

# Contents

<b>Contents</b>	<b>vi</b>
<b>List of Figures</b>	<b>xi</b>
<b>List of Tables</b>	<b>xiii</b>
<b>1 Background and Perspective</b>	<b>1</b>
<b>References</b>	<b>5</b>
<b>2 Puromycin Oligonucleotides Reveal Steric Restrictions For Ribosome Entry and Multiple Modes of Translation Inhibition</b>	<b>6</b>
2.1 Introduction . . . . .	7
2.2 Results and Discussion . . . . .	10
2.2.1 Puromycin versus 30P . . . . .	10
2.2.2 Potency of Puromycin and Puromycin Oligonucleotides . . . . .	10
2.2.3 Elongation Factor Dependence . . . . .	18
2.2.4 Product Distribution . . . . .	19
2.2.5 Puromycin Entry At Ribosome Pause Sites . . . . .	24
2.2.6 Preincubation and Carboxypeptidase Analysis . . . . .	24
2.2.7 Role of the Free Amine . . . . .	27
2.2.8 Revised Model for Puromycin Action: Multiple Modes of Inhibition . . . . .	28

2.2.9	Size, Rather than Affinity, Determines Potency of Puromycin Conjugates . . . . .	31
2.3	Conclusions . . . . .	32
2.4	Experimental Procedures . . . . .	33
2.4.1	Reagents . . . . .	33
2.4.2	Oligonucleotides . . . . .	33
2.4.3	IC <sub>50</sub> Determination . . . . .	34
2.4.4	Lysate Enrichment Assay . . . . .	35
2.4.5	TLC Assay for Detection of Met-puromycin . . . . .	36
2.4.6	Monomeric Avidin- and Streptavidin-capture of Biotinylated Puromycin Conjugates . . . . .	36
2.4.7	Preincubation Assay with Biotin-2P . . . . .	37
2.4.8	Carboxypeptidase Y Assay . . . . .	38
	References . . . . .	39
3	A General Approach to Detect Protein Expression <i>In Vivo</i> Using Fluorescent Puromycin Conjugates . . . . .	44
3.1	Introduction . . . . .	45
3.2	Results . . . . .	47
3.2.1	Design of Puromycin Conjugates . . . . .	47
3.2.2	Analysis of Puromycin-conjugate Activity <i>In Vitro</i> . . . . .	49
3.2.3	Analysis of Puromycin-conjugate Activity <i>In Vivo</i> . . . . .	53
3.2.4	Mechanism of Puromycin Conjugate Activity <i>In Vivo</i> . . . . .	57
3.2.5	Western Blot Analysis of Puromycin Conjugate Labeling in Live Cells . . . . .	60
3.2.6	Imaging of NIH 3T3 Cells Treated with Fluorescent Puromycin Conjugates . . . . .	62
3.3	Discussion . . . . .	62

3.4	Significance . . . . .	66
3.5	Experimental Procedures . . . . .	67
3.5.1	Reagents and Materials . . . . .	67
3.5.2	Puromycin Conjugates . . . . .	67
3.5.3	<i>In Vitro</i> Potency Determination for Puromycin Conjugates . .	68
3.5.4	Neutravidin Capture of <i>In Vitro</i> Translated Protein-puromycin-conjugate Products . . . . .	68
3.5.5	Preparation of MIG <sub>PAC</sub> Infected 16610D9 Cells . . . . .	69
3.5.6	Enrichment of GFP(+) 16610D9 Cells using Puromycin and Puromycin Conjugates . . . . .	69
3.5.7	Detection of Protein Synthesis Events <i>In vivo</i> using Flow Cytometry . . . . .	70
3.5.8	Western Blot Analysis of 16610D9 Cells Treated with Puromycin Conjugates . . . . .	70
3.5.9	Confocal Microscopy of NIH 3T3 cells Treated with Fluorescent Puromycin Conjugates . . . . .	71
	<b>References</b>	<b>72</b>
<b>4</b>	<b>The Puromycin Route to Assess Stereo- and Regiochemical Constraints on Peptide Bond Formation in Eukaryotic Ribosomes</b>	<b>76</b>
4.1	Introduction . . . . .	76
4.2	Results and Discussion . . . . .	77
4.3	Conclusions . . . . .	81
4.4	Experimental Procedures . . . . .	85
4.4.1	General Information . . . . .	85
4.4.2	General Procedure for Preparation of Puromycin Analogs . . .	86
4.4.3	IC <sub>50</sub> Determination . . . . .	89
4.4.4	Carboxypeptidase Assay . . . . .	90

<b>References</b>	<b>91</b>
<b>5 The Puromycin Route to Assess Amine Substitution Constraints on Peptide Bond Formation</b>	<b>96</b>
5.1 Background . . . . .	97
5.2 Results and Discussion . . . . .	99
5.3 Conclusions . . . . .	107
5.4 Experimental Procedures . . . . .	108
5.4.1 General Information . . . . .	108
5.4.2 General Procedure for Preparation of Puromycin Analogs . . .	109
5.4.3 IC <sub>50</sub> Determination . . . . .	109
<b>References</b>	<b>111</b>
<b>6 Stereoselectivity of Translation in Live Cells</b>	<b>114</b>
6.1 Background . . . . .	115
6.2 Results and Discussion . . . . .	116
6.3 Conclusions . . . . .	125
6.4 Experimental Procedures . . . . .	125
6.4.1 General information . . . . .	125
6.4.2 Procedure for Preparation of Fmoc-D-biotin . . . . .	126
6.4.3 General Procedure for Preparation of Puromycin Analogs . . .	126
6.4.4 <i>In vivo</i> Analysis of Puromycin Analogs . . . . .	127
6.4.5 Preparation of MIG <sub>PAC</sub> Infected 16610D9 Cells . . . . .	127
6.4.6 Enrichment of GFP(+) 16610D9 Cells using Puromycin and Puromycin Conjugates . . . . .	128
6.4.7 IC <sub>50</sub> Determination . . . . .	128
<b>References</b>	<b>130</b>

<b>7 Puromycin Analogs and the Homochirality of Life</b>	<b>133</b>
7.1 Introduction . . . . .	133
7.2 Results and Discussion . . . . .	135
7.3 Conclusions . . . . .	142
7.4 Experimental Procedures . . . . .	143
7.4.1 General Information . . . . .	143
7.4.2 HPLC Analysis . . . . .	143
7.4.3 Solubility ( $K_{sp}$ ) Determination . . . . .	144
<b>References</b>	<b>145</b>

## List of Figures

1.1	Central Dogma of Biology . . . . .	1
1.2	Analogy to Depict the Macromolecular Concentration of Cells . . . . .	2
2.1	Puromycin versus Aminoacyl-tRNA . . . . .	8
2.2	IC <sub>50</sub> Determinations for Puromycin and 30P . . . . .	12
2.3	3'-Puromycin tRNA Mimics . . . . .	16
2.4	Effect of Ribosome-depleted Lysate . . . . .	17
2.5	Translation Inhibition and Product Formation for biotin-2P . . . . .	21
2.6	Analysis and Quantitation of Biotin-2P Translation Products . . . . .	23
2.7	Analysis of Puromycin-conjugated Globin . . . . .	26
2.8	Distribution of Biotin-2P Effects on Translation . . . . .	29
2.9	Revised Model for the Action of Puromycin-oligonucleotides . . . . .	30
3.1	Mechanism of Puromycin and Fluorescent-dC-puromycin . . . . .	46
3.2	Structure of Puromycin Conjugates . . . . .	48
3.3	<i>In vitro</i> IC <sub>50</sub> Determination for Puromycin Conjugates . . . . .	50
3.4	Protein Labeling with FB2P . . . . .	51
3.5	Analysis of Fluorescent-puromycin Conjugate Activity in D9 Cells .	54
3.6	Fluorescence Shift Analysis for Conjugates versus Negative Controls .	56
3.7	Mechanism of Action of Puromycin in D9 Cells . . . . .	58
3.8	Mechanism of Action of Biotinylated-puromycin Conjugates in D9 Cells	59
3.9	Western Blot Analysis of D9 Cells Treated with BF2P . . . . .	61

3.10 Confocal Microscopy of NIH 3T3 Cells Treated with FB2P and BF2P	63
3.11 Confocal Microscopy of NIH 3T3 Cells Treated with Cy <sub>3</sub> 2P	64
4.1 Structures of Puromycin Analogs	78
4.2 IC <sub>50</sub> determination for L- and D-puromycin	79
4.3 Carboxypeptidase Y Analysis	82
4.4 SDS-PAGE Analysis of Puromycin-labeled Protein	83
4.5 Model of D-puromycin in the 50S subunit	84
5.1 N-substituted amino Acids Inserted into Peptides and Protein	98
5.2 N-substituted Puromycin Analogs	100
5.3 IC <sub>50</sub> determination for L-Pro-PANS	101
5.4 Mechanism of Peptide Bond Formation in the Ribosome	104
5.5 The Rate of Aminolysis Correlated to IC <sub>50</sub> Value	105
5.6 Hydrogen Bonding Potential for Amines	107
6.1 Structures of Puromycin Analogs Used in Live Cells	116
6.2 Toxicity Analysis	117
6.3 HPLC Analysis of Puromycin Analogs	118
6.4 Constructs Used to Examine Puromycin Purity	119
6.5 GFP(+) Cell Enrichment Experiment	120
6.6 Effect of Amino Acid Stereochemistry on Cell Viability	121
6.7 <i>In vitro</i> Potency of Biocytin-PANS Derivatives	122
6.8 Analysis of Translation Selectivity	124
7.1 The Fragment Reaction	136
7.2 Riboadenosine Compounds with Amino Acid Moieties	138
7.3 A Primordial RNA-only Ribosome and the Fragment Reaction	139
7.4 HPLC Analysis of L- and D-puromycin Analogs	140

## List of Tables

2.1	IC <sub>50</sub> Values for Puromycin and Puromycin Oligonucleotides . . . . .	13
3.1	IC <sub>50</sub> Values for Fluorescent Puromycin Conjugates . . . . .	52
4.1	IC <sub>50</sub> Values for Puromycin Analogs . . . . .	80
5.1	IC <sub>50</sub> Values for <i>N</i> -substituted Puromycin Analogs . . . . .	102
5.2	Amine Partial Negative Charge for <i>N</i> -substituted Amino Acids . . . .	106
7.1	Solubility Determination for Puromycin Analogs . . . . .	141