

The Characterization of nucleoside transport in the  
intestinal protozoan parasite *Giardia intestinalis*.

Robert Andrew Davey

Department of Microbiology and Immunology

The University of Adelaide,

Adelaide, 5005

Australia

A thesis submitted for the Degree of Doctor of Philosophy.

August, 1993.

Awarded 1994

## Abstract

A rapid sampling technique has been adapted and used to measure nucleoside transport in a human-derived isolate of the protozoan parasite *Giardia intestinalis* (syn. *G. lamblia*). The study was based on published findings that *Giardia* cannot produce nucleotides by *de novo* synthesis; that labelled adenine, guanine and uracil are incorporated directly into the ribonucleotide pool by the action of specific phosphoribosyltransferases; that exogenously acquired ribonucleosides are degraded rapidly within the cells by specific hydrolases to yield the respective bases; and that the parasite lacks ribonucleotide reductase and is able to produce 2'-deoxynucleotides only by direct phosphorylation of acquired 2'-deoxynucleosides. As cell membranes are effective barriers to the diffusion of nucleosides, it was considered that transporters must exist in *Giardia* to mediate their uptake.

Transport assays were conducted at 0°C to prevent loss of trophozoites by adherence, to minimize intracellular metabolism of transported nucleosides, and to slow transport for more accurate measurements. Initially, the transport of [<sup>3</sup>H]thymidine was measured and this was shown to follow simple Michaelis-Menten kinetics, with an apparent  $K_m$  of 50  $\mu\text{M}$  and  $V_{\text{max}}$  of 70  $\text{pmol}\cdot\text{min}^{-1}\cdot(10^6 \text{ cells})^{-1}$ . Thymine and uracil were the most effective inhibitors of thymidine transport ( $K_i$ 's of 30  $\mu\text{M}$  and 45  $\mu\text{M}$  respectively), followed by thymidine, deoxyuridine and uridine ( $K_i$ 's between 64-96  $\mu\text{M}$ ). In contrast, cytosine, its ribonucleoside and 2'-deoxyribonucleoside derivatives, and adenosine, were not effective inhibitors, even at high concentrations. These results indicated that the transporter responsible for a major part of thymidine influx recognized the nucleoside base moiety. Recognition may require the presence of an oxygen attached to the carbon at position 4 of the pyrimidine ring, as occurs in thymine and uracil, to allow hydrogen bonding. The 5-methyl group of thymine apparently does not interfere with the recognition process. Although the furanose ring appeared not to be involved in recognition and binding to this transporter (uracil, uridine and deoxyuridine were all efficient inhibitors of thymidine transport), it may be required for the translocation step, as bases enter the cells via a separate carrier (see below).

A detailed analysis of inhibition of thymidine influx by adenosine and deoxycytidine, which exhibited a maximum (plateau) level of inhibition only 20-30% of that effected by either uracil or uridine, suggested the existence of a second nucleoside transporter with a lower affinity for thymidine. The presence of a second permease was confirmed by measuring influx of [<sup>3</sup>H]deoxycytidine, [<sup>3</sup>H]adenosine and [<sup>3</sup>H]guanosine. The transport of each of these nucleosides was inhibited strongly by all naturally-occurring nucleosides but only poorly or not at all by nucleobases. These findings were consistent with a transporter that recognized structural features on the furanosyl moiety of ribonucleosides and 2'-deoxyribonucleosides. Both 2'- and 5'-deoxyadenosine were potent inhibitors of influx (>95% inhibition at 2 mM), 3'-deoxyadenosine was less effective (≈70% inhibition), whereas 2',3'-dideoxycytidine and cytosine arabinoside were virtually inactive (0-20% inhibition). These observations suggest that the 2' and 5' hydroxyl groups of the furanosyl ring are not necessary for recognition of nucleosides by the second transporter, whilst the 3' hydroxyl appears to be important. Michaelis-Menten constants ( $K_m$ ) were calculated for the influx at 0°C of deoxycytidine ( $220 \pm 116 \mu\text{M}$ ) and adenosine ( $45 \pm 24 \mu\text{M}$ ), with respective  $V_{\text{max}}$  values of  $13 \pm 4$  and  $11 \pm 2 \text{ pmol} \cdot \text{min}^{-1} \cdot (10^6 \text{ cells})^{-1}$ . Thymidine exhibited a  $K_i$  of  $205 \pm 90 \mu\text{M}$  against [<sup>3</sup>H]deoxycytidine influx.

The second transporter, which has a broad specificity, would appear to be sufficient to mediate the uptake of all nucleosides and 2'-deoxynucleosides required by the parasite. The thymine/uracil-specific thymidine transporter, which has high affinity for uridine and thymidine, may be important when these compounds are scarce. No evidence could be found for the existence of any other nucleoside transporter under the assay conditions used. In work extending these studies, the presence of a distinct base transporter which has specificity for purines and pyrimidines was demonstrated.

In order to clone the genes encoding the *Giardia* nucleoside transporters, a nucleoside transport deficient strain of *E. coli* (SΦ1660) was obtained. This enabled a strategy of complementation to be employed, by selection for transformants able to utilize pyrimidine nucleosides (uridine or cytidine) as the source of carbon. Two genes, designated *bc2* and *10ca*, were cloned from a *Giardia* genomic DNA library by this technique. Sequence analysis of the cloned fragments that had been isolated by complementation revealed an open reading

frame (ORF) of 1,134 bp and 984 bp, encoding a putative 43-kDa polypeptide and a 29-kDa polypeptide for *bc2* and *10ca* respectively. Transcripts of *bc2* and *10ca* were detected as a 1.6-kb and 1.1 kb RNA species respectively on Northern blots using total RNA extracted from *Giardia* trophozoites.

The likely secondary structure of the *BC2* polypeptide determined from a consensus of the structures predicted by eight algorithms is consistent with a known class of transporter, i.e. the porins, which have been characterized from mitochondria and bacterial outer membranes. The structural model contains 12 hydrophobic or amphiphilic  $\beta$ -strands, each sufficiently long to span a lipid bilayer. These are connected by hydrophilic loops, which contain the 7 predicted  $\alpha$ -helices, all comprised predominantly of hydrophilic residues. Southern hybridizations confirmed that *bc2* was of giardial and not bacterial origin, and that it was present in trophozoites as a single gene. This class of transporter may provide the structural stability required for proteins that are exposed to the luminal environment of the gut.

In contrast, the *IOCA* polypeptide shares significant sequence homology to DNA-binding proteins. This indicates that although complementation in *E. coli* strain S $\Phi$ 1660 has enabled the cloning of a putative transporter gene (*bc2*), other genes may be able to complement the bacterial mutation. It will therefore be important to further analyse the genes (and their encoded polypeptide products) isolated by this technique, to confirm their role as transporters in *Giardia*.

In summary, this study has led to the discovery and kinetic characterization of two distinct nucleoside transporters in *Giardia intestinalis* and to the isolation and characterization of a gene encoding a porin-like transporter. The data provide an insight into the pathways involved in nucleoside and nucleobase transport in this organism, and of the features of the substrate molecules that are necessary for recognition in this parasite. Further study of the putative nucleoside transporter gene and its polypeptide product(s) will be needed to confirm its role as a nucleoside transporter in *Giardia* and may provide an insight into the structure and function of the transporters. The data have important implications for future design of chemotherapeutic drugs that may be used in the treatment of giardiasis.

# Contents

<b>Chapter 1</b>	<b>Introduction</b>	<b>1</b>
1.1	Historical background	1
1.2	Nomenclature	2
1.3	Evolutionary history	2
1.4	Morphology	3
1.4.1	The trophozoite	3
1.4.2	The cyst	4
1.5	Life cycle	5
1.6	Attachment	5
1.7	Determination of <i>Giardia</i> species	6
1.8	Prevalence and epidemiology	9
1.9	Biochemistry of trophozoites	10
1.9.1	Growth in culture	10
1.9.2	Energy metabolism in <i>Giardia</i>	10
1.9.3	Nucleic acid metabolism	12
1.9.3.1	Purine metabolism of <i>G. intestinalis</i>	13
1.9.3.2	Pyrimidine metabolism	17
1.9.3.3	Deoxynucleotide biosynthesis	21
1.10	Nucleoside transport	22
1.11	Treatment of giardiasis by chemotherapy	24
1.11.1	Action of standard drugs used to combat giardiasis	25
1.11.2	Development of drug resistant strains of <i>G. intestinalis</i>	26
1.11.3	Design of novel drugs for use in treatment	27
1.12	Infection, immunity and vaccine development	28
1.13	Project definition	30
<b>Chapter 2</b>	<b>Materials and Methods</b>	<b>32</b>
2.1	Transport Assays	32
2.1.1	Chemicals and reagents	32

2.1.2	<i>Giardia</i> cultures	32
2.1.3	Harvesting trophozoites	33
2.1.4	[ <sup>3</sup> H]Nucleoside stock solutions	33
2.1.5	Manufacture of <sup>14</sup> C-acetylated bovine serum albumin	34
2.1.6	Sampling assay	34
2.1.7	Liquid scintillation counting and data analysis	35
2.1.7.1	Calculation of <sup>3</sup> H and <sup>14</sup> C content	35
2.1.7.2	Calculation of extracellular fluid contamination	35
2.1.8	Analysis of transport kinetics	36
2.1.8.1	Calculation of IC <sub>50</sub> values	36
2.1.8.2	Calculation of K <sub>m</sub> and K <sub>i</sub> values	36
2.2	Molecular biology and recombinant DNA techniques	36
2.2.1.	Chemicals and reagents	36
2.2.2	Enzymes	37
2.2.3	Growth media	37
2.2.4	Bacterial strains and plasmids	38
2.2.4.1	Maintenance of bacterial strains	38
2.2.5	Preparation of competent bacteria	38
2.2.6	Transformation of <i>Escherichia coli</i> K-12 strains	39
2.2.7	DNA extraction procedures	39
2.2.7.1	Plasmid DNA isolation	39
2.2.7.2	Preparation of genomic DNA	41
2.2.8	Analysis and manipulation of DNA	41
2.2.8.1	Nucleic acid quantitation	41
2.2.8.2	Restriction endonuclease digestion of DNA	42
2.2.8.3	Analytical and preparative separation of restriction fragments	42
2.2.8.4	Calculation of restriction fragment size	42
2.2.8.5	Dephosphorylation of DNA	42
2.2.8.6	End-filling with Klenow fragment	43

2.2.8.7	Ligation of DNA	43
2.2.8.8	In-gel ligation of DNA fragments	43
2.2.8.9	Preparation of DNA and RNA probes	44
2.2.8.10	Southern transfer and hybridization	45
2.2.8.11	Colony Blots	47
2.2.9	Construction of genomic DNA libraries	47
2.2.9.1	Selection of genes encoding putative nucleoside transporters	47
2.2.9.2	Detection of genomic fragments containing full length genes	48
2.2.10	Sequencing using dye labelled primers	48
2.2.11	DNA sequencing gels	49
2.2.12	Analysis of DNA sequences	49
2.2.13	Analysis and manipulation of RNA	50
2.2.13.1	Preparation of RNA	50
2.2.13.2	Northern Transfer	51
<b>Chapter 3</b>	<b>Development of an assay for measuring transport kinetics in</b>	
	<b><i>Giardia</i> trophozoites</b>	52
3.1	Introduction	52
3.2	Materials and methods	54
3.2.1	Composition of the dense oil layer	54
3.2.2	Density of oil phase components	54
3.2.3	Recovery of cells	54
3.2.4	Preparation of <sup>125</sup> I-labelled bovine serum albumin (BSA)	55
3.2.5	Extracellular drag volume	55
3.2.5.1	Use of <sup>125</sup> I-labelled BSA to determine extracellular volume	55
3.2.5.2	Use of <sup>14</sup> C-labelled BSA to determine extracellular volume	56
3.2.6	Assessment of non-specific binding of <sup>14</sup> C-labelled BSA to trophozoites	57
3.2.7	Quench monitoring	57
3.2.7.1	Quenching characteristics of solutions	57
3.2.7.2	Counting efficiency versus 'H' number	57

3.2.7.3	Determination of the energies of $\beta$ -particle emissions	58
3.2.7.4	Spreadsheet calculations	58
3.2.8	Osmolarity of assay medium	61
3.3	Results	61
3.3.1	Density of the oil phase	61
3.3.2	Sedimentation of cells through oil	64
3.3.3	Radioactive labelling of bovine serum albumin	65
3.3.3.1	Iodination of BSA	65
3.3.3.2	$^{14}\text{C}$ -acetylation of BSA	65
3.3.4	Extracellular fluid volume	68
3.3.5	Quenching of radioactive emissions	68
3.3.5.1	Dibutylphthalate reduces counting efficiency	68
3.3.5.2	Quench correction	72
3.3.6	Osmolarity of assay medium	75
3.4	Discussion	75
3.4.1	Assay design	75
3.4.2	Extracellular drag volume	76
3.4.3	Determining the amount of [ $^3\text{H}$ ]substrate transported in cells	78
3.4.3.1	Measurement of the $^3\text{H}$ and $^{14}\text{C}$ content of samples	78
3.4.3.2	Measuring quench	79
3.4.3.3	Spreadsheet software design	79
3.4.4	Assay medium	80
3.5	Summary	81
<b>Chapter 4</b>	<b>Detection and characterization of thymidine transport</b>	
83		
4.1	Introduction	83
4.2	Materials and Methods	85
4.2.1	Temperature dependence of efflux from cells pre-loaded with [ $^3\text{H}$ ]thymidine	85



4.2.2	Measurement of efflux from pre-loaded cells at 0°C	85
4.2.3	Intracellular metabolism of transported [ <sup>3</sup> H]thymidine	86
4.2.3.1	Phosphorylation	86
4.2.3.2	Hydrolysis	86
4.3	Results	87
4.3.1	Efflux of preloaded [ <sup>3</sup> H]thymidine	87
4.3.2	The effect on transport capability of incubating trophozoites in assay medium	89
4.3.3	Uptake of thymidine at 0°C	92
4.3.3.1	Choice of uptake period for kinetic studies	92
4.3.3.2	Saturation of uptake	95
4.3.3.3	Effect of glucose on thymidine uptake	95
4.3.4	Metabolism of intracellular thymidine	98
4.3.5	Specificity of the thymidine transporter	100
4.3.6	Kinetic analyses	100
4.3.6.1	IC <sub>50</sub> curves	102
4.4	Discussion	109
4.4.1	Measurement of thymidine transport	109
4.4.1.1	Efflux of thymidine	109
4.4.1.2	Transport capability after prolonged incubation of cells in medium	109
4.4.1.3	Influx and efflux of thymidine	110
4.4.1.4	Uptake, metabolism and influx of thymidine	111
4.4.2	Specificity of thymidine influx	111
4.5	Summary	119
<b>Chapter 5</b>	<b>The detection and characterization of a second nucleoside transporter having specificity for ribonucleosides and 2'-deoxyribonucleosides</b>	<b>120</b>
5.1	Introduction	120
5.2	Materials and Methods	121

5.2.1	Intracellular metabolism of transported [ <sup>3</sup> H]2'-deoxycytidine and [ <sup>3</sup> H]adenosine	121
5.2.2	Inhibition of thymidine transport by uracil and deoxycytidine	121
5.3	Results	122
5.3.1	Measurement of 2'-deoxycytidine influx	122
5.3.2	Metabolism of deoxycytidine	122
5.3.3	Specificity of the deoxycytidine transporter	126
5.3.3.1	Inhibition of deoxycytidine influx by thymidine	127
5.3.4	Transport of other nucleosides	130
5.3.4.1	Specificity of adenosine and guanosine uptake	130
5.3.4.2	Relative involvement of the broad-specificity (deoxycytidine) transporter and the thymine/uracil-specific transporter in thymidine transport	132
5.4	Discussion	134
5.4.1	Deoxycytidine uptake is mediated by a specific permease	135
5.4.2	Calculation of intracellular aqueous volume	136
5.4.3	Intracellular metabolism of deoxycytidine	136
5.4.4	Inhibition of nucleoside influx	137
5.4.4.1	Specificity of the deoxynucleoside transporter	137
5.4.4.2	Transport of other nucleosides	137
5.4.4.3	Thymidine transport via the broad-specificity nucleoside transporter	138
5.4.5	Recognition of substrate	139
5.5	Summary	142
<b>Chapter 6</b>	<b>Cloning of genes encoding putative nucleoside transporters</b>	<b>148</b>
6.1	Introduction	148
6.2	Materials and Methods	151
6.3	Results	151
6.3.1	Construction of a <i>Sau3A</i> genomic library	151
6.3.2	Isolation of clones	154

6.3.3	Restriction analysis of p10CA and p10UD	157
6.3.4	Nucleotide sequence determination of the p10CA and p10UD genomic inserts	157
6.3.4.1	Detection of open reading frames	160
6.3.5	Southern analysis	170
6.3.6	Northern analysis	175
6.3.7	Efficiency of re-transformation and complementation for nucleoside uptake with p10CA and pBC2	182
6.3.7.1	Transformation and complementation with pBC2 constructs	182
6.3.7.2	Transformation and complementation with p10CA	183
6.4	Discussion	189
6.4.1	Detection of the <i>10ca</i> and <i>bc2</i> sequences in trophozoite DNA	190
6.4.2	Analysis of the <i>bc2</i> and <i>10ca</i> nucleotide sequences	191
6.4.3	Potential recognition sites for polyadenylation of primary RNA transcripts	192
6.5	Summary	194
<b>Chapter 7</b>	<b>Structural prediction of the <i>bc2</i>-encoded polypeptide by analysis of primary structure</b>	195
7.1	Introduction	195
7.2	Methods	197
7.2.1	Prediction of polypeptide secondary structure	197
7.3	Results	198
7.3.1	Searches of DNA and protein sequence databases for sequences homologous to <i>bc2</i> or its deduced polypeptide products	198
7.3.2	Secondary structure prediction of the <i>BC2</i> polypeptide sequence	199
7.4	Discussion	207
7.4.1	<i>BC2</i> forms a porin-like transport protein	207

7.4.2	Reliability of the secondary structure predicted for <i>BC2</i>	208
7.5.	Summary	212
<b>Chapter 8</b>	<b>Predictions of the structure and function of the polypeptide encoded by the <i>10ca</i> gene of <i>G. intestinalis</i></b>	214
8.1	Introduction	214
8.2	Methods	215
8.3	Results	216
8.3.1	Searches for sequences homologous to <i>10ca</i> and its polypeptide product(s)	216
8.3.2	Analysis of hydrophobic and charged amino acids in the deduced amino acid sequence of <i>10CA</i>	219
8.3.3	Secondary structure prediction for <i>10CA</i>	222
8.4	Discussion	225
8.5	Summary	230
<b>Chapter 9</b>	<b>General Discussion</b>	231
9.1	Detection and characterization of nucleoside transport	231
9.2	Isolation of genes encoding potential nucleoside transporters	236
<b>Chapter 10</b>	<b>Bibliography</b>	239
<b>Appendix</b>	<b>Published work arising from this study</b>	266