B) is equal to or less than the sum of the van der Waals radii of atoms A & B, that is the total bond length contraction caused by H-bond formation is equal to or is greater than twice the van der Waals radius of the hydrogen atom. For intramolecular hydrogen-bonding, interaction only occurs when A-H and B are in a favourable spatial configuration and the distance between the H of the donor group and the acceptor site is between 1.4 and 2.5Å . The importance of hydrogen-bonding in chemical and biological situations and details of the numerous methods of detection available for hydrogen-bonding have been reported on several occasions[207,208]. An intramolecular hydrogen-bond has already been observed in the X-ray structure of CMCIT between the carbamoyl -NH and the tetrazinone ring nitrogen N-1 (See Section 2.1.3.1). If one assumes the presence of a similar bond in solution, then 8-carbamoyl derivatives possessing an amidic hydrogen may exhibit an intramolecular hydrogen-bond which diminishes the tendency of these compounds to ring-open in solution. This led to the proposal (Section 2.1.2.1) that compounds such as CMCIT and MCMCIT may experience increased stability, leading to enhanced activity relative to the disubstituted 8-carbamoylimidazo-tetrazinones such as DCMCIT. Chemical stability studies have indicated

that this is not the case, CMCIT, MCMCIT and DCMCIT having similar half-life values (See Section 2.1.2.2).

3.2 Tautomerism in triazenes

There have been many studies employing ir and nmr spectroscopic methods as a means of studying the tautomerism in triazene compounds. Hadzi and Jan[209] demonstrated that infra-red spectra of monoalkyltriazenes generally exhibit two -NH stretching vibration bands, one at 3480-3440 cm^{-1} assigned to tautomer (114a), and the other near 3338 cm^{-1} assigned to (114b). Unambiguous assignment of these bands was achieved by observing the shift of the low-frequency band to lower frequency when ^{15}N was introduced at N-1. The relative intensities of these bands are governed by the substituent (X), suggesting that the substituent influences the tautomeric equilibrium: electron-withdrawing substituents shift the equilibrium in favour of form (114b). Infra-red measurement has also shown a similar substituent effect in 1,3-diaryltriazenes[210,211].

The effect of substitution in the aryl group on the tautomerism of monoalkyltriazenes has also been demonstrated using nmr spectroscopy, the tautomerism also showing a strong dependence on the solvent used[212]. The substituent effect varied according to the position of the substituent, and whether it was electron-withdrawing or electron-donating. Thus, 1-aryl-

$X-C_6H_4-N{=}N-NHR \rightleftharpoons X-C_6H_4-NH-N{=}NR$

114a **114b**

OC_2H_5

$C{=}O \cdots H-N-N{=}N-CH_3$

115

3-methyltriazenes with strongly electron-withdrawing p-substituents were found to be in a state of tautomeric equilibrium, Ar.N=N.NHMe⇌Ar.NH.N=NMe. However, a similar study of 3-methyl-1-p-tolyltriazene indicated that the conjugated form predominated, whereas arylmethyltriazenes with strongly electron-withdrawing ortho-substituents were shown to exist as the unconjugated tautomer, Ar.NH.N=NMe.

It was reported that the equilibrium was shifted in favour of the unconjugated tautomer by the presence of electron-withdrawing substituents in the aryl group, and that the preference was total when the substituent was in the ortho-position. The hypothesis was that the absolute preference for the unconjugated tautomeric form in ortho-substituted aryltriazenes was due to the presence of a strong intramolecular hydrogen-bond (e.g. 115). This intramolecular hydrogen-bonding was considered much less likely in the conjugate tautomer of (115) and is not possible in the meta- and para-derivatives.

The triazenes formed following hydrolytic ring-opening of imidazotetrazinones should also display tautomerism. By analogy to the alkyltriazenes, the 8-carbamoyl group is thought to stabilise the unconjugated tautomer by hydrogen-bonding (27)[11]. However, this cannot be the sole requirement for activity since the three 8-carbamoyl derivatives

CMCIT, MCMCIT and DCMCIT would all be able to produce such a intramolecular bond and hence should be equiactive. Differences in cytotoxicity observed between these compounds cannot be explained by this theory.

From present findings showing the requirement of an amidic hydrogen for high activity, it is also feasible that the -NH group may also affect the tautomerism of the corresponding ring-opened triazenes (116a, 116b), giving rise to differences in the activity of these compounds. Participation of the -NH moiety in an intramolecular hydrogen-bonding interaction with the imidazole nitrogen is also possible (117). The existence of these intramolecular hydrogen-bonds in triazenes from CMCIT and MCMCIT may theoretically lead to an increased stability of these compounds relative to the corresponding triazene from the disubstituted derivative DCMCIT (118), in which there is only one type of intramolecular bond possible. As outlined in Section 2.1.1.2.7, many attempts were made to prepare the corresponding triazenes from CMCIT, MCMCIT, DCMCIT, the methyl ester (65a) and the ethyl ester (65b), for subsequent stablity studies and ^{1}H nmr studies to examine tautomerism in these compounds. Unfortunately, only reaction with CMCIT gave an isolatable product; however, when analysed by ^{1}H nmr the product (MCTIC, 3) was shown to be contaminated with a small amount of AIC (17). This contamination made interpretation of the ^{1}H nmr spectrum extremely difficult with assignment of -NH peaks impossible. Further batches of MCTIC prepared by the

NHR
C=O H
N N=N CH2CH2Cl ⇌
N NH
116a

R
CON-H
H
N=N-N CH2CH2Cl
N NH
116b

a) R=H
b) R=CH_3

RN—C=O H
H N N=N CH2CH2Cl
N NH
117

N(CH3)2
C=O H
N N=N CH2CH2Cl
N NH
118

a) $R_1=R_2=H$
b) $R_1=H$, $R_2=CH_3$
c) $R_1=R_2=CH_3$

N
O_2N
$CONR_1R_2$
NO_2
119

coupling reaction between Diazo-IC and 2-chloroethylamine[9] also showed the presence of decomposition products in the spectrum. The solvolytic instability of MCTIC precluded ^1H nmr analysis, as during sample preparation a gas (presumably nitrogen) evolved on addition of d_6 DMSO, indicating extremely rapid degradation of MCTIC in the solvent. For future stability experiments with these triazene derivatives it appears futile to synthesise the compounds via the ring-opening route. Diazotisation of the corresponding AIC derivatives followed by coupling with chloroethylamine will probably be more useful for preparation of the triazene derivatives[9].

3.3 Presence of the carbamoyl moiety in other antitumour agents

An earlier hypothesis suggested the existence of a 'receptor' for imidazotetrazinones with a requirement for a coplanar oxygen atom in the 8-substituent for optimum activity (Section 2.1.4). Although interesting, this conjecture was based upon differences observed in X-ray structures which are not necessarily representative of the situation within the biological environment. However, it is still conceivable that disubstitution of the 8-carbamoyl group, with disruption of planarity, may prevent binding of 8-carbamoylimidazotetrazinones to the 'receptor site', thus leading to low cytotoxicity. A similar hypothesis was proposed for

5-carbamoyl-2,4-dinitrophenylaziridine, CB 1954 (119)[213,214]. This compound exhibited a very high chemotherapeutic index against the Walker Carcinoma 256 in rats: however, a complete loss of activity was found when both hydrogens of the amide were substituted. Structure-activity relationships gave no correlation between the rates of alkylation of DNA and antitumour activity, and no overall correlation between partition coefficients and activity. The ability to bind to a theoretical receptor site was suggested as being essential for the antitumour activity of CB 1954. The carbamoyl derivative (119a), and the monosubstituted carbamoyl deravitive (119b), were seen to be able to maintain the presentation of a planar face to this hypothetical receptor. This was not the case for the disubstituted carbamoyl deravitive (119c). Studies of reversible binding to bovine serum albumin were used as a model for a possible drug-receptor interaction and to correlate biological activity with the ability of these compounds (119a) (119b) (119c) to bind to a specific receptor site. Comparison of the compounds showed that disubstitution lowered their affinity for the protein, and this correlated with a reduced antitumour activity.

Recent advances in molecular biology have inspired great interest in the role of drug binding to DNA. Many DNA binding agents have been discovered which utilise hydrogen-bonding interactions to stabilise their binding to the DNA structure[215]. The presence of the carbamoyl group

seems ubiquitous in these compounds such as CC 1065 (120)[216], and especially the antitumour antibiotic derivatives netropsin (121)[217] and distamycin (122)[218]. Netropsin and distamycin bind in the minor groove of DNA where they require binding sites consisting of four or five base pairs respectively. This firm and site-specific binding, which seems to underlie biological activity, is the net result of specific hydrogen-bonding, electrostatic attraction and van der Waals interactions. These agents have a characteristic structure containing a number of heterocycles linked by amide bonds. The molecules require a close approach to planarity and utilise the carbamoyl groups for hydrogen-bonding interactions. The role of the hydrogen-bonds in the binding of netropsin is thought to be involved with alignment of the drug with respect to the DNA structure[219]. The distances of the hydrogen-bonds formed vary, with some bond lengths being considerably longer than standard hydrogen-bond lengths[220]. A DNA binding agent containing a triazene linkage has also been reported, i.e. berenil (123)[221]. This compound exhibits potent antitumour activity, although charged amidinium groups probably play an important part in the activity of this compound.

Knowledge of how DNA binding drugs exert their antitumour effect has been used to speculate that a more specific 'receptor site' for imidazo-

120

121

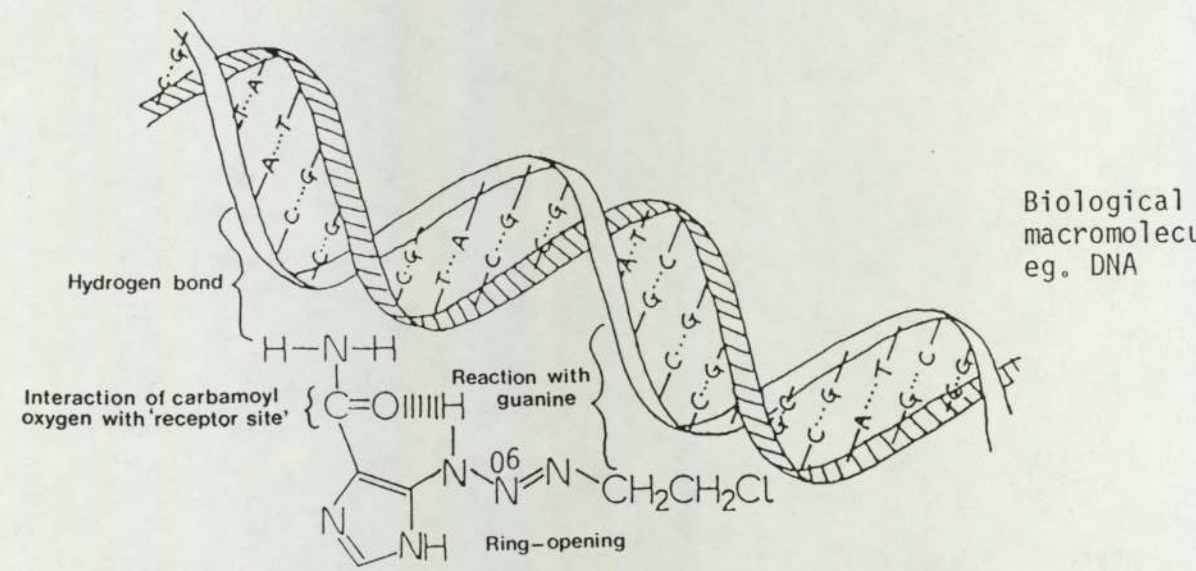
122

123

tetrazinones may exist. The requirement for planarity suggested earlier, coupled with the possibility of hydrogen-bonding via the carbamoyl -NH group, indicates that, as with the DNA binding drugs above, binding of imidazotetrazinones to DNA is feasible. It is suggested that imidazotetrazinones ring-open and alkylate DNA by reaction of the chloroethyl substituent with the O^6 position of guanine. In order to make such interaction possible the drug must presumably gain access to the major groove of DNA. Imidazotetrazinones possessing a coplanar oxygen atom in the 8-substituent may then achieve increased stability and/or activity by interaction with a biological macromolecule. Either alone, or in addition to the above, an intermolecular hydrogen-bonding interaction between the carbamoyl -NH moiety and other bindings sites may occur with 8-carbamoylimidazotetrazinones. It is suggested that the interaction of the 8-substituent with biological macromolecules, e.g. DNA, may be required to align the imidazotetrazinones in a favourable position for subsequent attack by guanine O^6 residues (Fig 31). From this one may speculate that 8-carbamoylimidazotetrazinones containing a carbamoyl -NH for donation to a hydrogen-bond might achieve increased stability/interaction with DNA, leading to enhanced antitumour activity/cytotoxicity compared with the corresponding disubstituted analogues.

There is no evidence available to support this conjecture, but there are

Figure 31 Postulated interaction of CMCIT with DNA



numerous methods which may provide the information required to substantiate this theory. The introduction of two dimensional correlated (COSY) and nuclear Overhauser effect (NOESY) nmr techniques has provided useful methods for examining drug-DNA interactions. Many reports have appeared giving details of nmr analyses in which a drug is mixed with an assigned piece of DNA or an assigned oligonucleotide, and the association between the two molecules investigated using COSY and NOESY techniques[217,222]. Co-crystallisation of drugs with DNA fragments followed by X-ray analysis has also been used to investigate binding of drugs to DNA[220]. Use of these powerful tools to study interaction of imidazotetrazinones with DNA holds exciting prospects and should provide vital information concerning the role of the 8-substituent in imidazotetrazinones.

3.4 Prelude to molecular modelling studies with imidazotetrazinones

Computer-graphics techniques have also provided valuable information regarding drug interactions with biological molecules[223,224,225]. Using a CHEMGRAF computer package (courtesy of May & Baker Ltd., Dagenham), attempts were made to investigate a possible binding site in DNA for CMCIT. However, after considerable efforts, it was decided that the approximations made in energy calculations, coupled with the difficulty in visualising drug-DNA interactions using this system, were preventing

adequate progress in this area. Instead, molecular modelling investigations with imidazotetrazinones were initiated in collaboration with Dr. S Neidle & Dr. A Beveridge, Institute Cancer Research, Sutton, Surrey. These studies, using an IRIS 2400 system, demonstrated that insertion of the intact molecule of CMCIT into the DNA structure , such that the chloroethyl group was within reaction distance of guanine O^6, was only possible in sequences of bases where guanine was followed by a thymine residue. Visual consideration of this "fit" was encouraging with a number of hydrogen-bonds with the DNA structure seemingly possible. The apparent requirement for a specific sequence of bases might suggest targeted alkylation of DNA by CMCIT since use of other base pair sequences caused huge electrostatic repulsive forces. It is hoped that these studies may be extended to elucidate the role of the 8-substitutent in 8-carbamoylimidazotetrazinones.

4 SUMMARY

Firm conclusions concerning the role of the 8-substitutuent in imidazotetrazinones cannot be drawn from the present studies. However, this work has highlighted a crucially important and intriguing requirement for an amidic hydrogen in 8-carbamoylimidazotetrazinones for optimum antitumour activity/cytotoxicity. During these investigations several proposals have been made concerning the role of the amidic hydrogen, and for possible mechanisms of action of these compounds, the most exciting theory envisaging possible targeted alkylation of DNA. The possibility that imidazotetrazinones interact with DNA, and the knowledge that certain sequence-specific agents possess pyrrole and/or imidazole moieties, connected by carbamoyl linkages[219,226], has implications for the design of further imidazotetrazinone derivatives. For example, it might be possible to synthesise imidazotetrazinones incorporating "sequence-specific chains" capable of recognising and binding to sequences of DNA base pairs specific to cancer genes, by attaching the sequence-specific moiety to the 8-carbamoyl group of CMCIT. Such compounds would perhaps provide targeted delivery of the chloroethyltriazene species to a specific oncogene, achieving increased selectivity relative to that of current imidazotetrazinone derivatives.

Caution must be exercised in making generalisations from results obtained with 8-carbamoylimidazotetrazinones, since various compounds possessing other 8-substituents are also extremely active. Testing of 8-sulphonamoyl derivatives (Table 2, xxviii, xxx, xxxi; Table 12, xi, x, iv) has shown that all of these compounds exhibit potent antitumour activity and high cytotoxicity. The 8-methyl sulphone (Table 2, xxxiii; Table 12, ix) also demonstrates high in vivo activity and high cytotoxicity. It is difficult to explain the discrepancies in cytotoxicity between these compounds and the 8-carbamoylimidazotetrazinones. A possible explanation is that the sulphur containing compounds (Table 12, iv, ix, x, xi) still retain coplanarity of an oxygen atom attached to the 8-position (Section 2.1.3.2). Maintenance of coplanarity may enable these compounds to fit the theoretical receptor site described for imidazotetrazinones putatively suggested as DNA. Of course, it is conceivable that the sulphonamoyl and sulphonyl groups are important for different reasons than those suggested so far. The dimethyl-sulphonamoyl grouping is present in the very promising antitumour agent TCNU (124)[227], but as yet no theories have been proposed for why this group is so important to the activity of this compound.

Earlier workers suggested that the sulponamide group acted as a good carrier group for antitumour agents, conferring an optimum pKa value for uptake into tumour cells[228,229]. Very limited success was achieved with

$(CH_3)_2N-S(=O)_2-CH_2-CH_2-NH-C(=O)-N(N=O)-CH_2-CH_2-Cl$

124

compounds synthesised based on this rationale, and hence it is unlikely that this is a reason for the excellent antitumour activity of imidazotetrazinones substituted with 8-sulphonamoyl or 8-sulphonyl groups.

The antitumour imidazotetrazinones have an extremely complex and interesting mode of action which yet defies interpretation. It is unfortunate that these compounds have experienced the classic dilemma of very potent activity correlating with severe toxicity, but perhaps this work will provide the insight towards further 8-substituted derivatives with improved therapeutic index.

5 MATERIALS

5.1 Chemicals and investigational compounds

5.1.1 Purchased chemicals

Chemicals used in this work were generally purchased from The Aldrich Chemical Co. Ltd., Gillingham, England, with the exception of the following:

Nicotinamide adenine dinucleotide phosphate, reduced form, (tetrasodium salt) was purchased from Sigma Chemical Co., Poole, England, and magnesium chloride hexahydrate was purchased from BDH Chemicals, Poole, England.

5.1.2 Gifts

CMCIT, together with any imidazotetrazinone derivatives not mentioned in the experimental section, and all HPLC solvents, were generously donated by May and Baker Ltd., Dagenham, England; S-S-dimethylsulphoximide was a gift of G. Pitchen, Centre de Recherche de Vitry, Rhône Poulenc, Vitry-sur-Seine, France.

5.1.3 Synthesised chemicals

CMEIT was synthesised in this department by Dr. G.U. Baig. Serine benzyl ester was synthesised in this department by Dr. S. Tendler.

5.2 Biological

5.2.1 Tissue culture vessels

Tissue culture (twenty-four multi-well) dishes (133x88 mM) were purchased from Gibco Ltd., Paisley, Scotland.

5.2.2 Media

RPMI (Rosewell Park Memorial Institute) 1640 media (containing hepes (25 μMol) and L-glutamine) and horse serum (mycoplasma screened) were purchased from Gibco Ltd., Paisley, Scotland.

5.2.3 Animals

CBA/CA female mice (18-22 g) were supplied by Bantim & Kingman, Hull, England.

6. EXPERIMENTAL DETAILS

6.1 Chemical methods

NMR spectra were recorded on a Varian EM360A nmr spectrometer in DMSO-d_6 as solvent. Tetramethylsilane (TMS) was incorporated as an internal standard. All peaks are assigned in terms of δ values. Abbreviations used in the interpretation of the nmr spectra are: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet.

Mass spectra were measured at 70 eV on a V.G. Micromass 12B single focusing spectrometer with a source temperature in the range 300°C; m/z signifies the molecular ion peak observed. My thanks to Mrs. K. Farrow, this department, for supplying these data.

C,H,N analyses were kindly performed by A. Stevens, May & Baker Ltd., Dagenham, Essex.

All melting points are recorded uncorrected.

Recrystallisation of imidazotetrazinone derivatives was achieved using a mixture of acetone and water (9:1), unless otherwise stated.

6.1.1 Preparation of 3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxylic acid

a) Using concentrated sulphuric acid as solvent

CMCIT (10.0g, 0.04mol) was treated with concentrated sulphuric acid (50ml) with stirring. When the solid had dissolved, the solution was carefully treated with a solution of sodium nitrite (10g, 0.145mol) in water (25ml) over 30 minutes, the temperature being maintained below 60°C. The resulting yellow solution was stirred at 35°C for 2.5 hours and was then poured onto ice. The solid was filtered off, washed well with water and dried in vacuo, to give 3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo-[5,1-d]-1,2,3,5-tetrazine-8-carboxylic acid (53) as a buff coloured powder (8.34g, 85.8%), m.p.166°C; νmax (KBr) 1760, 1710, cm^{-1}; m/z 243/245.

b) Using trifluoroacetic acid as solvent

CMCIT (5.0g, 0.02mol) was treated with trifluoroacetic acid (20ml) with stirring. When the solid had dissolved the solution was carefully treated with a solution of sodium nitrite (5.0g, 0.07mol) in water (10ml) over 30 minutes, the temperature being maintained below 60°C. The resulting yellow solution was stirred at 35°C for 2.5 hours and was then poured onto ice. The solid was filtered off, washed well with water and dried in vacuo.

to give 3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxylic acid (53) as a buff coloured powder (4.62g, 95.01%) m.p. 166°C.

6.1.2 Preparation of 3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo-[5,1-d]-1,2,3,5-tetrazine-8-carbonyl chloride

Thionyl chloride (80ml) was added to 3-(2-chloroethyl)-2,3-dihydro-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxylic acid (10g, 0.04 mol) together with a catalytic amount of dimethylformamide (five drops). The mixture was heated under reflux at 80°C for 2.5 hours, before distillation of the excess thionyl chloride under reduced pressure. The residue was treated with toluene (50ml), and evaporation gave 3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-d]-1.2.3.5-tetrazine-8-carbonyl chloride (57) as a light brown crystalline solid (1.03 g, 87.17%). m.p. 69°C; νmax (KBr) 1730, 1520 cm^{-1}; m/z 261/263.

6.1.3 Preparation of 8-methylcarbamoyl-3-(2-chloroethyl)imidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one monohydrate

Methylamine (40% aqueous solution)(0.95g, 12.2mmol) in tetrahydrofuran (5ml) was added, dropwise, to the acid chloride (57) (1.6g, 6.13mmol) in

tetrahydrofuran (5ml). The mixture was stirred at ambient temperature for 30 minutes and a precipitate which formed was collected, washed on the filter with distilled water (10ml) and recrystallised from aqueous acetone to afford 8-methylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one monohydrate (64a) in the form of a white solid (0.88g, 56.6%) m.p.121°C. (Found: C,35.0; H,3.84; N,30.8; $C_8H_9N_6O_2Cl.H_2O$ requires C,34.98; H,4.04; N,30.60%); νmax (KBr) 1750, 1640, cm^{-1}; [60MHz; $(CD_3)_2SO$], 2.8(3H,d,CH_3), 4.0(2H,t,CH_2), 4.6(2H,t,CH_2), 8.4(1H,broad band,NH), 8.9 (1H,s,CH). m/z 257.

6.1.4 Preparation of 8-dimethylcarbamoyl-3-(2-chloroethyl)imidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Dimethylamine (40% aqueous solution) (1.38g, 12.2mmol) in tetrahydrofuran (2ml) was added, dropwise, to the acid chloride (57) (1.6g, 6.13mmol) in tetrahydrofuran (5ml). The mixture was stirred at ambient temperature for 20 minutes. The resulting solution was treated with petroleum (b.p. 60-80°C) (10ml), and the precipitate which formed was collected and recrystallised from aqueous acetone to give 8-dimethylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (64b) in the form of a white solid (0.69g, 41.7%), m.p. 116-117°C. (Found: C,39.5; H,4.00; N,30.9, $C_9H_{11}N_6O_2Cl$ requires C,39.94; H,4.09; N,31.05%); νmax (KBr): 1720, 1610, cm^{-1}; [60MHz; $(CD_3)_2SO$], 3.1(6H,s,$(CH_3)_2$), 4.0(2H,t,CH_2), 4.6(2H,t,CH_2), 8.85(1H,s,CH).

6.1.5 Preparation of 8-propylcarbamoyl-3-(2-chloroethyl)-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Propylamine (0.24g, 4.0mmol) in dichloromethane (3ml) was added, dropwise, to the acid chloride (57) (0.52g, 2mmol) in dichloromethane (2ml). The mixture was stirred at ambient temperature for two hours, followed by addition of distilled water (2ml) and petroleum ether (b.p. 60-80°C) (5ml). The precipitate formed was collected and recrystallised from aqueous acetone to afford 8-propylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (64c) as a pale purple powder (0.36g, 64.1%), m.p.111.2°C. (Found: C.42.1; H,4.7; N,29.6, $C_{10}H_{13}N_6O_2Cl$ requires C,42.19; H,4.60; N,29.52%); νmax (KBr) 1760, 1650, cm^{-1}; [60MHz; $(CD_3)_2SO$] 0.8(3H,t,CH_3), 1.5(2H,q,CH_2), 3.3(3H,q,CH_3), 4.0(2H,t,CH_2), 4.6(2H,t,CH_2), 8.45(1H,s,NH), 8.85 (1H,s,CH).

6.1.6 Preparation of 8-isopropylcarbamoyl-3-(2-chloroethyl)imidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Isopropylamine (0.45g, 7.6mmol) in dichloromethane was added, dropwise, to the acid chloride (57) (1.0g, 3.8mmol). The mixture was stirred at ambient temperature for 5 hours followed by addition of distilled water (2ml) and petroleum ether (b.p. 60-80°C) (5ml). The resulting precipitate was collected and recrystallised from aqueous acetone to yield

8-isopropylcarbamoyl-3-(2- chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (64d) in the form of a cream coloured powder (0.75 g, 68.8%), m.p.119-120°C. (Found: C,42.4; H,4.5; N,29.2, $C_{10}H_{13}N_6O_2Cl$ requires C,42.19; H,4.60; N,29.52%); νmax (KBr) 1760, 1690, cm^{-1}; [60MHz; $(CD_3)_2SO$] 1.29 (6H,d,$(CH_3)_2$), 4.10(3H,q,CH_2,CH), 4.65 (2H,t,CH_2), 8.15 (1H,d,NH), 8.90(1H,s,CH). m/z 285.

6.1.7 Preparation of 8-allylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Allylamine (0.44g, 7.7mmol) in dichloromethane (10ml) was added, dropwise, to the acid chloride (57) (1.0g, 3.8mmol). The mixture was stirred at ambient temperature for 3 hours followed by addition of distilled water (2ml) and petroleum ether (b.p. 60-80°C) (5ml). The resulting precipitate was collected and recrystallised from aqueous acetone to yield 8-allylcarbamoyl-3-(2-chloroethyl) imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (64e) in the form of a pale purple powder (0.54g, 50.2%), m.p.121°C. (Found: C,42.5; H,3.8; N,29.9, $C_{10}H_{11}N_6O_2Cl$ requires C,42.49; H,3.92; N,29.73%); νmax (KBr) 1780, 1660, cm^{-1}; [60MHz; $(CD_3)_2SO$] 4.15 (4H,q,$(CH_2)_2$), 4.75 (2H,t,CH_2), 5.25 (2H,t,CH_2), 5.95 (1H,s,CH), 8.6(1H,s,NH),8.8(1H,s,CH). m/z 283.

6.1.8 Preparation of 8-cyclopropylcarbamoyl-3-(2-chloroethyl)imidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one monohydrate

Cyclopropylamine (0.44g, 3.83mmol) in dichloromethane (10ml) was added, dropwise, to the acid chloride (57) (0.5g, 1.9mmol). The mixture was stirred at ambient temperature for two hours and a precipitate which formed was collected and recrystallised from aqueous acetone to afford 8-cyclopropylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one monohydrate (64f) in the form of a white solid (0.33g, 62.0%), m.p. 132°C. (Found: C,39.6; H,4.21; N,28.2. $C_{10}H_9N_6OCl.H_2O$ requires C,39.94; H,4.36; N,27.94%); νmax (KBr): 1760, 1660, cm^{-1}; [60MHz; $(CD_3)_2SO$] 0.7 (5H,d,$CH(CH_2)_2$), 4.1(2H,t,CH_2), 4.6(2H,t,CH_2), 8.5(1H,d,NH), 8.8(1H,s,CH), m/z 283.

6.1.9 Preparation of 8-phenylcarbamoyl -3-(2-chloroethyl)imidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Aniline (0.73g, 7.7mmol) in dichloromethane (5ml) was added, dropwise, to the acid chloride (57) (1.0g, 3.83mmol). The mixture was stirred at ambient temperature for 5 hours followed by treatment with distilled water (2ml) and petroleum ether (b.p. 60-80°C) (5ml). The precipitate formed

was collected and recrystallised from aqueous acetone to give 8-phenyl-carbamoyl–3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (64g) in the form of a deep yellow solid (0.80g, 65.7%), m.p.166-167°C. (Found: C,48.4; H,3.3; N,26.1, $C_{13}H_{11}N_6O_2Cl$ requires C,48.9; H,3.48; N,26.3%); νmax (KBr) 1750, 1700, cm^{-1}; [60MHz; $(CD_3)_2SO$] 4.05 (2H,t,CH_2), 4.65 (2H,t,CH_2), 7.25 (5H,m,C_6H_5), 9.0 (1H,s,CH), 10.35 (1H,s,NH). m/z 319.

6.1.10 Preparation of 8-cyclohexylcarbamoyl-3-(2-chloroethyl)imidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Cyclohexylamine (0.75g, 7.56mmol) in pyridine (3.5ml) was added, dropwise, to the acid chloride (57) (1.53g, 5.86mmol) in tetrahydrofuran (5ml). The mixture was stirred at ambient temperature for 10 minutes and then poured into ice-cold dilute hydrochloric acid (100 ml). The mixture was stirred and a precipitate formed which was collected and recrystallised from aqueous acetone to afford 8-cyclohexylcarbamoyl-3-(2-chloro-ethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (64h) in the form of a white solid (1.26g, 66.1%), m.p. 130°C. (Found: C,48.3; H,5.3; N,25.8, $C_{13}H_{17}N_6O_2Cl$ requires C,48.08; H,5.3; N,25.88%); νmax (KBr): 1750, 1660, cm^{-1}; [60MHz; $(CD_3)_2SO$], 1.5 (11H,broad band, C_6H_{11}), 4.0 (2H,t,CH_2), 4.6(2H,t,CH_2), 8.8 (1H,s,CH).

6.1.11 Preparation of 8-neopentylcarbamoyl-3-(2-chloroethyl)imidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

A stirred solution of the acid chloride (57) (1.6g, 6.13mmol) in dry tetrahydrofuran (5ml) was treated with neopentylamine (1.06g, 12.2mmol) in tetrahydrofuran (5ml). The mixture was stirred at ambient temperature for 2 hours and a precipitate formed, which was collected and recrystallised from aqueous acetone to afford 8-neopentylcarbamoyl-3-(2-chloroethyl)-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (64i) as a cream coloured powder (1.00g, 52.1%), m.p. 148°C. (Found: C, 45.7; H,5.2; N,26.8. $C_{12}H_{17}N_6O_2Cl$ requires C,46.08; H, 5.48; N,26.87%); νmax (KBr) 1730, 1660, cm^{-1}; [60MHz; $(CD_3)_2SO$] 0.9 (9H,s,$(CH_3)_3$), 3.1 (2H,t,CH_2), 4.0 (2H,t,CH_2), 4.6 (2H,t,CH_2), 8.1 (1H,broad,NH), 8.8 (1H,s,CH).

6.1.12 Preparation of 8-methoxycarbonyl-3-(2-chloroethyl)imidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Methyl alcohol (5ml) was added to the acid chloride (57) (1.0g, 3.8mmol) and the resulting mixture was stirred at ambient temperature for 2.5 hours. The precipitate formed was collected and recrystallised from aqueous acetone to give 8-methoxycarbonyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (65a) as a white crystalline solid (0.67g,

67.5%), m.p. 113°C. (Found: C,37.1; H,3.00; N,27.0, $C_8H_8N_5O_3Cl$ requires C,37.3; H,3.13; N,27.18%); νmax (KBr) 1780, 1750, cm^{-1}; [60MHz; $(CD_3)_2SO$] 4.00(5H,t,CH_2, CH_3), 4.65 (2H,t,CH_2), 8.9 (1H,s,CH). m/z 258.

6.1.13 Preparation of 8-ethoxycarbonyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Absolute ethanol (6ml) was added to the acid chloride (57) (0.52g, 2.0mmol). The mixture was stirred at ambient temperature for one hour. The precipitate formed was collected and recystallised from aqueous acetone to give 8-ethoxycarbonyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (65b) as a white solid (0.38g, 69.8%), m.p.96°C. (Found: C,39.6; H,3.65; N,25.9, $C_9H_{10}N_5O_3Cl$ requires C,39.79; H,3.71; N,25.78%); νmax (KBr) 1780, 1740, cm^{-1}; [60 MHz; $(CD_3)_2SO$] 1.35 (3H,t,CH_3), 4.0 (2H,t,CH_2), 4.35 (2H,t,CH_2), 4.65 (2H,t,CH_2), 8.9 (1H,s,CH).

6.1.14 Preparation of 8-propoxycarbonyl-3-(2-chloroethyl)imidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Propyl alcohol (4ml) was added to the acid chloride (57) (1.0g, 3.8mmol). The mixture was stirred at ambient temperature for two hours. The precipitate formed was collected and recrystallised from aqueous acetone to give 8-propoxycarbonyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-

4(3H)-one (65c) as a fine white powder (0.69g, 63.4%), m.p.92°C. (Found: C,42.1; H,4.15; N,24.6, $C_{10}H_{12}N_5O_3Cl$ requires C,42.04; H,4.23; N,24.51%); νmax (KBr) 1760, 1730, cm^{-1}; [60MHz; $(CD_3)_2SO$] 1.0 (3H,t,CH_3), 1.7 (2H,q,CH_2), 4.05 (2H,t,CH_2), 4.3 (2H,t,CH_2), 4.65 (2H,t,CH_2), 8.9 (1H,s,CH). m/z 285.

6.1.15 Preparation of 8-isopropoxycarbonyl-3-(2-chloroethyl)imidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Isopropyl alcohol (5ml) was added to the acid chloride (57) (1.0g, 3.8mmol) and the resulting mixture was stirred at ambient temperature for two hours. The precipitate formed was collected and recrystallised from aqueous acetone to give 8-isopropoxycarbonyl-3-(2-chloroethyl)imidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (65d) as a white crystalline solid (0.63g, 57.9%), m.p.113°C. (Found: C,42.2; H,4.18; N,24.6; Cl,12.3, $C_{10}H_{12}N_5O_3Cl$ requires C,42.04; H,4.23; N,24.51; Cl,12.4%); νmax (KBr) 1760, 1720, cm^{-1}; [60MHz; $(CD_3)_2SO$] 1.35 (6H,d,$(CH_3)_2$), 4.00 (2H,t,CH_2), 4.65 (2H,t,CH_2), 5.20 (2H,m,CH), 8.85 (1H,s,CH). m/z 285.

6.1.16 Preparation of S,S-dimethyl-N-[3-(2-chloroethyl)-[3H]-4-oxoimidazo[5,1-d]-1,2,3,5-tetrazin-8ylcarbonyl]sulphoximide

A solution of the acid chloride (57) (1.5g, 5.7mmol) in dichloromethane (5ml) was treated dropwise with a solution of S,S-dimethylsulphoximine (0.53g, 5.7mmol) and triethylamine (0.58g, 5.7mmol) in dichloromethane (5ml). The reaction flask was suspended in an ice/acetone bath and the reaction was allowed to proceed for 20 minutes. A precipitate formed which was filtered off and recrystallised from aqueous acetone to give S,S-dimethyl-N-[3-(2-chloroethyl)-[3H]-4-oxoimidazo[5,1-d]-1,2,3,5-tetrazin-8ylcarbonyl]sulphoximide (66) in the form of a buff coloured solid (1.42g, 77.60%) m.p.165°C. (Found: C,34.1; H,3.38; N,26.3. $C_9H_{11}N_6O_3ClS$ requires C,33.91; H,3.48; N,26.4%); νmax (KBr) 1730, 1660, cm^{-1}; [60MHz; $(CD_3)_2SO$] 3.5 (6H,s,$(CH_3)_2$), 4.0 (2H,t,CH_2), 4.6 (2H,t,CH_2), 8.7 (1H,s,CH).

6.1.17 Preparation of 8-azidocarbonyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Sodium azide (0.25g, 3.8mmol) was added portionwise, to the acid chloride (57) (1.0g, 3.8mmol) in aqueous acetone (10ml). The mixture was stirred at ambient temperature for 3.5 hours, followed by addition of petroleum ether (b.p. 60-80°C) (10ml). The precipitate formed was

collected and recrystallised from aqueous acetone to give 8-azidocarbonyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (67) as a white crystalline solid (0.83g, 81.0%), m.p.115°C. (Found: C,31.3; H,1.72; N,41.7, $C_7H_5N_8O_2Cl$ requires C,31.3; H,1.88; N,41.7%); νmax (KBr) 2175 (N=N=N), 1760, 1720, cm^{-1}; [60 MHz; $(CD_3)_2SO$] 4.05 ($2H,t,CH_2$), 4.65 ($2H,t,CH_2$), 8.95 (1H,s,CH). m/z 269.

6.1.18 Preparation of 3-(2-chloroethyl)-8-diazoacetylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one monohydrate

Diazomethane (1.0g, 23.8mmol) in ether (75ml) was added slowly to the acid chloride (57) (1.0g, 3.83mmol). The reaction vessel was suspended in an icebath and the reaction was allowed to proceed at this temperature, in the dark, for a period of 30 minutes. A yellow suspension formed which was collected and recrystallised from aqueous acetone to give 3-(2-chloroethyl)-8-diazoacetyl imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one monohydrate (68) in the form of a buff coloured solid (0.67g, 65.1%), m.p. 69°C. (Found: C,34.1; H,2.9; N,34.8, $C_8H_5N_7O_2Cl.H_2O$ requires C,33.6; H,2.8; N,34.3%); νmax (KBr): 2100 ($RCOCH=\overset{+}{N}=\overset{-}{N}$), 1750, 1730, cm^{-1}; [60MHz; $(CD_3)_2SO$], 3.96 ($2H,t,CH_2$), 4.56 ($2H,t,CH_2$), 6.76 (1H,s,CH), 8.8 (1H,s,CH). m/z 268.

6.1.19 Preparation of 8-phenylaminocarbamoyl-3-(2-chloroethyl)-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Phenylhydrazine (0.81g, 7.52mmol) in pyridine (3.5ml) was added, dropwise, to the acid chloride (57) (1.54g, 5.9mmol) in tetrahydrofuran (5 ml). The mixture was stirred at ambient temperature for 10 minutes and then poured into ice cold dilute hydrochloric acid (100ml). The solution was stirred and a precipitate which formed was collected and recrystallised from aqueous acetone to afford 8-phenylaminocarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4-one (69a) in the form of a bright yellow solid (1.40g, 71.2%), m.p.156°C. (Found: C,47.1; H,3.63; N,29.2. $C_{13}H_{12}N_7O_2Cl$ requires C,47.1; H,3.62; N,29.38%); νmax (KBr): 1750, 1680, cm^{-1}; [60MHz; $(CD_3)_2SO$] 4.0 (2H,t,CH_2), 4.63 (2H,t,CH_2), 7.0 (5H,m,C_6H_5), 7.9 (1H,s,NH), 8.85 (1H,s,CH), 10.4 (1H,s,NH). m/z 335.

6.1.20 Preparation of 3-(2-chloroethyl)-8-(3,5-difluorophenylamino-carbamoyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

2,4-Difluorophenylhydrazine (1.08g, 7.5mmol) in pyridine (3.5ml) was added, dropwise, to the acid chloride (57) (1.53g, 5.86mmol) in tetrahydrofuran (5ml). The mixture was stirred at ambient temperature for 10 minutes and then poured into cold dilute hydrochloric acid (100ml). The

mixture was stirred and a precipitate which formed was collected and recrystallised from aqueous acetone to give 3-(2-chloroethyl)-8-(3,5-difluorophenylaminocarbamoyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (69b) as a bright yellow solid (1.08g, 39.4%) m.p.168°C. (Found: C,41.6; H,2.40; N,26.3, $C_{13}H_{10}N_7O_2ClF_2$ requires C,42.2; H,2.72; N,26.5%); νmax (KBr) 1740, 1650, cm^{-1}; [60MHz; $(CD_3)_2SO$] 4.0 (2H,t,CH_2), 4.6 (2H,t,CH_2), 7.0 (5H,m,C_6H_4NH), 8.9 (1H,s,CH), 10.4 (1H,s,NH).

6.1.21 Preparation of 1-[3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-d]-1,2,3,5-tetrazin-8-ylcarbonyl]-4-phenylsemicarbazide monohydrate

A solution of the acid chloride (57) (1.53g, 5.86mmol) in tetrahydrofuran (5ml) was added, dropwise, to 4-phenylsemicarbazide (1.13g, 7.5mmol) in pyridine (3.5ml) and tetrahydrofuran (5ml). The mixture was stirred at ambient temperature for 15 minutes and then poured into cold dilute hydrochloric acid (100ml). The solution was stirred and a precipitate which formed was collected and recrystallised from aqueous acetone to give 1-[3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-d]-1,2,3,5-tetrazin-8ylcarbonyl]-4-phenylsemicarbazide monohydrate (69c) as a buff solid (1.56g, 70.8%), m.p.169°C. (Found: C,42.6; H,3.78; N,28.4, $C_{14}H_{13}N_8O_3Cl.H_2O$ requires C,42.59; H,3.80; N,28.40%); νmax (KBr) 1745, 1665, cm^{-1}; [60 MHz; $(CD_3)_2SO$] 4.06 (2H,t,CH_2), 4.68 (2H,t,CH_2), 7.28 (5H,m,C_6H_5), 8.26 (1H,s,NH), 8.84 (1H,s,NH), 8.96 (1H,s,CH), 10.24 (1H,s,NH).

6.1.22 Preparation of 8-methoxycarbonylaminocarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

A solution of the acid chloride (57) (1.53g, 5.86mmol) in dry tetrahydrofuran (5ml) was added, dropwise, to methyl carbazate (0.99g, 11.0mmol) in pyridine (3.5ml) and tetrahydrofuran (5ml). The mixture was stirred at ambient temperature for 10 minutes and then poured into cold dilute hydrochloric acid (100ml) with stirring. The precipitate formed was collected and recrystallised from aqueous acetone to afford 8-methoxycarbonylaminocarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (70) in the form of a white powder (0.97g, 52.4%), m.p. 188°C. (Found: C,34.6; H,3.12; N,30.7. $C_9H_{10}N_7O_4Cl$ requires C,34.24; H,3.19; N,31.06%); νmax (KBr) 1730, 1680, cm^{-1}; [60MHz $(CD_3)_2SO$] 3.6 (3H,s,CH_3), 4.0 (2H,t,CH_2), 4.6 (2H,t,CH_2), 8.8 (1H,s,CH), 9.2 (1H,s,NH), 10.26 (1H,s,NH).

6.1.23 Preparation of 8-[N-(ethoxycarbonylmethyl)carbamoyl]-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

A solution of the acid chloride (57) (0.52g, 2mmol) in dichloromethane (8ml) was treated with a mixture of glycine ethyl ester hydrochloride (0.28g, 2mmol) and triethylamine (0.28g, 2.8mmol) in dichloromethane (10ml). The mixture was stirred at ambient temperature for 1 hour, and then the dichloromethane solvent was removed by evaporation. The

residue was treated with water and the resulting solution was stirred for 30 minutes. The precipitate was filtered off and recystallised from ethyl acetate to give 8-[N-(ethoxycarbonylmethyl)carbamoyl]-3-(2-chloroethyl)-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (71) in the form of a pink solid (0.28g, 43.0%) m.p.114°C. (Found: C,39.7; H,4.05; N,24.3, $C_{11}H_{13}N_6O_4Cl$ requires C,40.1; H,3.99; N,25.5%); νmax (KBr) 1750, 1670, cm^{-1}; [60 MHz; $(CD_3)_2SO$] 1.2 (2H,m,CH_2), 4.05 (5H,q,C_2H_5), 4.65 (2H,t,CH_2), 8.85 (1H,s,CH). m/z 329.

6.1.24 Preparation of 8-ethylthiocarbonyl-3-(2-chloroethyl)-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Ethane thiol (4ml) was added to the acid chloride (57) (0.7g, 2.6mmol). The mixture was stirred at ambient temperature overnight (16 hours). The precipitate formed was collected and recrystallised from aqueous acetone to give 8-ethylthiocarbonyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (72) as a white solid (0.37g, 49.4%), m.p. 111-112°C. (Found; C,37.4; H,3.32; N,24.2, $C_9H_{10}N_5O_2SCl$ requires C,37.57; H,3.50; N,24.34%); νmax (KBr) 1760, 1660, cm^{-1}; [60MHz; $(CD_3)_2SO$] 1.3 (3H,t,CH_3), 3.05 (2H,q,CH_2), 3.3 (3H,s,CH_3) 4.05 (2H,t,CH_2), 4.65 (2H,t,CH_2), 8.95 (1H,s,CH). m/z 287.

6.1.25 Ultra-violet determination of half-life values for decomposition of 8-carbamoylimidazotetrazinones in RPMI 1640 media

Solutions of each derivative CMCIT, MCMCIT and DCMCIT were prepared in DMSO (10 g/L) and aliquots (10 μL) of these stock solutions were added to silica cuvettes (1 cm pathlength) containing the buffer (3 ml). The cuvettes were maintained at 37°C in a Hewlett Packard 8451A Diode Array spectrophotometer and the solutions were sampled at pre-set time intervals. The decrease in absorbance with time was measured at 325 nm against a RPMI 1640 blank. Half-life values were calculated using a plot of the natural logarithm concentration of drug versus time.

6.1.26 HPLC analysis of imidazotetrazinones

As mentioned in section 2.1.2.2, separation of CMCIT, MCMCIT and DCMCIT was achieved using a 250 Lichrosorb RP select B column and an acetic acid (0.5% in water)/methanol mobile phase. Quantification was determined from the peak height ratio of the sample peaks to the internal standard, CMEIT.

A 40 μl aliquot of the internal standard (in DMSO) was added to a test-tube followed by addition of 0.5 ml HCl (1N) and 0.5 ml of the drug incubation mixture (10 μl of 10 g/L solution in DMSO, in 5 ml of RPMI 1640 media containing 17% horse serum). Ethyl acetate (3.0 ml) was

added and the contents vortexed. The layers were separated by centrifugation in a Hereus 6000 Labofuge at 2,000 g for ten minutes. An aliquot (2 ml) of the organic layer was removed and the ethyl acetate was evaporated using a Savant Speed Vac Concentrator SVC 100H and a Refrigerated Condensation Trap RT-100A. The residue was dissolved in 0.5% acetic acid in water (125 µl) and methanol (125 µl). The solutions were vortexed and transferred into low volume inserts for use in the assay. Standard solutions were prepared over a concentration range of 0.5 mg/L - 25 mg/L by the addition of DMSO solutions of drug to test-tubes with simultaneous preparation with the sample solutions.

The injection volume was typically 40 µl, this being taken using a Waters WISP 710B Automatic Injection Sampler and the solvent delivery system was a Waters Model 510 Chromatography Pump. Detection was at 325 nm using a Lamba-max Variable Wavelength Model 480 LC Spectrophotometer. The HPLC conditions were controlled by a Digital Professional 350 Data Control System via a Waters System Interface Module. Typical conditions were a flow rate of 1.3 ml/min with a run time of 8 minutes.

6.1.27 Extraction efficiency of the HPLC method

To determine the efficiency of the ethyl acetate extraction calibration solutions of CMCIT, MCMCIT and DCMCIT were prepared by adding

aliquots of stock solutions of drug (10 g/L) to mobile phase to give concentrations of 0.5– 25 mg/L. Tubes containing equal amounts of drug were prepared and extracted with ethyl acetate as outlined in section 6.1.26. Results obtained for the extracted samples were compared with values obtained from the calibration tubes (Table 25).

6.1.28 Reproducibility of the HPLC method

The reproducibility of the HPLC assay was appraised by preparing six separate stock solutions of each compound CMCIT, MCMCIT and DCMCIT (0.25 mg/L and 12.5 mg/L) and analysing 0.5 ml aliquots from each of these solutions (Table 26).

6.1.29 Reproducibility of the instrument used for the HPLC method

The reproducibility of the instrument was established by analysing multiple injections from stock solutions (0.5 mg/L and 25 mg/L) prepared from each compound CMCIT, MCMCIT and DCMCIT. Results obtained are given in Table 27.

6.1.30 Infra-red measurement of -NH stretching frequencies in imidazotetrazinones

Approximately 5 mg of drug was treated with dry chloroform and if necessary the suspension was allowed to stand overnight to rensure a

saturated solution. The mixture was filtered through glass wool into a 3 cm ir grade cell. The -NH stretching frequency was measured using a Perkin Elmer SP4000 ir spectrophotometer by scanning the range between 3600-3100 cm^{-1} using chloroform as a reference. The shift in the -NH stretching frequency (H2) was determined by comparing values obtained against the averaged value from the carbamoyl -NH stretching frequencies produced by CMCIT (Appendix 2).

6.2 Biological methods

6.2.1 Description and origin of the TLX5S lymphoma cell line

The TLX5 lymphoma is a rapidly growing invasive murine tumour which was originally induced in the thymus of a CBA mouse by X-ray irradiation[230].

6.2.2 Extraction of TLX5 mouse ascites cells

Ascitic fluid was removed from the peritoneal cavity of CBA/CA mice bearing a routine passage of TLX5 lymphoma and was mixed with sterile saline (37°C). If necessary, saline (5ml) was syringed into the peritoneal cavity, removed and added to the above cell solution. The cells were sedimented on a Hereus 6000 Labofuge bench centrifuge at approximately 500G and washed with erythrocyte lysis buffer, to remove red cell debris. The tumour cells were then centrifuged and resuspended in RPMI 1640 media containing 17% horse serum.

6.2.3 Maintenance of TLX5 lymphoma cells

TLX5 cells were maintained in exponential phase at a density of $2x10^4$ cells/ml under an atmosphere of 10% CO_2 in air provided by a Flow Laboratories 210 CO_2 gassing incubator, at 37°C. RPMI 1640 media supplemented with 17% horse serum was used as the culture medium.

6.2.4 *In vitro* cytotoxicity assays against TLX5 lymphoma cells

All tissue culture work was concluded under aseptic conditions provided by a Gelaire B5B3 laminar air flow cabinet. Aliquots (2ml) of logarithmically growing TLX5 cells were plated out at approximately $2x10^4$/ml into wells of twenty-four multi-well dishes and drug solutions (in DMSO) added at concentrations of 0.625-20 mg/L. The drug concentrations were added to the cells in amounts such that the final concentration of DMSO did not exceed 0.2%. After 72 hours incubation at 37°C under an atmosphere of air/CO_2 (90:10), the cells were counted using a Coulter Laboratories ZM or ZB1 electronic coulter counter. The cytotoxicity was calculated as % inhibition of TLX5 cell growth by expressing the number of cells remaining in the test wells as a percentage of the number of control cells remaining after 72 hours incubation.

6.2.5 Preparation of mouse hepatic microsomes

Female CBA mice were routinely used for obtaining microsomes, the animals were sacrificed by cervical dislocation and the livers removed. The whole procedure for preparation of microsomes was always performed under aseptic conditions. The livers were homogenised at 4°C in approximately 4 vols of potassium phosphate buffer 0.1M, pH 7.4. The suspension obtained was centrifuged for 10 minutes at 10,000g in a Pegasus AP65 centrifuge. The supernatant was decanted off and

centrifuged at 100,000g for 60 minutes. The pellet obtained was homogenised with RPMI 1640 media supplemented with 17% horse serum, to give a suspension of microsomes equivalent to 1g liver/ml.

6.2.6 Effect of the presence of microsomes upon in vitro cytotoxicity of imidazotetrazinones against TLX5 cells

Hepatic microsomes were prepared as outlined in section 6.2.5. The cytotoxicity assay was similar to the procedure given in section 6.2.4. However, in these experiments the drug was initially added to a sealed sterile tube containing TLX5 cells at 4×10^4 cells/ml in RPMI 1640 media with 17% horse serum, containing NADPH (48μMol), magnesium chloride hexahydrate (325μMol) and microsomes (50 mg/ml). The drug incubation mixture was incubated for 90 minutes at 37°C with occasional exposure to an atmosphere of 10% CO_2 in air. The contents of the tube were then vortexed and aliquots (2 ml) were plated out and incubated at 37°C for 72 hours as in section 6.2.4.

6.2.7 Effect of the presence of NADPH upon metabolic activation of 8-dimethylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Experiments were conducted in a similar manner to those outlined in Section 6.2.6. However, DCMCIT was also incubated with microsomes in the absence of NADPH. The effect upon metabolic activation of DCMCIT by incubation with liver microsomes in the presence or absence of NADPH

was demonstrated graphically by constructing plots of % inhibition of TLX5 cell growth versus time (Fig 14).

6.2.8 Preincubation with inactivated microsomes and its effect upon metabolic activation of 8-dimethylcarbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Liver microsomes were prepared as outlined in Section 6.2.5. Heat-inactivation of microsomes was achieved by boiling the microsomal suspension in a sealed bijou tube for ten minutes followed by homogenisation under aseptic conditions. DCMCIT was incubated in the presence of microsomes and inactivated microsomes in a similar manner to Section 6.2.6. Results obtained are given in Table 20.

6.2.9 Determination of formaldehyde cytotoxicity against TLX5 lymphoma cells

Data obtained from HPLC experiments (Section 2.2.2.2) indicated that 28% metabolism occured during metabolic activation of DCMCIT. To ensure that resultant release of formaldehyde (10 nm/ml) was not responsible for the increased cytotoxicity of DCMCIT when incubated with microsomes, cytotoxicity assays of formaldehyde against TLX5 cells were performed. These assays were performed in an identical manner to those outlined in Section 6.2.4, using formaldehyde at concentrations of 5 nm/ml, 10 nm/ml and 20 nm/ml (Table 21).

APPENDIX 1 Results from structure-activity studies on 8-carbamoylimidazotetrazinones (substituent set 1) correlating activity (ID_{50}) with hydrophobicity (Π) and theoretical hydrogen-bonding capacity (DV)

a)

SUBSTITUENT	DV	RESULT
CONHOBZ	1.00	¯.75
CONH2	1.00	¯.36
CONHCYC	1.00	¯.58
CONHPH	1.00	¯.52
CONHISOPR	1.00	¯.29
CONHPR	1.00	¯.55
CONHTBU	1.00	¯.30
CONHAL	1.00	¯.29
CONHME	1.00	¯.51
CONHNEO	1.00	¯.87
CONHOME	1.00	¯.44
CONHNHPH	1.00	¯.26
CONHCH2CH2	1.00	¯.29
CONHCYHEX	1.00	¯.65
CONHOH	1.00	¯1.09
CONME2	.00	¯1.19
CONMEPH	.00	¯1.30
CONPIP	.00	¯1.28

b)

	REGRESSION COEFFICIENT	STANDARD ERROR	T VALUES	P
CONSTANT	¯1.2567			
DV	.7400	.144	5.121	.0001

N = 18 S = .228 F = 26.226 R = .78809 P = .00012

c)

SUBST.	OBS	CALC	DIFF	DIFF/SE
CONHOBZ	¯.750	¯.517	¯.233	¯1.021
CONH2	¯.360	¯.517	.157	.686
CONHCYC	¯.580	¯.517	¯.063	¯.277
CONHPH	¯.520	¯.517	¯.003	¯.015
CONHISOPR	¯.290	¯.517	.227	.992
CONHPR	¯.550	¯.517	¯.033	¯.146
CONHTBU	¯.300	¯.517	.217	.948
CONHAL	¯.290	¯.517	.227	.992
CONHME	¯.510	¯.517	.007	.029
CONHNEO	¯.870	¯.517	¯.353	¯1.546
CONHOME	¯.440	¯.517	.077	.336
CONHNHPH	¯.260	¯.517	.257	1.123
CONHCH2CH2	¯.290	¯.517	.227	.992
CONHCYHEX	¯.650	¯.517	¯.133	¯.584
CONHOH	¯1.090	¯.517	¯.573	¯2.509
CONME2	¯1.190	¯1.257	.067	.292
CONMEPH	¯1.300	¯1.257	¯.043	¯.190
CONPIP	¯1.280	¯1.257	¯.023	¯.102

d)

SUBSTITUENT	AR.PI	AR.PI.SQ	DV	RESULT
CONHOBZ	.56	.31	1.00	¯.75
CONH2	¯1.49	2.22	1.00	¯.36
CONHCYC	¯.69	.47	1.00	¯.58
CONHPH	.74	.55	1.00	¯.52
CONHISOPR	¯.21	.04	1.00	¯.29
CONHPR	.01	.00	1.00	¯.55
CONHTBU	.19	.04	1.00	¯.30
CONHAL	¯.53	.28	1.00	¯.29
CONHME	¯1.27	1.61	1.00	¯.51
CONHNEO	.81	.66	1.00	¯.87
CONHOME	¯1.33	1.77	1.00	¯.44
CONHNHPH	¯.34	.11	1.00	¯.26
CONHCH2CH2	¯.46	.21	1.00	¯.29
CONHCYHEX	.98	.96	1.00	¯.65
CONHOH	¯1.92	3.69	1.00	¯1.09
CONME2	¯1.46	2.13	.00	¯1.19
CONMEPH	.30	.09	.00	¯1.30
CONPIP	¯.11	.01	.00	¯1.28

CORRELATION COEFFICIENT MATRIX

e)

	AR.PI	AR.PI.SQ	DV
AR.PI	1.000	¯.728	.041
AR.PI.SQ	¯.728	1.000	.044
DV	.041	.044	1.000

f)

	REGRESSION COEFFICIENT	STANDARD ERROR	T VALUES	P
CONSTANT	¯1.1756			
AR.PI	¯.2120	.068	¯3.140	.0070
AR.PI.SQ	¯.2298	.057	¯4.023	.0013
DV	.7869	.106	7.442	.0000

N = 18 S = .166 F = 21.965 R = .90817 P = .00002

g)

SUBST.	OBS	CALC	DIFF	DIFF/SE
CONHOBZ	¯.750	¯.579	¯.171	¯1.032
CONH2	¯.360	¯.583	.223	1.342
CONHCYC	¯.580	¯.350	¯.230	¯1.382
CONHPH	¯.520	¯.672	.152	.915
CONHISOPR	¯.290	¯.353	.063	.381
CONHPR	¯.550	¯.391	¯.159	¯.959
CONHTBU	¯.300	¯.438	.138	.832
CONHAL	¯.290	¯.341	.051	.305
CONHME	¯.510	¯.490	¯.020	¯.121
CONHNEO	¯.870	¯.712	¯.158	¯.951
CONHOME	¯.440	¯.513	.073	.442
CONHNHPH	¯.260	¯.342	.082	.493
CONHCH2CH2	¯.290	¯.339	.049	.297
CONHCYHEX	¯.650	¯.817	.167	1.006
CONHOH	¯1.090	¯.830	¯.260	¯1.567
CONME2	¯1.190	¯1.356	.166	.997
CONMEPH	¯1.300	¯1.260	¯.040	¯.242
CONPIP	¯1.280	¯1.155	¯.125	¯.755

h)

SUBSTITUENT	AR.PI	AR.PI.SQ	RESULT
CONHOBZ	.56	.31	¯.75
CONH2	¯1.49	2.22	¯.36
CONHCYC	¯.69	.47	¯.58
CONHPH	.74	.55	¯.52
CONHISOPR	¯.21	.04	¯.29
CONHPR	.01	.00	¯.55
CONHTBU	.19	.04	¯.30
CONHAL	¯.53	.28	¯.29
CONHME	¯1.27	1.61	¯.51
CONHNEO	.81	.66	¯.87
CONHOME	¯1.33	1.77	¯.44
CONHNHPH	¯.34	.11	¯.26
CONHCH2CH2	¯.46	.21	¯.29
CONHCYHEX	.98	.96	¯.65
CONHOH	¯1.92	3.69	¯1.09
CONME2	¯1.46	2.13	¯1.19
CONMEPH	.30	.09	¯1.30
CONPIP	¯.11	.01	¯1.28

CORRELATION COEFFICIENT MATRIX

i)

	AR.PI	AR.PI.SQ
AR.PI	1.000	¯.728
AR.PI.SQ	¯.728	1.000

j)

	REGRESSION COEFFICIENT	STANDARD ERROR	T VALUES	P
CONSTANT	¯.5398			
AR.PI	¯.1586	.144	¯1.099	.2894
AR.PI.SQ	¯.1842	.122	¯1.508	.1491

N = 18   S = .357   F = 1.137   R = .36284   P = .34797

k)

SUBST.	OBS	CALC	DIFF	DIFF/SE
CONHOBZ	¯.750	¯.686	¯.064	¯.180
CONH2	¯.360	¯.712	.352	.986
CONHCYC	¯.580	¯.517	¯.063	¯.177
CONHPH	¯.520	¯.758	.238	.668
CONHISOPR	¯.290	¯.514	.224	.627
CONHPR	¯.550	¯.541	¯.009	¯.024
CONHTBU	¯.300	¯.577	.277.	.776
CONHAL	¯.290	¯.507	.217	.608
CONHME	¯.510	¯.635	.125	.351
CONHNEO	¯.870	¯.790	¯.080	¯.224
CONHOME	¯.440	¯.655	.215	.601
CONHNHPH	¯.260	¯.506	.246	.689
CONHCH2CH2	¯.290	¯.505	.215	.603
CONHCYHEX	¯.650	¯.872	.222	.622
CONHOH	¯1.090	¯.915	¯.175	¯.490
CONME2	¯1.190	¯.700	¯.490	¯1.370
CONMEPH	¯1.300	¯.604	¯.696	¯1.949
CONPIP	¯1.280	¯.524	¯.756	¯2.116

APPENDIX 2 Results from structure-activity studies on 8-carbamoylimidazotetrazinones (substituent set 2) correlating activity (ID_{50}) with hydrophobicity (Π) and measured hydrogen-bonding capacity (H_2)

a)

SUBSTITUENT	AR.PI	AR.PI.SQ	RESULT
CONHOBZ	.56	.31	‾.75
CONH2	‾1.49	2.22	‾.36
CONHCYC	‾.69	.47	‾.58
CONHPH	.74	.55	‾.52
CONHISOPR	‾.21	.04	‾.29
CONHPR	.01	.00	‾.55
CONHTBU	.19	.04	‾.30
CONHAL	‾.53	.28	‾.29
CONHME	‾1.27	1.61	‾.51
CONHNEO	.81	.66	‾.87
CONHOME	‾1.33	1.77	‾.44
CONHNHPH	‾.34	.11	‾.26
CONHCH2CH2	‾.46	.21	‾.29
CONHCYHEX	.98	.96	‾.65
CONHOH	‾1.92	3.69	‾1.09

CORRELATION COEFFICIENT MATRIX

b)

	AR.PI	AR.PI.SQ
AR.PI	1.000	‾.692
AR.PI.SQ	‾.692	1.000

c)

	REGRESSION COEFFICIENT	STANDARD ERROR	T VALUES	P
CONSTANT	‾.3700			
AR.PI	‾.2064	.069	‾3.006	.0106
AR.PI.SQ	‾.2493	.059	‾4.219	.0012

N = 15 S = .167 F = 8.909 R = .77302 P = .00428

d)

SUBST.	OBS	CALC	DIFF	DIFF/SE
CONHOBZ	¯.750	¯.563	¯.187	¯1.123
CONH2	¯.360	¯.616	.256	1.535
CONHCYC	¯.580	¯.345	¯.235	¯1.411
CONHPH	¯.520	¯.660	.140	.839
CONHISOPR	¯.290	¯.337	.047	.280
CONHPR	¯.550	¯.372	¯.178	¯1.067
CONHTBU	¯.300	¯.419	.119	.715
CONHAL	¯.290	¯.330	.040	.243
CONHME	¯.510	¯.510	.000	¯.001
CONHNEO	¯.870	¯.702	¯.168	¯1.010
CONHOME	¯.440	¯.537	.097	.581
CONHNHPH	¯.260	¯.327	.067	.404
CONHCH2CH2	¯.290	¯.327	.037	.225
CONHCYHEX	¯.650	¯.812	.162	.970
CONHOH	¯1.090	¯.894	¯.196	¯1.178

e)

SUBSTITUENT	AR.PI	AR.PI.SQ	H2	RESULT
CONHOBZ	.56	.31	12.00	¯.75
CONH2	¯1.49	2.22	.00	¯.36
CONHCYC	¯.69	.47	¯10.00	¯.58
CONHPH	.74	.55	8.00	¯.52
CONHISOPR	¯.21	.04	¯6.00	¯.29
CONHPR	.01	.00	¯16.00	¯.55
CONHTBU	.19	.04	.00	¯.30
CONHAL	¯.53	.28	¯16.00	¯.29
CONHME	¯1.27	1.61	¯28.00	¯.51
CONHNEO	.81	.66	¯22.00	¯.87
CONHOME	¯1.33	1.77	14.00	¯.44
CONHNHPH	¯.34	.11	¯10.00	¯.26
CONHCH2CH2	¯.46	.21	¯16.00	¯.29
CONHCYHEX	.98	.96	¯6.00	¯.65
CONHOH	¯1.92	3.69	¯22.00	¯1.09

CORRELATION COEFFICIENT MATRIX

f)

	AR.PI	AR.PI.SQ	H2
AR.PI	1.000	¯.692	.228
AR.PI.SQ	¯.692	1.000	¯.139
H2	.228	¯.139	1.000

g)

	REGRESSION COEFFICIENT	STANDARD ERROR	T VALUES	P
CONSTANT	¯.3363			
AR.PI	¯.2234	.067	¯3.318	.0067
AR.PI.SQ	¯.2513	.057	¯4.410	.0010
H2	.0048	.003	1.381	.1925

N = 15   S = .161   F = 7.024   R = .81057   P = .00667

h)

SUBST.	OBS	CALC	DIFF	DIFF/SE
CONHOBZ	¯.750	¯.482	¯.268	¯1.669
CONH2	¯.360	¯.561	.201	1.252
CONHCYC	¯.580	¯.348	¯.232	¯1.443
CONHPH	¯.520	¯.602	.082	.507
CONHISOPR	¯.290	¯.328	.038	.237
CONHPR	¯.550	¯.415	¯.135	¯.839
CONHTBU	¯.300	¯.389	.089	.552
CONHAL	¯.290	¯.365	.075	.465
CONHME	¯.510	¯.592	.082	.508
CONHNEO	¯.870	¯.788	¯.082	¯.508
CONHOME	¯.440	¯.417	¯.023	¯.144
CONHNHPH	¯.260	¯.336	.076	.472
CONHCH2CH2	¯.290	¯.363	.073	.453
CONHCYHEX	¯.650	¯.825	.175	1.090
CONHOH	¯1.090	¯.940	¯.150	¯.934

i)

SUBSTITUENT	H2	RESULT
CONHOBZ	12.00	¯.75
CONH2	.00	¯.36
CONHCYC	¯10.00	¯.58
CONHPH	8.00	¯.52
CONHISOPR	¯6.00	¯.29
CONHPR	¯16.00	¯.55
CONHTBU	.00	¯.30
CONHAL	¯16.00	¯.29
CONHME	¯28.00	¯.51
CONHNEO	¯22.00	¯.87
CONHOME	14.00	¯.44
CONHNHPH	¯10.00	¯.26
CONHCH2CH2	¯16.00	¯.29
CONHCYHEX	¯6.00	¯.65
CONHOH	¯22.00	¯1.09

	REGRESSION COEFFICIENT	STANDARD ERROR	T VALUES	P
CONSTANT	¯.4846			
H2	.0041	.005	.786	.4511

j) N = 15   S = .247   F = .618   R = .21304   P = .45114

k)

SUBST.	OBS	CALC	DIFF	DIFF/SE
CONHOBZ	¯.750	¯.436	¯.314	¯1.274
CONH2	¯.360	¯.485	.125	.505
CONHCYC	¯.580	¯.525	¯.055	¯.222
CONHPH	¯.520	¯.452	¯.068	¯.275
CONHISOPR	¯.290	¯.509	.219	.888
CONHPR	¯.550	¯.550	.000	¯.001
CONHTBU	¯.300	¯.485	.185	.749
CONHAL	¯.290	¯.550	.260	1.053
CONHME	¯.510	¯.599	.089	.359
CONHNEO	¯.870	¯.574	¯.296	¯1.199
CONHOME	¯.440	¯.428	¯.012	¯.050
CONHNHPH	¯.260	¯.525	.265	1.076
CONHCH2CH2	¯.290	¯.550	.260	1.053
CONHCYHEX	¯.650	¯.509	¯.141	¯.571
CONHOH	¯1.090	¯.574	¯.516	¯2.091

REFERENCES

Number

1 Finger.J. J.Prakt.Chem. 1888. 37. 431-445.

2 Ege.G.; Gilbert.K. Tetrahedron Lett. 1979. 44. 4253-4256.

3 Ege.G.; Gilbert.K. German Patent. 2932305. 1981.

4 Palmer.M.H.; Gaskell.A.J.; Findlay.R.H. J.Chem.Soc. Perkin Trans. II. 1974. 778-784.

5 Wiley.P.F. In "Chemistry of 1.2.3-Triazines and 1.2.4-Triazines. Tetrazines and Pentazines"; Wiley: New York. 1978. 1296-1300.

6 Stevens.M.F.G. Progr.Med.Chem. 1976. 13. 205-269.

7 Gescher.A.G.; Stevens.M.F.G.; Turnbull.C.P. J.Chem.Soc. Perkin Trans. I. 1977. 103-106.

8 Baig.G.U.; Stevens.M.F.G.; Stone.R.; Lunt.E. J.Chem.Soc. Perkin Trans. I. 1982. 1811-1819.

9 Shealy.Y.F.; O'Dell.C.A.; Krauth.C.A. J.Pharm.Sci. 1975. 64. 177-180.

10 Stone.R. Ph.D. Thesis. Aston University. 1981.

11 Stevens.M.F.G.; Hickman.J.A.; Stone.R.; Gibson.N.W.; Baig.G.U.; Lunt.E.; Newton.C.G. J.Med.Chem. 1984. 27. 196-201.

12 Padwa.A.; Kumajai.T. Tetrahedron Lett. 1981. 22. 1199-1202.

13 Horton.J.K.; Stevens.M.F.G. J.Chem.Soc. Perkin Trans. I. 1981. 1433-1436.

14 Erickson.J.G.; Wiley.P.F.; Wystrach.V.P. In "The Chemistry of Heterocyclic Compounds. Vol. X. The 1.2.3- and 1.2.4-Triazines. Tetrazines and Pentazines"; Wiley; New York. 1957.

15 Horgan.C.M.T. Ph.D. Thesis. Aston University. 1985.

16 Goddard.C. Ph.D. Thesis. Aston University. 1986.

17 Baig.G.U.; Stevens.M.F.G. J.Chem.Soc. Perkin Trans. I. 1987. 665-670.

REFERENCES

Number

18 Lunt.E.; Stevens.M.F.G.; Graham.M.; Stone.R.; Wooldridge.K.R.H. British Patent Application. 2104522A. 1983.

19 Lunt.E.; Newton.C.G.; Smith.C.; Stevens.G.P.; Stevens.M.F.G.; Straw.C.G.; Walsh.R.J.A.; Warren.P.J.; Fizames.C.; Lavelle.F.; Langdon.S.P.; Vickers.L.M. J.Med.Chem 1987. 30. 357-366.

20 Stevens.M.F.G. In "New Avenues in Developmental Cancer Chemotherapy"; Harrap.K.R.; Connors.T.A..Eds.; Academic Press: 1987. 335-354.

21 Stevens.M.F.G.; Hickman.J.A.; Stone.R.; Gibson.N.W.; Lunt.E. Br.J.Cancer 1983. 48. 120.

22 Hickman.J.A.; Stevens.M.F.G.; Gibson.N.W.; Langdon.S.P.; Fizames.C.; Lavelle.F.; Atassi.G.; Lunt.E.; Tilson.R.M. Cancer Res. 1985. 45. 3008-3013.

23 Fodstad.Ø.; Aamdal.S.; Pihl.A.; Boyd.M.R. Cancer Res. 1985. 45. 1778-1786.

24 Goldin.A.; Venditti.J.M.; MacDonald.J.S.; Muggia.F.M.; Henney.J.E.; Devita.V.T..Jr. Eur.J.Cancer 1981. 17. 129-142.

25 Newlands.E.S.; Blackledge.G.; Slack.J.A.; Goddard.C.; Brindley.C.J.; Holden.L.; Stevens.M.F.G. Cancer Treat. Rep. 1985. 69. 801-805.

26 Joss.R.A.; Ryssel.H.J.; Bischoff.A.K.; Goldhirsch.A.; Brunner.K.W. Cancer Treat.Rep. 1986. 70. 797-798.

27 Harding.M.; Kaye.S.B.; Dorward.A.; Mackie.R.; Smythe.J.; Blackledge.G. Proc.Brit.Assoc.Cancer Res. 28th Meeting. 1987.

28 Gundersen.S.; Aamdal.S.; Fodstad.Ø. Br.J.Cancer. 1987. 55. 433-435.

29 Newton.C.G.. unpublished results.

30 Schein.P.S. In "Cancer and Chemotherapy 3. Antineoplastic Agents"; Crooke. S.T.; Prestayko. A.W.; Eds.. Academic Press: New York. 1981. 37-48.

REFERENCES

Number

31 Mitchell,E.P.; Schein,P.S. Cancer Treat.Rep. 1986, 70, 31-41.

32 Schmall,B.; Cheng,C.J.; Fujimura,S.; Gersten,N.; Grunberger,D.; Weinstein,B.I. Cancer Res. 1973, 33, 1921-1924.

33 Wheeler,G.P.; Bowdon,B.J.; Grimsley,J.A.; Lloyd,H.H. Cancer Res. 1974, 34, 194-200.

34 Wheeler,G.P.; Bowdon,B.J. Cancer Res. 1968, 28, 52-59.

35 Baril,B.B.; Baril,E.F.; Laszlo,J.; Wheeler,G.P. Cancer Res. 1975, 35, 1-5.

36 Chuang,R.Y.; Laszlo,J.; Keller,P. Biochem.Biophys.Acta 1976, 425, 463-468.

37 Kann,H.E.,Jr.; Kohn,K.W.; Lyles,J.M. Cancer Res. 1974, 34, 398-402.

38 Babson,J.R.; Reed,D.J. Biochem.Biophys.Res.Commun. 1978, 83, 754-762.

39 Brown,W.E.; Wold,F. Biochemistry 1973, 12, 835-840.

40 Babson,J.R.; Reed,D.J.; Sinkey,M.A. Biochemistry 1977, 16, 1584-1589.

41 Brodie,A.E.; Babson,J.R.; Reed,D.J. Biochem.Pharmacol. 1980, 29, 652-654.

42 Kann,H.E.,Jr.; Kohn,K.W.; Widerlite,L.; Gullion,D. Cancer Res. 1974, 34, 1982-1988.

43 Kann,H.E.,Jr.; Schott,M.A.; Petkas,A. Cancer Res. 1980, 40, 50-55.

44 Kann,H.E.,Jr.; Blumenstein,B.A.; Petkas,A.; Schott,M.A. Cancer Res. 1980, 40, 771-775.

45 Anderson,T.; McMenamin,M.G.; Schein,P.S. Cancer Res. 1975, 35, 761-765.

46 Panasci,L.C.; Green,D.; Nagourney,R.; Fox,P.; Schein,P.S. Cancer Res. 1977, 37, 2615-2618.

REFERENCES

Number

47 Heal,J.M.; Fox,P.A.; Schein,P.S. Cancer Res. 1979, 39, 82-89.

48 Panasci,L.C.; Green,D.; Schein,P.S. J.Clin.Invest. 1979, 64, 1103-11.

49 Ahlgren,J.D.; Green,D.C.; Tew,K.D.; Schein,P.S. Cancer Res. 1982, 42, 2605-2608.

50 Horikoshi,A.; Smith,R.; Murphy,M.J., Jr. Chemotherapy 1983, 29, 135-144.

51 Gibson,N.W.; Hickman,J.A. Biochem.Pharmacol. 1982, 31, 2795-2800.

52 Johnston,T.P.; Montgomery,J.A. Cancer Treat.Rep. 1986, 70, 13-30.

53 Ludlum,D.B. In "Handbook of Experimental Pharmacology"; Sartorelli,A.C.; Johns,D.G.; Eds.; Springer-Verlag: Berlin: 1975, 6-17.

54 Price,C.C. In "Antineoplastic and Immunosuppressive Agents Part II"; Sartorelli,A.C.; Johns,D.G.; Eds.; Springer-Verlag: Berlin: 1975, 1-5.

55 Roberts,J.J. In "The Interaction of Drugs and Subcellular Components in Animal Cells"; Campbell,P.M., Ed.; Churchill : London, 1968, 5-27.

56 Loveless,A.; Hampton,C.L. Mut.Res. 1969, 7, 1-12.

57 Eadie,J.S.; Conrad,M.; Toorchen,D.; Topal,M.D. Nature (London) 1984, 308, 201-204.

58 Lawley,P.D.; Martin,C.N. Biochem.Journ. 1975, 145, 85-91.

59 Singer,B. J. Natl.Cancer Inst. 1979, 62, 1329-1339.

60 Rajewsky,M.F. In "Chemical Carcinogenesis"; Nicolini,C., Ed.; Plenum Press: New York, 1982, 363-379.

61 Guttenplan,J.B. Carcinogenesis 1984, 5, 155-159.

62 Goth,R.; Rajewsky,M.F. Proc.Nat.Acad.Sci.USA 1974, 71, 639-643.

REFERENCES

Number

63 Tong,W.P.; Ludlum,D.B. Cancer Res. 1981, 41, 380-382.

64 Tong,W.P.; Kirk,M.C.; Ludlum,D.B. Cancer Res. 1982, 42, 3102-3105.

65 Kohn,K.W. Cancer Res. 1977, 37, 1450-1454.

66 Lown,J.W.; McLaughlin,C.W.; Chang,Y.M. Biorgan.Chem. 1978, 7, 97-110.

67 Ewig,R.A.G.; Kohn,K.W. Cancer Res. 1977, 37, 2114-2122.

68 Ewig,R.A.G.; Kohn,K.W. Cancer Res. 1978, 38, 3197-3203.

69 Erickson,L.C.; Bradley,M.D.; Ducore,J.M.; Ewig,R.A.G.; Kohn,K.W. Proc.Nat.Acad.Sci.USA. 1980, 77, 467-471.

70 Olsson,M.; Lindahl,T. J.Biol.Chem. 1980, 255, 10569-10571.

71 Harris,A.L.; Karran,P.; Lindahl,T. Cancer Res. 1983, 43, 3247-3252.

72 Hora,J.F.; Eastman,A.; Bresnick,E. Biochemistry 1983, 22, 3759-3763.

73 Sedgwick,B.; Lindahl,T. J.Mol.Biol. 1982, 154, 169-175.

74 Lindahl,T. Br.J.Cancer 1987, 56, 91-95.

75 Mehta,J.R.; Ludlum,D.B.; Renard,A.; Verly,W.G. Proc.Nat.Acad.Sci.USA 1981, 78, 6766-6770.

76 Erickson,L.C.; Laurent,G.; Sharkey,N.A.; Kohn,K.W. Nature (London) 1980, 288, 727-729.

77 Day,R.S.; Ziolkowski,C.H.J.; Scudiero,D.A.; Meyer,S.A.; Mattern,M.R. Carcinogenesis 1980, 1, 21-32.

78 Day,R.S.,III. Bioscience 1981, 31, 807-813.

79 Scudiero,D.A.; Meyer,S.A.; Clatterbuck,B.E.; Mattern,M.R.; Ziolkowski,C.; Day,R.S.,III. Cancer Res. 1984, 44, 2467-2474.

REFERENCES

Number

80 Gibson,N.W.; Hartley,J.; La France,R.J.; Vaughan,K. Carcinogenesis 1986, 7, 259-265.

81 Margison,G.P.; Brennand,J., in press.

82 Brennand,J.; Margison,G.P. Carcinogenesis 1986, 7, 2081-2084.

83 Margison,G.P. Proc.Brit.Assoc.Cancer Res. 28th Meeting, 1987.

84 Fox,M.; Brennand,J.; Margison,G.P. Proc.Brit.Assoc.Cancer Res. 28th Meeting, 1987.

85 Strauss,B.S. Life Sci. 1974, 15, 1685-1693.

86 Skibba,J.L.; Bryan,G.T. Toxicol.App.Pharmacol. 1971, 18, 707-719.

87 Bull,V., personal communication.

88 Bull,V. Ph.D. Thesis, Aston University, in preparation.

89 Horgan,C.M.T.; Stevens,M.F.G.; Tisdale,M.J. Br.J. Cancer 1983, 48, 132.

90 Horgan,C.M.T.; Tisdale,M.J. Biochem.Pharmacol. 1984, 33, 2185-2192.

91 Dive,C.; Workman,P.; Watson,J. Proc.Brit.Assoc.Cancer Res./EASR/RSM Joint Winter Meeting, 1986.

92 Dive,C.; Workman,P.; Watson,J. Proc.Brit.Assoc.Cancer Res. 28th Meeting, 1987.

93 Gibson,N.W.; Erickson,L.C.; Hickman,J.A. Cancer Res. 1984, 44, 1767-1771.

94 Gibson,N.W.; Hickman,J.A.; Erickson,L.C. Cancer Res. 1984, 44, 1772-1775.

95 Hartley,J.A.; Gibson,N.W.; Kohn,K.W.; Mattes,W.B. Cancer Res. 1986, 46, 1943-1947.

96 Karran,P.; Williams,S.A. Carcinogenesis 1985, 6, 789-792.

REFERENCES

Number

97 Clarke,D.A.; Barclay,R.K.; Stock,C.C.; Rondesvedt,C.S.,Jr. Proc.Soc.Exp.Biol.Med. 1955, 90, 484-488.

98 Shealy,Y.F.; Krauth,C.A.; Montgomery,J.A. J.Org.Chem. 1962, 27, 2150-2154.

99 Carter,S.K.; Freidman,M.A. Eur.J.Cancer 1972, 8, 85-92.

100 Comis,R.L. Cancer Treat.Rep. 1976, 60, 165-176.

101 Spassova,M.K.; Golovinsky,E.V. Pharmac. & Ther. 1985, 27, 333-352.

102 Shealy,Y.F.; Struck,R.F.; Holum,L.B.; Montgomery,J.A. J.Org.Chem. 1961, 26, 2396-2401.

103 Kreis,W. In "Cancer, A Comprehensive Treatise - 5. Hydrazines and Triazines"; Becker,F.F.; Ed.; Plenum Press: New York, 1977.

104 Schulman,M.P.; Buchanan,J.M. J.Biol.Chem. 1952, 196, 513-526.

105 Hano,K.; Akashi,A. Gann 1964, 55, 25-36.

106 Skibba,J.L.; Ramirez,G.; Beal,D.D.; Bryan,G.T. Biochem.Pharmacol. 1970, 19, 2043-2051.

107 Vaughan,K.; Stevens,M.F.G. Chem.Soc.Rev. 1978, 7, 377-397.

108 Skibba,J.; Beal,D.D.; Ramirez,G.; Bryan,G.T. Cancer Res. 1970, 30, 147-150.

109 Hill,D.L. Cancer Res. 1975, 35, 3106-3110.

110 Luce,J.K.; Thurman,W.G.; Isaacs,B.L.; Talley,R.W. Cancer Chemother.Rep. 1970, 54, 119-124.

111 Wilman,D. Biochem.Soc.Trans. 1986, 14, 377.

112 Baig,G.U. Ph.D. Thesis, Aston University, 1986.

113 Stevens,J., personal communication.

114 Bueler,C.A.; Pearson,D.E. In "Survey of Organic Synthesis"; Wiley: New York, 1970, 861-862.

REFERENCES

Number

115 Lee,J.B. J.Am.Chem.Soc. 1966, 88, 3440-3441.

116 Fieser,L.F.; Peters,M.A. J.Am.Chem.Soc. 1932, 54, 4376.

117 Peters,D. J.Chem.Soc. 1960, 1832-1837.

118 Fieser,L.F.; Fieser,M.A. In "Reagents for Organic Synthesis"; Wiley: New York, 1967, 26.

119 Mann,F.G.; Porter,J.W.G. J.Chem.Soc.Trans. II, 1945, 148, 751-760.

120 Fuhrhop,J.; Penzlin,G. In "Organic Synthesis"; Verlag Chemie: Berlin, 1983, 129-132.

121 Fisher,M.H.; Nicholson,W.H.; Stuart,R.S. Can.J.Chem. 1961, 39, 1336-1339.

122 Washburne,S.S.; Peterson,W.R.,Jr. Synth.Commun. 1972, 2, 227-230.

123 Kricheldorf,H.R. Synthesis 1972, 551-553.

124 MacMillan,J.H.; Washburne,S.S. J.Org.Chem. 1973, 38, 2982-2984.

125 Warren,J.D.; Press,J.B. Synth.Commun. 1980, 10, 107-110.

126 Moore,H.W.; Goldish,D.M. In "Chemistry of Halides, Pseudohalides, Azides"; Patai,S.; Rappaport,Z., Eds.; Wiley: London, 1983, 358.

127 Smith,P.A.S. In "Organic Reactions Vol. III"; Adams,R.; Bachmann,W.E.; Fieser,L.F.; Johnson,J.R.; Snyder,H.R., Eds.; Wiley: New York, 1946, 338.

128 Snyder,H.R.; Elston,C.T.; Kellom,D.B. J.Am.Chem.Soc. 1953, 75, 2014-2015.

129 Weinstock,J. J.Org.Chem. 1961, 26, 3511.

130 Wiersum,U.E. Recl.Trav.Chim. Pays-Bas 1982, 101, 317-332.

131 Wiersum,U.E. Aldrichimica Acta. 1984, 17, 31-41.

REFERENCES

Number

132 Black,H.T. Aldrichimica Acta. 1983, 16, 3-11.

133 Smith,P.A.S. In "Molecular Rearrangements Pt. 1"; de Mayo, P., Ed.; Wiley: New York, 1963, 528-564.

134 Bachmann,W.E.; Struve,W.S. In "Organic Reactions, Vol.I"; Adams,R.; Bachmann,W.E.; Fieser,L.F.; Johnson,J.R.; Snyder,H.R., Eds.; Wiley: New York, 1954, 38-63.

135 Marquisee,M.J.; Kauer,J.C. J.Med.Chem. 1978, 21, 1188-1194.

136 Baldwin,R.W. Eur.J.Cancer and Clin.Oncol. 1985, 21, 1281-1285.

137 Embleton,M.J. Br.J.Cancer 1987, 55, 227-231.

138 Shioiri,T.; Ninomiya,K.; Yamada,S. J.Am.Chem.Soc. 1972, 94, 6203-6205.

139 Warren,M., personal communication.

140 Wallis,E.S.; Lane,J.F. In "Organic Reactions Vol. III"; Adams,R.; Bachmann,W.E.; Fieser,L.F.; Johnson,J.R.; Snyder,H.R., Eds.; Wiley: New York, 1946, 268-307.

141 Fuller,W.D.; Goodman,M.; Verlander,M.S. J.Am.Chem.Soc. 1985, 107, 5821-5822.

142 Loudon,G.M.; Radhakrishna,A.S.; Almond,M.R.; Blodgett,J.K.; Boutin,R.H. J.Org.Chem. 1984, 49, 4272-4276.

143 Boutin,R.H.; Loudon,G.M. J.Org.Chem. 1984, 49, 4279-4284.

144 Lunt,E., personal communication.

145 Wolff,H. In "Organic Reactions Vol. III"; Adams,R.; Bachmann,W.E.; Fieser,L.F.; Johnson,J.R.; Snyder,H.R., Eds.; Wiley: New York, 1949, 307-337.

146 Bryant Bachman,G.; Goldmacher,J.E. J.Org.Chem. 1964, 29, 2576-2579.

147 Blum,J. Tetrahedron Letts. 1966, 15, 1605-1608.

REFERENCES

Number

148 Blum,J.; Oppenheimer,E.; Bergmann,E.D. J.Am.Chem.Soc. 1967, 89, 2338-2341.

149 Lunt, E., personal communication.

150 Slack,J.A.; Stevens,M.F.G.; Goddard,C.; Khan,A. Proc. Am.Assoc.Cancer Res. 1983, 24, 291.

151 Slack,J.A.; Goddard,C. J.Chromatogr. 1985, 337, 178-181.

152 Lowe,P.R.; Schwalbe,C.H.; Stevens,M.F.G. J.Chem.Soc. Perkin Trans. II. 1985, 357-361.

153 Freeman,H.C.; Hutchinson,N.D. Acta.Crystallogr. 1979, B35, 2051-2054.

154 Edwards,S.L.; Sherfinski,J.S.; Marsh,R.E. J.Am.Chem.Soc. 1974, 96, 2593-2597.

155 Lowe,P.R., personal communication.

156 Clark,J.; Cunliffe,A.E. Chem.& Ind. 1973, 281-283.

157 Albert,A.; Serjeant,E.P. In "The Determination of Ionisation Constants, 3rd Edition"; University Press: Cambridge, 1984, 29.

158 Dolfini,E.; Martini,A.; Donelli,M.G.; Morasca,L.; Garattini,S. Eur.J.Cancer 1973, 9, 375-378.

159 Connors,T.A.; Cox,P.J.; Farmer,P.B.; Foster,A.B.; Jarman,M. Biochem.Pharmacol. 1974, 23, 115-129.

160 Takamizawa,A.; Matsumoto,S.; Iwata,T.; Tochino,Y.; Katagiri,K.; Yamaguchi,K. J.Med.Chem. 1975, 18, 376-383.

161 Phillips,B.J. Biochem.Pharmacol. 1974, 23, 131-138.

162 Rutty,C.J.; Connors,T.A. Biochem.Pharmacol. 1977, 26, 2385-2391.

163 Rutty,C.J.; Abel,G. Chem.Biol.Interactions 1980, 29, 235-246.

164 Gescher,A.; Hickman,J.A.; Stevens,M.F.G. Biochem.Pharmacol. 1979, 28, 3235-3238.

REFERENCES

Number

165 Nash,T. Biochemistry 1953, 55, 416-421.

166 Farmer,P.B., personal communicaton.

167 Preusmann,R.; Von Hodenberg,A.; Hengy,H. Biochem.Pharmacol. 1969, 18, 1-13.

168 Pool,B.L. J.Cancer Res.Clin.Oncol. 1979, 93, 221-231.

169 Gescher,A.; Hickman,J.A.; Simmonds,R.J.; Stevens,M.F.G.; Vaughan,K. Biochem.Pharmacol. 1981, 30, 89-93.

170 Vaughan,K.; Tang,Y.; Llanos,G.; Horton,J.K.; Simmonds,R.J.; Hickman,J.A.; Stevens,M.F.G. J.Med.Chem. 1984, 27, 357-363.

171 Kolar,G.F.; Maurer,M.; Wildschütte,M. Cancer Lett. 1980, 10, 235-241.

172 Mizuno,N.S.; Decker,R.W. Biochem.Pharmacol. 1976, 25, 2643-2647.

173 Saunders,P.P.; De Chang,W.; Chao,L. Chem.Biol.Interactions 1986, 58, 319-331.

174 Farina,P.; Torti,L.; Urso,R.; Horton,J.K.; Gescher,A.; D'Incalci,M. Biochem.Pharmacol. 1986, 35, 209-215.

175 Ross,D.; Farmer,P.B.; Gescher,A.; Hickman,J.A.; Threadgill,M.D. Biochem.Pharmacol. 1981, 31, 3621-3627.

176 Ross,D.; Farmer,P.B.; Gescher,A.; Hickman,J.A.; Threadgill,M.D. Biochem.Pharmacol. 1983, 32, 1773-1781.

177 Quarterman,C., personal communication.

178 Crum-Brown,A.; Fraser,T. Trans.Roy.Soc.Edinburgh 1868-1869, 25, 151.

179 Crum-Brown,A.; Fraser,T. Trans.Roy.Soc.Edinburgh 1868-1869, 25, 693.

180 Hansch,C. In "International Encyclopedia of Pharmacology and Therapeutics, Section 5: Structure-activity Relationships"; Cavallito,C.J., Ed.; Pergamon Press: Oxford, 1973, Vol.1, 75-110.

REFERENCES

Number

181 Redl,G.; Cramer,R.D.,III.; Berkoff,C.E. Chem.Soc.Rev. 1974, 3, 273-292.

182 Martin,Y.C. "Quantitative Drug Design. A Critical Introduction"; Marcel Dekker: New York, 1978.

183 Hansch,C.; Fujita,T. J.Am.Chem.Soc. 1964, 86, 1616-1626.

184 Fujita,T.; Iwasa,J.; Hansch,C. J.Am.Chem.Soc. 1964, 86, 5175-5180.

185 Hansch,C. Acc.Chem.Res. 1969, 2, 232-239.

186 Craig,P.N. J.Med.Chem. 1971, 14, 680-684.

187 Wooldridge,K.R.H., personal communication.

188 Wooldridge,K.R.H. Eur.J.Med.Chem. 1980, 15, 63-66.

189 Hansch,C.; Leo,A.J. "Substituent Constants For Correlation Analysis in Chemistry and Biology"; Wiley: New York, 1979.

190 Baker,A.W.; Shulgin,A.T. J.Am.Chem.Soc. 1958, 80, 5358-5363.

191 Baker,A.W.; Kaeding,W.W. J.Am.Chem.Soc. 1959, 81, 5904-5907.

192 Reeves,L.W.; Allan,E.A.; Strømme,K.O. Cancer J.Chem. 1960, 38, 1249-1254.

193 Broughton,B.J.; Chaplen,P.; Knowles,P.; Lunt,E.; Marshall,S.M.; Pain,D.L.; Wooldridge,K.R.H. J.Med.Chem. 1975, 18, 1117-1122.

194 Connors,T.A.; Goddard,P.M.; Merai,K.; Ross,W.C.J.; Wilman,D.E.V. Biochem.Pharmacol. 1976, 25, 241-246.

195 Rondestvedt,C.S.,Jr.; Davis,S.J. J.Org.Chem. 1957, 22, 200-203.

196 Hansch,C.; Smith,N.; Engle,R.; Wood,H. Cancer Chemother.Rep. 1972, 56, 443-456.

REFERENCES

Number

197 Lin,Y.; Loo.T.L.; Vadlamudi,S.; Goldin,A. J.Med.Chem. 1972, 15, 201-203.

198 Noell,C.W.; Cheng,C.C. J.Med.Chem. 1969, 12, 545-546.

199 Shealy,Y.F.; O'Dell,C.A. J.Pharm.Sci. 1971, 60, 554-560.

200 Hatheway,G.J.; Hansch,C.; Kim,K.H.; Milstein,S.R.; Schmidt,C.L.; Smith,R.N.; Quinn,F.R. J.Med.Chem. 1978, 21, 563-577.

201 Hansch,C.; Hatheway,G.J.; Quinn,F.R. J.Med.Chem. 1978, 21, 574-577.

202 Hebborn,P.; Danielli,J.F. Nature 1956, 177, 25-27.

203 Hebborn,P.; Danielli,J.F. Biochem.Pharmacol. 1958, 1, 19-24.

204 Dalton,C.; Hebborn,P. Biochem.Pharmacol. 1965, 14, 1567-1572.

205 Carl,P.L.; Chakravarty,P.K.; Katzenellenbogen,J.A.; Weber,M.J. Proc.Nat.Acad.Sci.USA. 1980, 77, 2224-2228.

206 Chakravarty,P.K.; Carl,P.L.; Weber,M.J.; Katzenellenbogen,J.A. J.Med.Chem. 1983, 26, 638-644.

207 Pimentel,G.C.; McClellan,A.L. In "The Hydrogen-bond"; Pauling, L., Ed.; Freeman: New York, 1960.

208 Vinogradov,S.N.; Linnell,R.H. In "Hydrogen-bonding", Van Nostrand Reinhold: New York, 1971.

209 Hadzi,D.; Jan,J. Spectroscopy Lett. 1968, 1, 139-143.

210 Weckherlin,S.; Luttke,W. Tetrahedron Letts. 1964, 1711.

211 Mitsuhashi,T.; Osamura,Y.; Simamura,O. Tetrahedron Letts. 1965, 2593.

212 Vaughan,K. J.Chem.Soc.Perkin Trans.II 1977, 17-20.

213 Hickman,J.A. Biochem.Pharmacol. 1974, 23, 2833-2838.

REFERENCES

Number

214 Hickman,J.A.; Melzach,D.H. Biochem.Pharmacol. 1975, 24, 1947-1952.

215 Rao,S.N.; Singh,C.U.; Kollman,P.A. J.Med.Chem. 1986, 29, 2484-2492.

216 Hurley,L.H.; Reynolds,V.L.; Swenson,D.H.; Petzold,G.L.; Scahill,T.A. Science 1984, 226, 843-844.

217 Patel,D.J.; Shapiro,L. J.Biol.Chem. 1986, 261, 1230-1240.

218 Dervan,P.B. Science 1986, 232, 464-471.

219 Goodsell,D.; Dickerson,R.E. J.Med.Chem. 1986, 29, 727-733.

220 Kopka,M.L.; Yoon,C.; Goodsell,D.; Pjura,P.; Dickerson,R.E. Proc.Nat.Acad.Sci.USA. 1985, 82, 1376-1380.

221 Pearl,L.H.; Skelly,J.V.; Hudson,B.D.; Neidle,S. Nuc.Acids Res. 1987, 15, 3469-3478.

222 Patel,D.L.; Shapiro,L. Biochimie 1985, 67, 887-915.

223 Gund,P.; Andose,J.D.; Rhodes,J.B.; Smith,G.M. Science 1980, 208, 1425-1431.

224 Hol,W.G.L. Angewante Chem.Int. Ed. 1986, 25, 767-779.

225 Fruhbeis,H.; Klein,R.; Wallmeier,H. Angewante Chem. Int. Ed. 1987, 26, 403-418.

226 Lown,J.W.; Krowicki,K.; Balzarini,J.; De Clercq,E. J.Med.Chem. 1986, 29, 1210-1214.

227 Workman,P. Eur.J.Cancer Clin.Oncol. 1987, in press.

228 Calvert,N.; Connors,T.A.; Ross,W.C.J. Eur.J.Cancer 1968, 4, 627-636.

229 Noyanalpan,N.; Ozden,S.; Ozden,T. J.Fac.Pharm.Ankara 1977, 7, 104-110.

230 Connors,T.A.; Jones,M. Recent Results in Cancer Res. 1970, 33, 181-187.