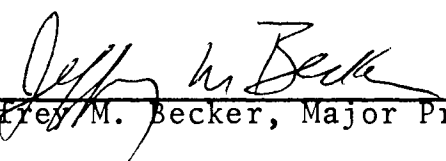


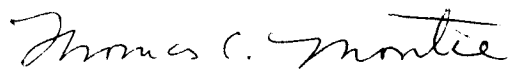
To the Graduate Council:

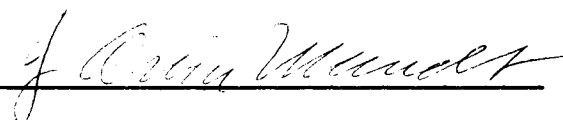
I am submitting herewith a thesis written by Ali Gulumoglu entitled "A Study of Drug-Peptide Conjugates Against the Opportunistic Yeast Candida albicans." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.



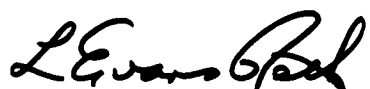
Jeffrey M. Becker, Major Professor

We have read this thesis
and recommend its acceptance:





Accepted for the Council:



Vice Chancellor
Graduate Studies and Research

Thesis
80
.G848
cop. 2

A STUDY OF DRUG-PEPTIDE CONJUGATES AGAINST
THE OPPORTUNISTIC YEAST CANDIDA ALBICANS

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Ali Gulumoglu
March 1980

1416222

DEDICATION

E'ûzübillâhi mineşşeytanirracim bismillâhirrahmanirrahim.

Eşhedü enlâ ilahe illâillâh ve eşhedü enne Muhammeden aabdühü ve resulüh.

Allahın birliğine, Hazreti Muhammed Allahın kulu ve elçisi olduğuna inandım.

The work herein is dedicated to my father for whom the sacrifices he has endured are finally bearing fruit. Also, for whom, the dreams he envisioned when we emigrated to the United States are finally coming true, proving once again that hard work is rewarded.

To my mother whose selfless attitude and endless encouragement have made even the most trying moments bearable. She has supported me in all I ever wanted to do.

To my brother from whom I have always received love, companionship and encouragement. I hope I can repay him by being an example that will always be worthy of his respect and emulation.

And to my grandparents Cafer, Hatice, Ramazan and Fatma, because of whom I have my family to thank. Allah rahmet eylesin.

I love you all.

ACKNOWLEDGMENTS

I would like to express my deep gratitude to my major professor, Dr. Jeffrey M. Becker. Dr. Becker's endless search for the truth and driving force for excellence has taught me more than I could relate. He has instilled in me a deep appreciation for research that I will carry with me in future endeavors. His guidance and encouragement are greatly appreciated.

I would like to thank members of my committee, Dr. Thomas C. Montie and Dr. J. Orvin Mundt, for their help and constructive criticism of my work.

I extend gratitude to friends and colleagues who have helped both academically as well as socially. They have made my tenure as a graduate student educationally challenging and enjoyable.

Thanks.

ABSTRACT

A series of orotyl and fluoro-orotyl-peptides were tested as possible drugs against the opportunistic yeast Candida albicans. None of the peptides were toxic; however, two seemed to be transported into the cells, based on competition of transport assays with radioactive peptide trimethionine. The most promising of the drug-peptides, fluoro-orotyl-leucyl-leucine, was a competitive inhibitor of trimethionine uptake with a K_I of 5×10^{-4} M. By this criterion fluoro-orotyl-leucyl-leucine seemed to be getting into the cells through the oligopeptide transport system. It was shown that even though fluoro-orotyl-leucyl-leucine was transported into cells, it was not toxic because the drug, fluoro-orotic acid, was not released from the peptide. Fluoro-orotic acid, by itself, was inhibitory to the pyrimidine biosynthetic enzyme activities of the yeast cell. It was concluded that fluoro-orotic acid is carried into cells via the oligopeptide transport system and if fluoro-orotic acid were released from the peptide, it should be inhibitory to the enzymes of the yeast. This was the first instance that a fluorinated pyrimidine was shown to be carried into cells by way of the oligopeptide permease.

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION.	1
II. MATERIALS AND METHODS	4
Organism.	4
Chemicals	4
Minimum Inhibitory Concentration Assays . . .	5
Growth of cells	5
Standardization of cell density	6
Blue broth.	6
Procedure	7
Transport Studies	8
Growth of cells	8
Assays.	9
Enzyme Assays	11
Growth of cells	11
Extraction of cells	12
Procedure (references 31, 32)	12
III. RESULTS	14
Minimum Inhibitory Concentration Assays . . .	14
Transport Studies	14
Trimethionine	14
Orotic acid	22
Fluoroorotic acid	26
Enzyme Assays	29
IV. DISCUSSION.	35
REFERENCES.	46
VITA.	50

LIST OF FIGURES

FIGURE	PAGE
1. Trimethionine Transport Assays--Orotyl-Peptides As Competitors	17
2. Trimethionine Transport Assays--Orotyl-Peptides Derivatized at the Carboxy-Terminus As Competitors.	19
3. Trimethionine Transport Assays--Fluoroorotyl- Peptide Conjugates As Competitors.	20
4. Kinetics of Trimethionine Uptake in the Presence (+FOL ₂) and Absence of FOL ₂ (Control).	23
5. Orotic Acid Transport Assays--The Competitors Were Orotyl-Peptides and Fluoroorotyl-Peptides Which Were Derivatized at the Carboxy- Terminus	24
6. Orotic Acid Transport Assays--The Competitors Shown Were the Fluoroorotyl-Peptides and Fluoroorotic Acid.	25
7. Fluoroorotic Acid Transport Assays--Cells Were Grown in Vogel's-Isoleucine.	28
8. Fluoroorotic Acid Transport Assays--Cells Grown in Yeast-N-Base with Isoleucine as the Nitrogen Source Were Assayed for 5-FO Uptake .	30
9. Fluoroorotic Acid Transport Assays--Cells Grown in Vogel's-NH ₄ NO ₃ Were Assayed for 5-FO Uptake at a Final Concentration of 1×10^{-5} M.	31
10. Time Course of Orotidine-5-monophosphoric Acid Production	32
11. Time Course of Uridine-5-monophosphate Production	34

CHAPTER I

INTRODUCTION

Species of Candida appear as common infectious agents in man. They are also one of the most prevalent micro-organisms responsible for infection in patients weakened by cancer, immunosuppressive therapy, radiation treatment or other debilitating conditions. According to one survey (22), 58 percent of all fungal infections in compromised patients was due to Candida. Progress in chemotherapy of deep-seated fungal infections, in general, and in candidosis, in particular, have lagged far behind the advances in the development of antibacterial antibiotics during the past decades. The most widely used anti-mycotic drugs have shortcomings. Amphotericin B is toxic to the kidneys of the host (29) and fluorocytosine (5-FC) results in the problem that resistant strains arise readily (5).

The toxicity of fluorinated pyrimidines such as 5-FC and fluorouracil (5-FU) to mammalian (10, 14, 23, 24), bacterial (11, 21, 23), and fungal cells (5, 6, 21, 23) is well known. Fluoroorotic acid (5-FO) is also toxic to mammalian and bacterial cells (7, 10, 14, 21, 23, 24); however, there has been only one report on the toxicity of 5-FO to fungal cells (21). It has been reported that 5-FO does not enter cells readily (7). This suggested that

capacity to penetrate may play a role in the poor toxicity of 5-F0 to fungal cells. It has also been reported that some compounds enter cells via the transport systems of other substances. Ames and co-workers (1) have shown that a substance that is not transported into cells entered the cells through the oligopeptide permease when presented as part of an oligopeptide. They have termed this phenomenon "illicit transport."

In the present work, a series of fluoroorotyl-peptide conjugates were used to explore this approach in fulfilling the great need for an anti-candidal agent. It was hoped these peptides would overcome the suggested lack of penetration of 5-F0 by carrying 5-F0 into the yeast through the peptide transport system.

The peptides were specifically designed for activity against Candida, using the known information on the oligopeptide transport system of Candida (27, 28). Besides being lethal to the pathogen, the drug-peptides should be relatively innocuous to the host. This has also been taken into account in the design of the peptides, based on the studies on the transport of peptides in mammals (12, 16). That is, the peptides should only be transported into the yeast and not into the host.

It was hoped that by using specifically designed drug-peptides the penetration capability of 5-F0 could be increased; in addition, the targeting of 5-F0 could be

achieved. This approach represented a unique and novel attempt to solving the problem of anti-candidal drugs.

In this study it has been shown that a peptide can carry a fluorinated pyrimidine into cells. However, the drug-peptides were found not to be toxic because 5-FU was not released from the peptide by the enzymatic activities of the yeast cell.

CHAPTER II

MATERIALS AND METHODS

Organism

Candida albicans WD 18-4, a methionine, lysine double auxotroph was used in all experiments. The organism was supplied by Dr. Alvin Sarachek of Wichita State University. The yeast was maintained on YEPD agar slants: 1 percent yeast extract (Difco), 2 percent Bacto-peptone (Difco), 2 percent dextrose and 2 percent agar (Difco). It was transferred each month to a fresh slant, incubated 24 hours at 37°C and stored at 4°C.

Chemicals

Refer to reference 3 for the synthesis of radioactive trimethionine (Met-Met-(¹⁴C)Met or Met₃). The Amersham Corp. of Arlington Heights, Ill. was the supplier of (6-¹⁴C) orotic acid monohydrate (OA), 59 Ci/m. Fluoro-orotic-2-¹⁴C-acid, 7.9 Ci/m, was purchased from ICN Pharmaceuticals, Inc., Irvine, Ca. 92715. Phosphoribosyl-1-pyrophosphate (PRPP), lithium chloride, orotidine-5-monophosphoric acid (OMP), 6-azauridine-5-monophosphoric acid (azaUMP) and dithiothreitol (DTT) were from Sigma. Uridine-5-monophosphate (UMP) was purchased from Calbiochem, La Jolla, Ca. Orotic acid and 5-FO were bought from P-L Biochemicals, Inc., Milwaukee, Wisconsin 53205.

Polygram Cel 300 Polyethyleneimine cellulose (PEI cellulose) plastic backed TLC plates (20 x 20 cm) were purchased from Brinkmann Instruments, Inc., Westbury, New York 11590. Fluorocytosine was a gift from Dr. Fred Naider, College of Staten Island, CUNY, Staten Island, New York. The following drug-peptides were synthesized by Drs. Ti and Naider at the College of Staten Island: orotyl-leucyl-leucine (OL₂), orotyl-glycyl-glycine (OG₂), orotyl-glycyl-glycine benzyl ester (OG₂OBzl), orotyl-glycyl-glycine tertiary butyl ester (OG₂OTB), orotyl-methionyl-methionine methyl ester (OM₂OMe), fluoroorotyl-leucyl-leucine (FOL₂), fluoroorotyl-leucine (FOL), N-ε-fluoroorotyl-lysyl-leucine (FOLL), fluoroorotyl-leucyl-leucine tertiary butyl ester (FOL₂OTB), fluoroorotyl-leucine tertiary butyl ester (FOLOTB), fluorotyl-glycyl-glycine benzyl ester (FOG₂OBzl). The manuscript describing the methods of syntheses is in preparation. Leucyl-leucyl-leucine (Leu₃) was from Bachem, Inc., Torrance, Ca. 90505. The amino acid residues of all peptides were all of the L configuration. Dimethyl formamide (DMF) was purchased from Fisher Scientific. All other chemicals were reagent grade or the purest commercially available.

Minimum Inhibitory Concentration Assays

A. Growth of cells. Medium used was Yeast-N-Base without amino acids (Difco) (67 mg/ml) containing 30 percent

dextrose, 300 µg/ml methionine and 300 µg/ml lysine. This was filter-sterilized and kept 4°C. For growth 0.5 ml of this was placed into 4.5 ml sterile dH₂O contained in a Klett tube, loop inoculated from a slant and grown at 37°C to at least 100 Klett Units. Growth was followed by the Klett-Summerson Photoelectric Colorimeter as an increase in turbidity at 400-420 nm (blue filter). Aeration was accomplished by vigorous shaking on a rotating shaker (200 rpm).

B. Standardization of cell density. For each assay (i.e., each microtiter plate), cell density had to be standardized in order to make valid comparisons among assays. A portion of the cells grown to at least 100 Klett Units were resuspended in blue broth (see C. below) according to the formula:

$$\frac{\text{total volume of cells, in blue broth, needed for one assay}}{\text{final Klett Unit of the cells}} =$$

the volume of cells to be resuspended in that volume of blue broth.

C. Blue broth. Yeast-N-Base without amino acids (13.4 mg/ml), 6 percent dextrose, 60 µg/ml methionine, 60 µg/ml lysine was prepared, adjusted to pH 7.2 and 8 µg/ml bromphenol blue was added. The medium was filter-sterilized and kept at 4°C. In some assays bromthymol blue was used as the pH indicator and the solution was adjusted to 7.0.

D. Procedure. Tissue Culture Microtiter (flat bottom) Plates (Costar, Cambridge, Mass.) were used in the assays. The plates have eight rows (A-H) and 12 wells per row. Sterile dH₂O (50 μ l) was placed in wells 1-11 of each row and 50 μ l of the drug solution was placed in the first well of each row. Serial 1:2 dilutions were then made across a row with a microdiluter. Duplicate rows were run for each drug to be tested. The drugs were standardized so that a drug-peptide contained in a well had the same molar concentration as that of the base compound to which toxicity was being compared. Base compound was either 5-FC or 5-FO. Blue broth (50 μ l), containing cells was then added to wells 1-11 of each row. The plates were incubated in a humid chamber at 37°C. Plates were read at 24 and 48 hours. The last wells of a pair of rows having no growth were reported as the minimum inhibitory concentration (M. I. C.).

In some assays where the solubility of the drug-peptide was in question, this modification of the above method was used. Wells 2-11 received 50 μ l of sterile dH₂O. Drug-peptide solution (100 μ l) was placed in well 1 of each row. Serial 1:2 dilutions were then made and blue broth, containing cells, was added to the wells. This modification allowed for the use of double the volume of a half-as-concentrated drug-peptide solution.

In all assays well 12 of each row was used for the

proper controls: a cell-free control to test for the sterility of the medium and a medium-containing-cells control to see if the cells were growing properly.

Transport Studies

A. Growth of cells.

1. Trimethionine and orotic acid assays. The medium used in all experiments was Vogel's-N-Medium (35) modified by the addition of inositol (36 $\mu\text{g/ml}$), pantothenic acid (2 $\mu\text{g/ml}$), pyridoxine HCl (4 $\mu\text{g/ml}$), thiamine HCl (4 $\mu\text{g/ml}$), biotin (0.5 ng/ml), and the exclusion of NH_4NO_3 . Isoleucine (Ile) (6.56 mg/ml) was added as the nitrogen source. The medium was autoclaved 15 minutes at 121°C and sterile solutions of methionine (10 $\mu\text{g/ml}$), lysine (10 $\mu\text{g/ml}$) and dextrose (2 percent) were added aseptically. This final growth medium was designated Vogel's-Ile. For starter culture 25 ml of Vogel's-Ile, contained in a 125 ml flask, was loop inoculated from a slant. Cells were grown 18-24 hours at 37°C and aerated by vigorous stirring with a magnetic stirring bar or a rotating shaker (200 rpm). A 7-10 percent inoculum was made from the starter culture into fresh medium contained in a 500 ml side-arm flask. This inoculum gave an initial Klett reading of 20-25 Klett Units. The culture was grown 6-8 hours (40-60 Klett Units) at 37°C and aerated as above.

2. Fluoroorotic acid assays. Vogel's-Ile was prepared as described above. Yeast-N-Base without amino acids and $(\text{NH}_4)_2\text{SO}_4$ (Difco) (1.7 mg/ml) supplemented with methionine (10 $\mu\text{g}/\text{ml}$), lysine (10 $\mu\text{g}/\text{ml}$), and dextrose (2 percent) was prepared and filter-sterilized. A sterile solution of Ile (6.56 mg/ml) was added aseptically as the nitrogen source. Vogel's- NH_4NO_3 was prepared exactly like Vogel's-Ile; however, isoleucine was excluded and NH_4NO_3 (2.05 mg/ml) was substituted. Cells were grown as described above for Vogel's-Ile.

B. Assays.

1. Trimethionine and competitors. Cells were harvested by filtration using a Millipore Apparatus with a filter of 47 mm diameter and 0.45 μm pore size, washed twice with cold dH_2O and resuspended in cold dH_2O . The density of the cells was adjusted so that a 1:10 dilution gave a Klett Unit of 30 (1.4 mg dry weight cells/ml). The cells were kept on ice until assayed. For the uptakes, cells were incubated 10 minutes at 37°C, with stirring, then added to an equal volume of a reaction mixture. The reaction mixture, which was likewise incubated, contained dextrose (2 percent), citrate-phosphate buffer (pH 3.5, 0.012 M), and radioactive Met_3 (6.06×10^{-5} M, 1 Ci/m). At various time intervals 500 μl portions were removed and applied to filters (Millipore Corp., 25 mm, 0.45 μm)

supported by a Millipore or a Yeda (Yeda Scientific Instruments, Rehovot, Israel) manifold. The filters were then washed twice with cold dH_2O . The reaction mixture with the cells in it was at 37°C and stirred during sampling. The filters were placed in Bray's Solution (8) and radioactivity was counted with a Searle IsoCap/300 6868 Liquid Scintillation System or a Beckman LS 7000.

Competition of transport assays was done the same way as with Met3 alone. However, the drug-peptide to be used as the competitor was included in the reaction mixture prior to the cells being added. The drug-peptides were dissolved either in water or DMF.

2. Orotic acid and competitors. Cells were prepared as outlined above. An equal volume of cells were added to a reaction mixture containing dextrose (2 percent), citrate-phosphate buffer (pH 3.5, 0.012 M). This was incubated, with stirring, at 37°C for 10 minutes. Radioactive orotic acid was added at a final concentration of 5×10^{-6} M and at various time intervals 500 μl portions were removed, applied to filters, washed twice and counted. In some experiments the specific activity of the orotic acid was reduced to conserve the radioactive stock.

Competition of transport assays was done exactly as outlined above except the competing drug-peptide was added a few seconds before orotic acid.

3. Fluoroorotic acid. Fluoroorotic acid (1 Ci/m) transport assays were done at 1×10^{-5} or 5×10^{-5} M final concentrations. Citrate-phosphate buffer was used at a range of pH values: 2.6, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 7.3, 8.0. At pH 5.5 sodium-phosphate buffer was used at a 0.05 M final concentration. In those assays where 5-FO was added last to the reaction mixture, the methods were identical to that of orotic acid. In one assay a reaction mixture composed of dextrose (2 percent), citrate-phosphate buffer (pH 3.5, 0.012 M) and 5-FO (1×10^{-4} M) was prepared. An equal volume of cells, which was incubated 10 minutes with stirring, was added to this and the procedure was the same as with Met₃ assays.

Enzyme Assays

A. Growth of cells. Yeast-N-Base without amino acids (Difco) (67 mg/ml) with 30 percent dextrose, methionine (300 μ g/ml), lysine (300 μ g/ml) was prepared at a volume of 100 ml and was filter-sterilized. This was added to 900 ml of sterile dH₂O, 100 ml was removed, placed in a 500 ml flask and loop-inoculated from a slant. The culture was grown 19 hours at 37°C with vigorous stirring with a magnetic stirring bar. This culture was used to inoculate the 900 ml of sterile medium, grown to mid-log (9 hours) at 37°C and aerated as above.

B. Extraction of cells. Cells were harvested by centrifugation (9000 x g), washed once with cold 0.9 percent NaCl, resuspended in 20 ml of Tris buffer (pH 8, 0.05 M). The cell suspension and glass beads (40 gm, Sigma Chemical Co., 75-100 μ m diameter) were added to the 50 ml size omnimixer stainless steel cup (Ivan Sorvall Corp.). The suspension was kept on ice and homogenized at top speed for five minutes, low speed for one minute, and then another 10 minutes at top speed. The glass beads, unbroken cells and cell debris were removed by centrifugation (25300 x g). The supernatant fluid was dialyzed overnight against Tris buffer (pH 8, 0.05 M) at 4°C. The resulting solution was used as a cell extract (CE). Protein determination was carried out by the BioRad Protein Assay (BioRad Labs, Richmond, Ca.). The protein content of the cell extract was 3.36 mg/ml. The cell extract was frozen until just prior to use and the remainder was immediately refrozen.

C. Procedure (References 31, 32). Reaction mixture containing, as final concentrations, PRPP 0.2 mM, $MgCl_2$ 4 mM, Tris buffer (pH 8) 45 mM, DTT 0.7 mM was prepared and CE was added at a final concentration of 0.56 mg/ml. The reaction mixture was incubated for 15 minutes at room temperature and at 37°C for an additional 10 minutes. Orotic acid, at 15 Ci/m, was added at a final concentration

of 0.3 mM. The final volume of the reaction mixture was 36 μ l. Samples (7 μ l) were removed at various time points and placed into 3 μ l of a 150 mM EDTA, 5 mM azaUMP solution to stop the enzymatic processes. These were kept in an ice water bath until spotted onto TLC plates.

To spot the samples 4 μ l of each time point was removed and spotted onto TLC plates. The spots were dried by gently blowing cool air with a hair drier. Each spot was then overlaid with 4 μ l of a solution containing 1 mM OMP, 1 mM UMP, 3 mM orotic acid.

Thin Layer Chromatography plates were developed in a glass chamber; solvent was 0.2 M lithium chloride. After developing, the plates were thoroughly air dried and spots were localized under ultraviolet light and outlined with a pencil. The spots corresponding to the known R_f values of OMP and UMP were cut out with a scalpel. The cut-outs were placed in 0.5 ml of a 0.1 M HCl, 0.2 M KCl solution contained in a minivial. The vials were shaken on a rotating shaker (200 rpm) for 30-60 minutes. Bray's Solution (5 ml) was added to each minivial and counted for radioactivity.

The procedures for competition experiments were identical to what is outlined above except that the competitor was added a few seconds prior to the addition of orotic acid. The competitors used were 5-FO and FOL₂ which were included in the assays at four, five and eight fold higher molar concentrations to that of orotic acid.

CHAPTER III

RESULTS

Minimum Inhibitory Concentration Assays

To determine the toxicity of the drug-peptide conjugates, the minimum inhibitory concentration assay was performed on representative peptides. The results are listed in Table 1. The fluorocytosine was toxic at 1.25 $\mu\text{g}/\text{ml}$. This is similar to the known toxicity of the drug (6). Fluoroorotic acid was not toxic to levels of 320 $\mu\text{g}/\text{ml}$, which is considered to be non-toxic (19). Orotyl-leucyl-leucine, FOL₂, FOL, FOLL, and FOG₂OBzl were also non-toxic. Fluoroorotic acid, which is known to be toxic in other systems, was not toxic to Candida, presumably because of poor capacity to penetrate. The fact that the drug-peptides were not toxic, however, served as a reason to determine whether these compounds were transported. We wanted to show that the peptides could be used to carry 5-FO into the cells.

Transport Studies

A. Trimethionine. A powerful method of determining if two substances enter a cell through the same transport system is to do competition of uptake assays. To decide whether any of the orotyl-peptides and fluoroorotyl-peptides

Table 1. Minimum inhibitory concentration assays of drugs and representative drug-peptides.^a

Drug	Minimum Inhibitory Concentration
Fluorocytosine	1.25
Fluoroorotic acid	320
Orotyl-leucyl-leucine	320
Fluoroorotyl-leucyl-leucine	320
Fluoroorotyl-leucine	320
N-ε-Fluoroorotyl-lysyl-leucine	320
Fluoroorotyl-glycyl-glycine benzyl ester ^b	320

^aData for fluorocytosine and fluoroorotic acid are expressed in micrograms per milliliter. Data for the drug-peptides are expressed in microgram equivalents per milliliter. The drug and drug-peptides were standardized so that there was the same molar concentration of the drug within assay wells.

^bI thank Sam V. Lewis for the data.

could enter Candida, competition of the transport of Met₃ with the peptides was carried out. The transport assay for Met₃ has been well characterized (28). The structures of the drug-peptides are an orotic acid conjugated to a dipeptide through the amine terminus of the dipeptide. The peptides were sufficiently similar to a tripeptide, and it was expected that they would compete with the transport of trimethionine. Accordingly, each of the drug-peptides was tested to see if they competed with Met₃. Competition would indicate that the peptides were entering the cells through the peptide transport system. For the competition experiments a control Met₃ uptake (no competitor) was done. Initial rates of uptake of Met₃ with and without competitor were calculated and percent inhibition of the initial rate of uptake of Met₃ was derived. Most of the drug-peptides were water soluble; however, all but one of the peptides that were blocked at the carboxy terminus needed to be solubilized in DMF. An appropriate DMF control was done for these uptakes. Figure 1 shows the results of the experiments with OL₂ and OG₂. Orotyl-leucyl-leucine inhibited the uptake of Met₃ while OG₂ did not. Orotyl-glycyl-glycine tertiary butyl ester, which was water soluble, did not inhibit the transport of Met₃ (Figure 2). Orotyl-glycyl-glycine benzyl ester and OM₂OMe were dissolved in DMF. In these two cases the final concentration of DMF

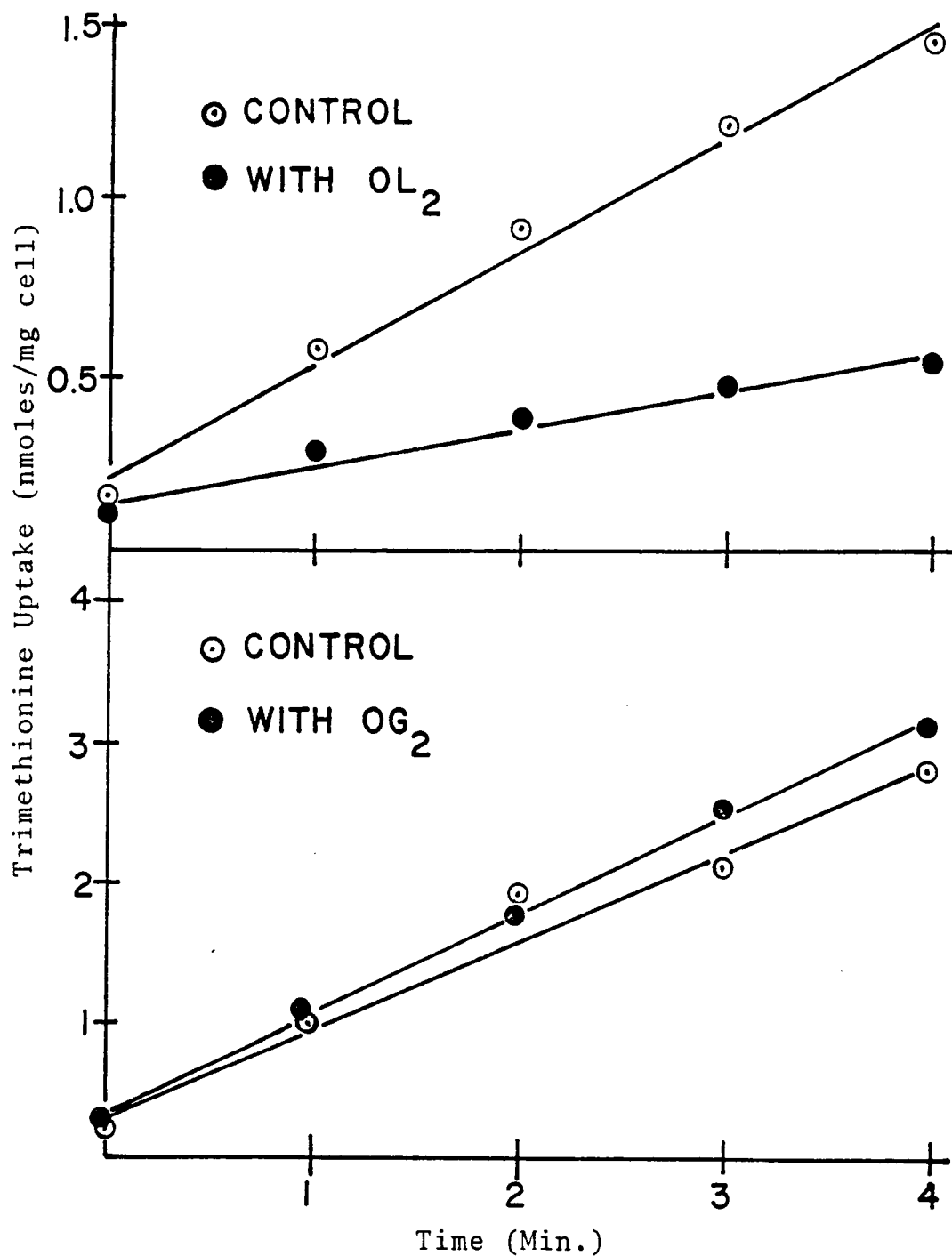


Figure 1. Trimethionine transport assays-- orotyl-peptides as competitors. OL₂ = Orotyl-leucyl-leucine; OG₂ = Orotyl-glycyl-glycine.

in the reaction mixture was 0.9 percent. Therefore, a 0.9 percent DMF control was done and gave an inhibition of 43 percent when compared with the control Met₃ uptake (Figure 2). The inhibition seen with the two peptides was no greater than the inhibition given by DMF. The inhibition observed was then attributed to the DMF; these peptides were considered to be non-inhibitors of tri-methionine transport (Figure 2).

The results of the fluoro-orotyl-peptide competition assays are found in Figure 3. Fluoro-orotyl-leucyl-leucine, FOL, FOLL, FOL₂OTB, FOLOTB, and FOG₂OBzl were tested against Met₃. Fluoro-orotyl-leucyl-leucine, FOL, and FOLL were water soluble. From the graphs it can be seen that FOL₂ inhibits Met₃ uptake, although not as well as OL₂ (Figure 1). Fluoro-orotyl-leucine and FOLL did not inhibit Met₃ transport. Fluoro-orotyl-leucyl-leucine tertiary butyl ester, FOLOTB and FOG₂OBzl were dissolved in DMF and the proper (0.7 percent DMF) control was performed. None of these peptides was shown to be inhibitory to Met₃ transport. The results are summarized in Table 2.

In Table 2 it can be seen that of the peptides tested, only OL₂ (67 percent competition) and FOL₂ (40 percent competition) seem to be getting into the cells by this criterion. Since FOL₂ is the peptide that contains the drug, 5-FO, a Lineweaver-Burk plot was constructed using Met₃ and FOL₂ as its competitor. Fluoro-orotyl-

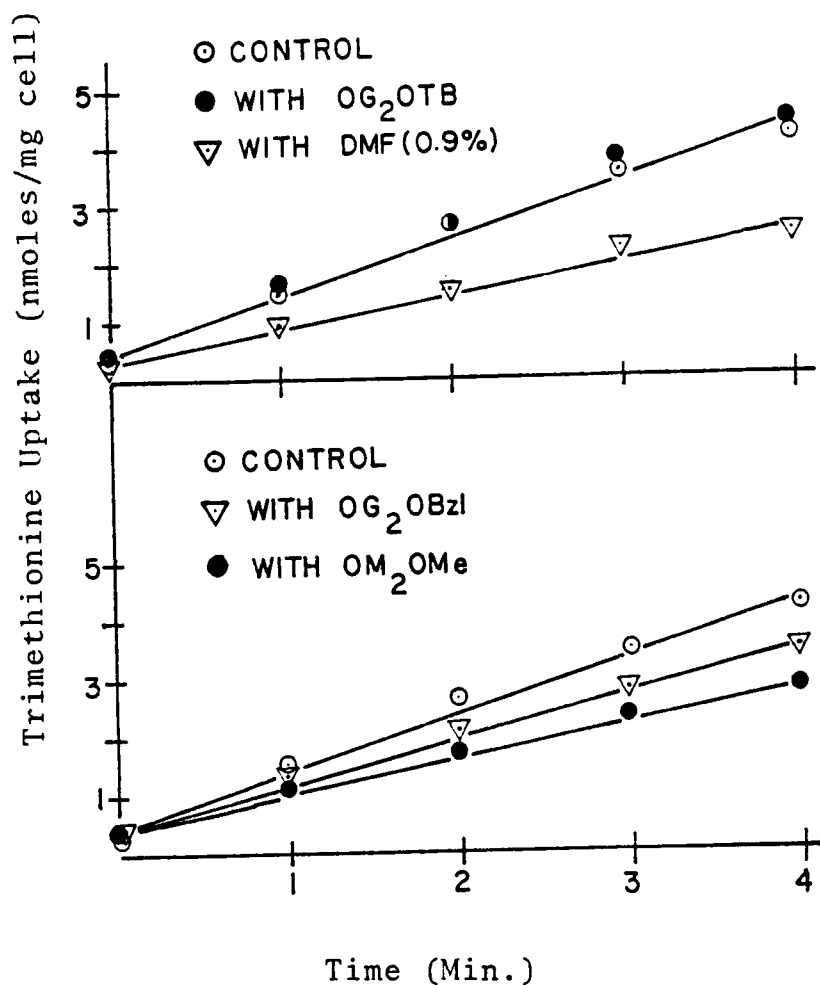


Figure 2. Trimethionine transport assays--orotyl-peptides derivatized at the carboxy-terminus as competitors. A control Met₃ uptake in the presence of DMF is included. OG₂OTB = Orotyl-glycyl-glycine tertiary butyl ester; DMF = Dimethyl formamide; OG₂OBzl = Orotyl-glycyl-glycine benzyl ester; OM₂OMe = Orotyl-methionyl-methionine methyl ester; Met₃ = Trimethionine.

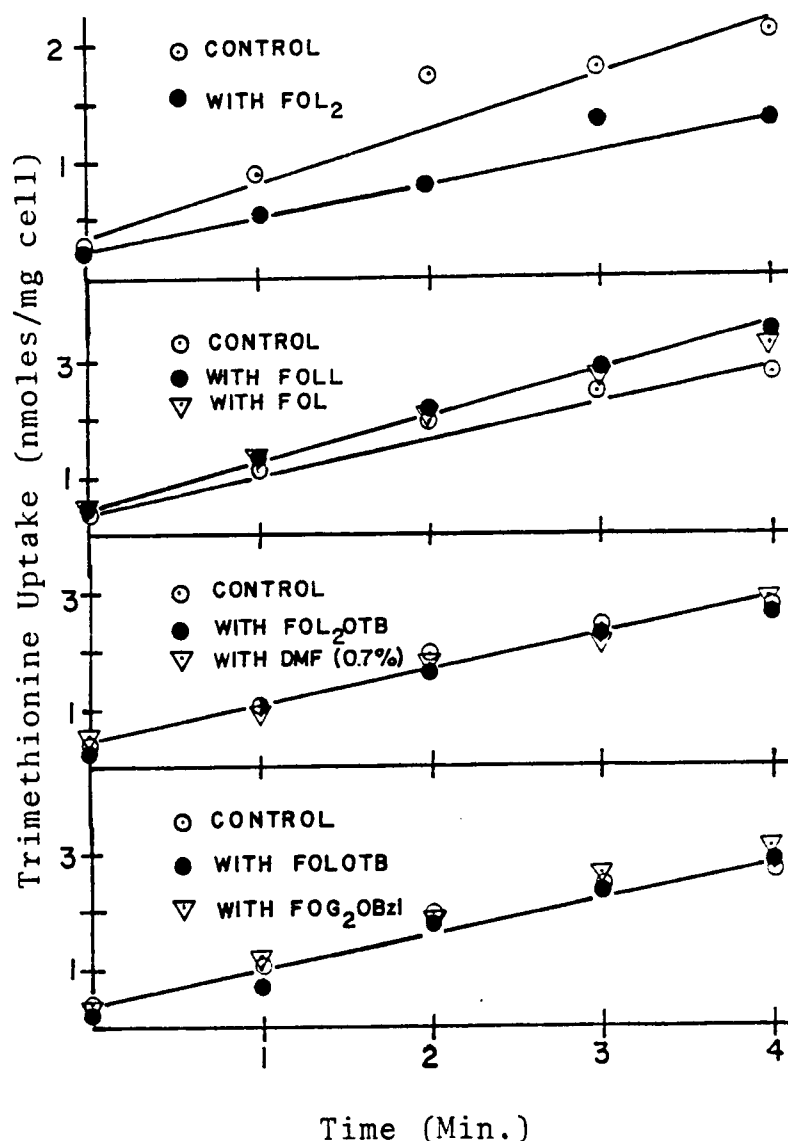


Figure 3. Trimethionine transport assays--fluoroorotyl-peptide conjugates as competitors. A control Met₃ uptake in the presence of DMF is shown. FOL₂ = Fluoroorotyl-leucyl-leucine; FOLL = N-ε-Fluoroorotyl-lysyl-leucine; FOL = Fluoroorotyl-leucine; FOL₂OTB = Fluoroorotyl-leucyl-leucine tertiary butyl ester; DMF = Dimethyl formamide; FOLOTB = Fluoroorotyl-leucine tertiary butyl ester; FOG₂OBzl = Fluoroorotyl-glycyl-glycine benzyl ester; Met₃ = Trimethionine.

Table 2. Control and competition of transport assays with Met₃ and the drug-peptides.

Competitor ^a	Control Uptake ^b	Competitor Uptake ^c	Percent Inhibition ^d
Orotyl-leucyl-leucine	0.36	0.12	67
Orotyl-glycyl-glycine	0.74	0.82	0
Orotyl-glycyl-glycine tertiary butyl ester	1.00	1.00	0
Orotyl-glycyl-glycine benzyl ester ^e	1.00	0.77	23
Orotyl-methionyl-methionine methyl ester ^e	1.00	0.60	40
Fluoroorotyl-leucyl-leucine	0.47	0.28	40
Fluoroorotyl-leucine	0.63	0.63	0
N-ε-Fluoroorotyl-lysyl-leucine	0.63	0.77	0
Fluoroorotyl-leucyl-leucine tertiary butyl ester ^f	0.63	0.63	0
Fluoroorotyl-leucine tertiary butyl ester ^f	0.63	0.63	0
Fluoroorotyl-glycyl-glycine benzyl ester ^f	0.63	0.63	0
Leucyl-leucyl-leucine	0.43	0.11	75

^aAll competitors are at 10-fold higher molar concentration to that of Met₃ in the final reaction mixture.

^bThe data given are the initial rate of uptake of Met₃ expressed in nanomoles of Met₃ taken up per milligram dry weight of cells per minute.

^cInitial rate of uptake of Met₃ in the presence of the competitor.

^dRatio of competitor uptake to control uptake subtracted from 100.

^eDissolved in DMF. The final concentration of DMF in reaction mixture was 0.9 percent.

^fDissolved in DMF. The final concentration of DMF in reaction mixture was 0.7 percent.

leucyl-leucine was at a 10-fold higher molar concentration to that of Met₃. By showing competitive inhibition of transport there would be further evidence that FOL₂ was entering the cells through the peptide transport system. The results of this experiment are in Figure 4. From the data it can be seen that FOL₂ is indeed a competitive inhibitor of trimethionine uptake. The K_m of Met₃ is 3.3×10^{-5} M and the K_I of FOL₂ is 5×10^{-4} M.

B. Orotic acid. To see if any of the drug-peptide conjugates could possibly enter the cells through the orotic acid transport system, competition studies were carried out. Control OA uptake containing no competitor in the reaction mixture was considered as 100 percent. As above, this was used to calculate percent inhibition of uptake by the drug-peptides. Orotyl-leucyl-leucine, OG₂, and OG₂OTB were the orotyl-peptides tested; none of them inhibited the transport of OA (Figure 5). Fluoroorotyl-leucyl-leucine tertiary butyl ester and FOLOTB were not inhibitors of orotic acid uptake. Again, these were dissolved in DMF and control transport assay (0.1 percent DMF) was done (Figure 5). Fluoroorotyl-leucyl-leucine, FOL, FOLL (Figure 6) were also non-inhibitors. The results indicated that none of the drug-peptides inhibited the transport of OA.

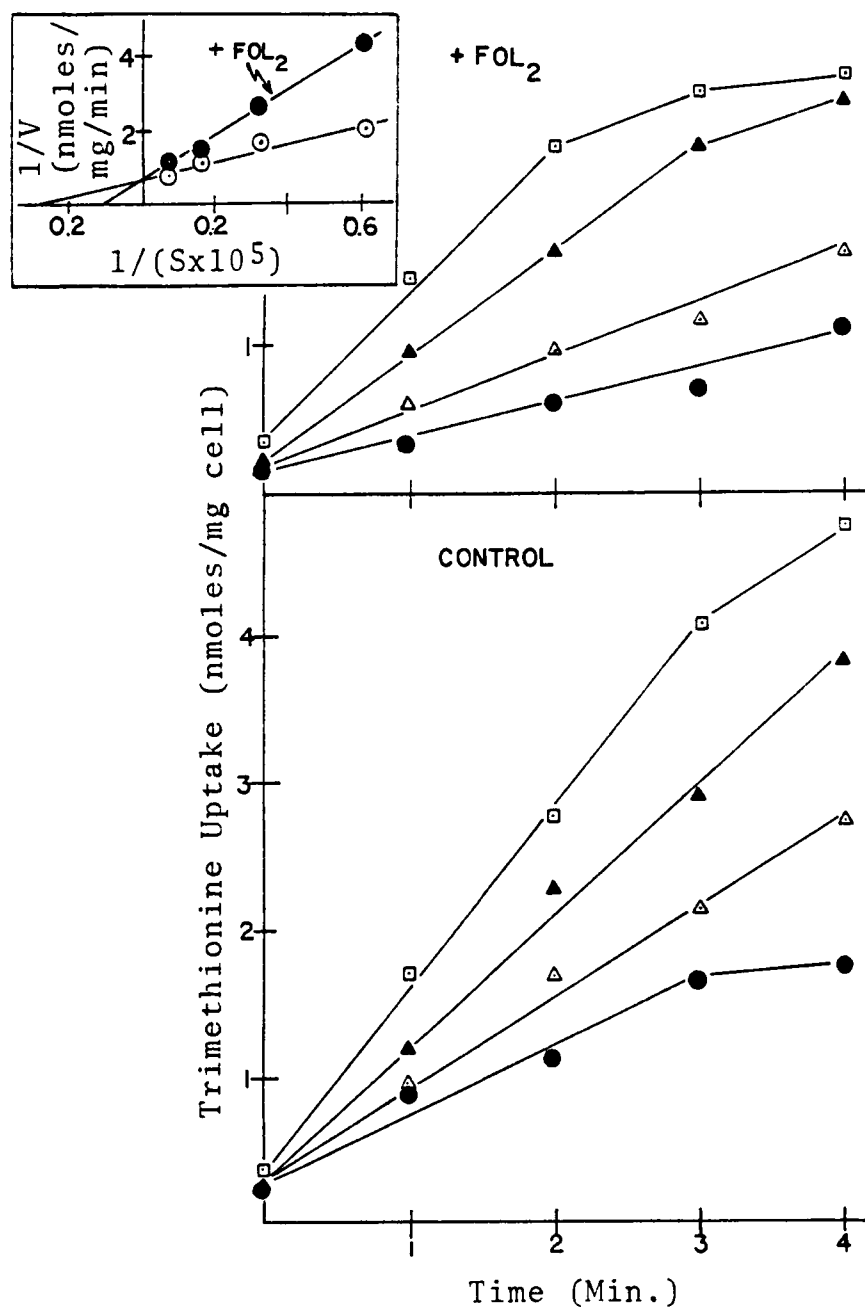


Figure 4. Kinetics of trimethionine uptake in the presence (+FOL₂) and absence of FOL₂ (control). The final concentrations of Met₃ in both assays were: (●) 1.6×10^{-5} M, (△) 3×10^{-5} M, (▲) 6×10^{-5} M, (◻) 12×10^{-5} M. The final concentration of FOL₂ was 3×10^{-4} M. The inset shows Lineweaver-Burk analysis of the data. The K_m of Met₃ transport was 3.3×10^{-5} M and K_I was 5×10^{-4} M. FOL₂ = Fluoro-orotyl-leucyl-leucine; Met₃ = Trimethionine.

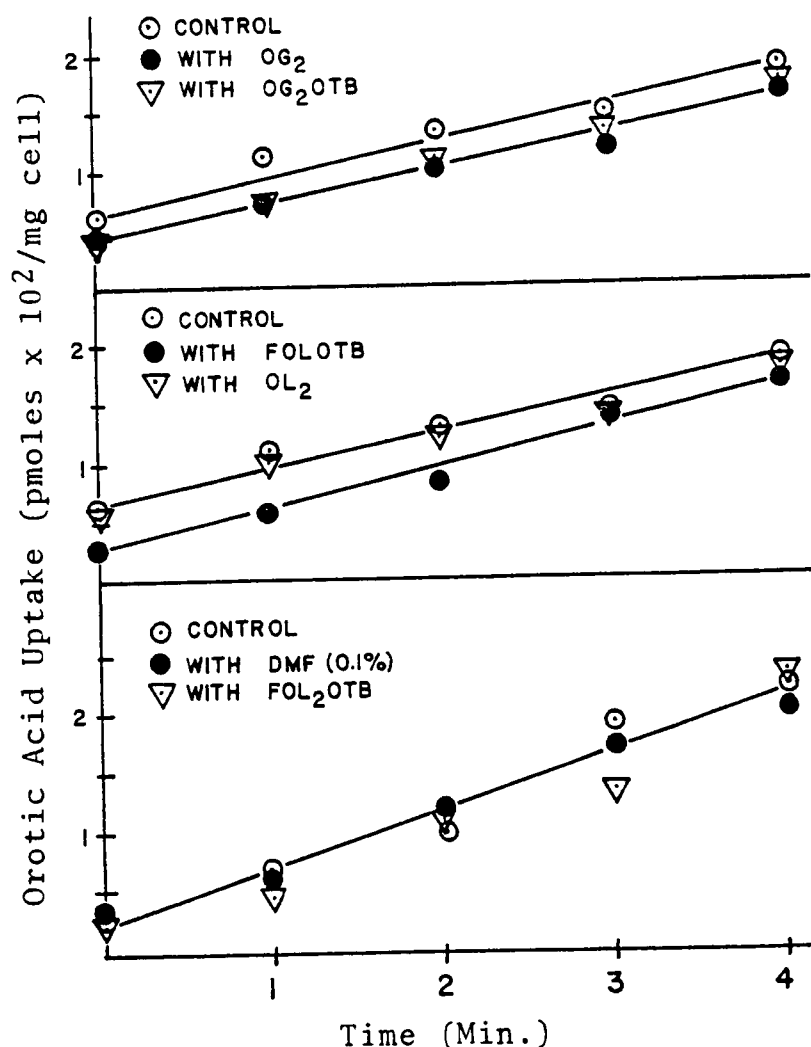


Figure 5. Orotic acid transport assays--the competitors were orotyl-peptides and fluoroorotyl-peptides which were derivatized at the carboxy-terminus. A control uptake in the presence of DMF is included. OG₂ = Orotyl-glycyl-glycine; OG₂OTB = Orotyl-glycyl-glycine tertiary butyl ester; FOLOTB = Fluoroorotyl-leucine tertiary butyl ester; OL₂ = Orotyl-leucyl-leucine; DMF = Dimethyl formamide; FOL₂OTB = Fluoroorotyl-leucyl-leucine tertiary butyl ester.

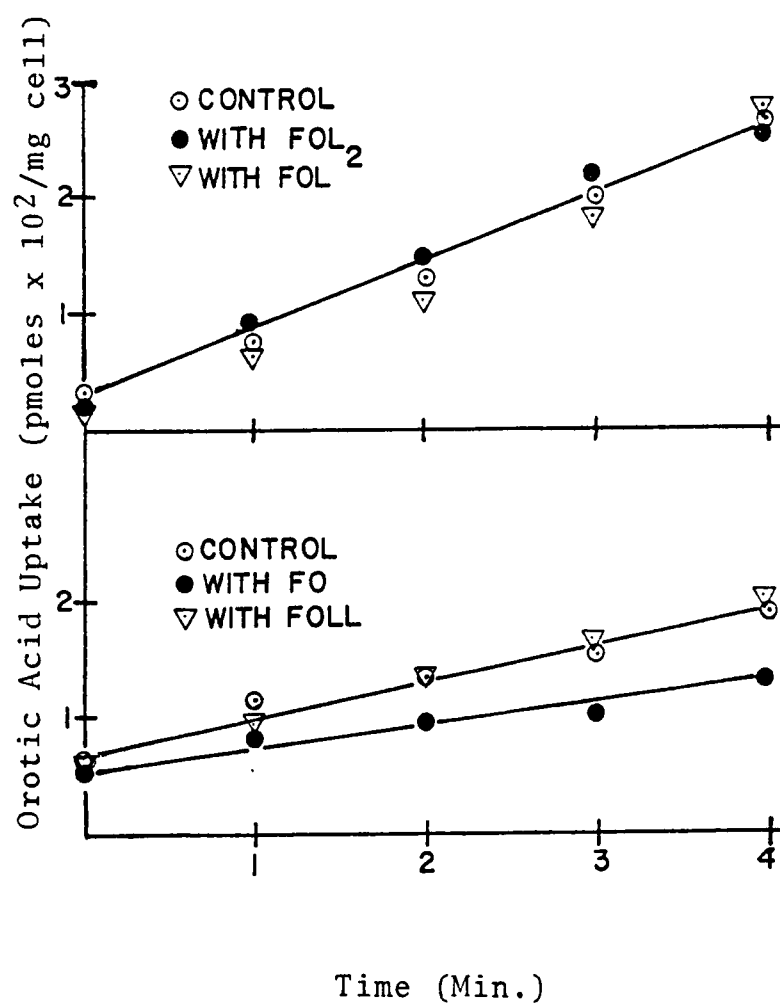


Figure 6. Orotic acid transport assays--the competitors shown were the fluoro-orotyl-peptides and fluoro-orotic acid. FOL₂ = Fluoro-orotyl-leucyl-leucine; FOL = Fluoro-orotyl-leucine; FO = Fluoro-orotic acid; FOLL = N-ε-Fluoro-orotyl-lysyl-leucine.

To answer the question whether 5-FO gets into cells, 5-FO was used as a competitor of OA. At 20-fold molar excess to orotic acid 5-FO competed only 33 percent with the uptake of OA. This indicated that 5-FO does not enter cells readily via the orotic acid transport system. This was an interesting result as it was already shown that 5-FO was not toxic to Candida. The reason may then be due to the poor penetration of 5-FO. The results are summarized in Table 3.

C. Fluoroorotic acid. To gain direct evidence for the transport of 5-FO, radioactive (1 Ci/m) was utilized. A range of pH values was used to get the best conditions for uptake. Cells grown in Vogel's-Ile were assayed at the various pH values found in Figure 7. The data indicate that 5-FO was not taken up under conditions where uptake of orotic acid, peptides, amino acids and sugars could readily be detected. The data points were scattered and no initial rates of uptake could be calculated. In order to see if different media could improve uptakes, cells grown in Yeast-N-Base with Ile as the nitrogen source were assayed. Uptakes were done at pH 3.5 with either 5-FO added last to the reaction mixture or with the cells added last. These were done to see if there was a difference in transport between the two methods. It was felt that there may be a need for the cells to be

Table 3. Control and competition uptakes with orotic acid and drug-peptides.

Competitor ^a	Control Uptake ^b	Competitor Uptake ^c	Percent Inhibition ^d
Orotyl-leucyl-leucine	30	30	0
Orotyl-glycyl-glycine	30	30	0
Orotyl-glycyl-glycine tertiary butyl ester	30	30	0
Fluoroorotic acid (20xM)	30	20	33
Fluoroorotyl-leucyl-leucine (7xM)	68	64	6
Fluoroorotyl-leucine (16xM)	68	58	15
N-ε-Fluoroorotyl-lysyl-leucine	30	30	0
Fluoroorotyl-leucyl-leucine tertiary butyl ester ^e	53	53	0
Fluoroorotyl-leucine tertiary butyl ester ^e	30	35	0

^aAll competitors are at 10-fold higher (unless otherwise stated) concentration to that of orotic acid in the final reaction mixture.

^bThese data given are initial rates of uptake of orotic acid expressed in picamoles of orotic acid taken up per milligram dry weight of cells per minute.

^cInitial rate of uptake of orotic acid in the presence of competitor.

^dRatio of competitor uptake to control uptake subtracted from 100.

^eDissolved in DMF. The final concentration of DMF in the reaction mixture was 0.1 percent.

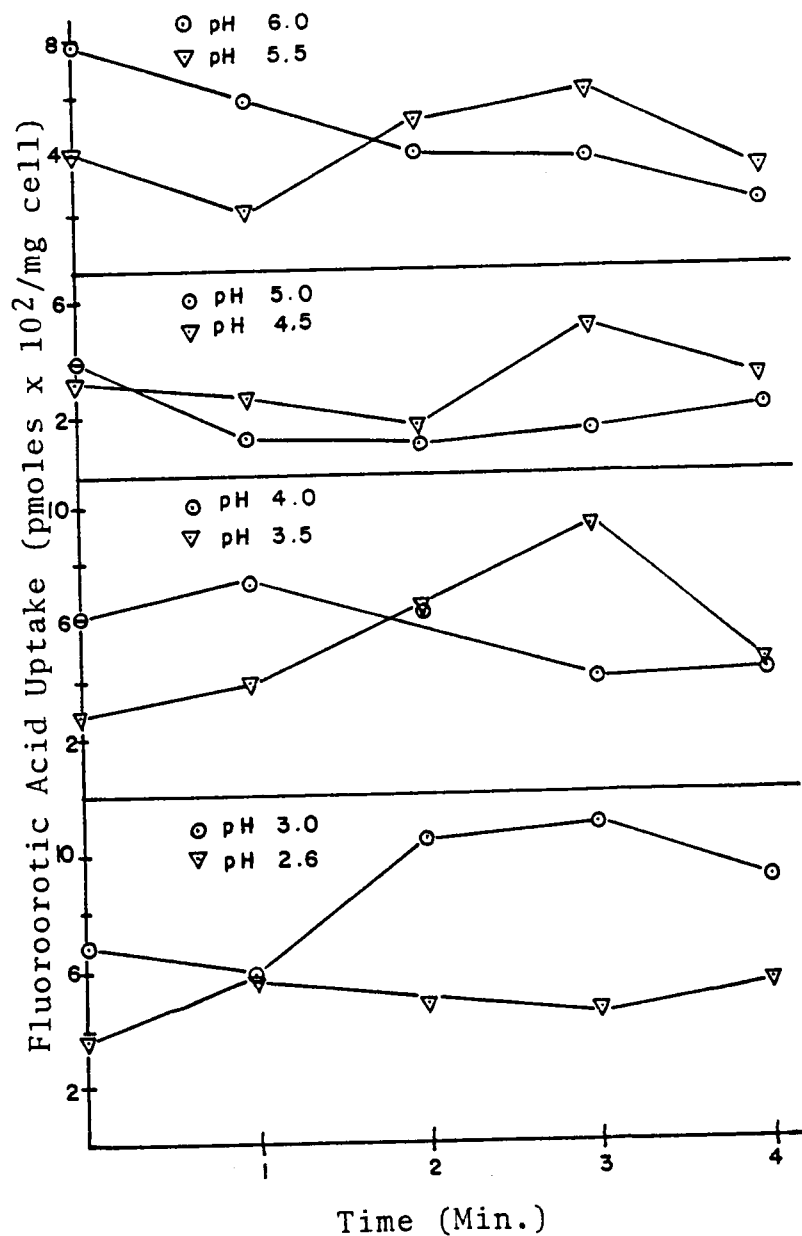


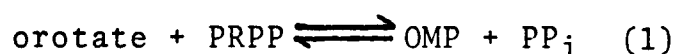
Figure 7. Fluoroorotic acid transport assays-- cells were grown in Vogel's-Isoleucine. Fluoroorotic acid was at a final concentration of 5×10^{-5} M and assays were done at pH values shown.

"energized" by being incubated with dextrose before receiving the substrate, 5-FO. The uptakes (Figure 8) were no better than those cells grown in Vogel's-Ile and assayed at pH 3.5 (Figure 7). The cells grown on Yeast-N-Base with Ile as the nitrogen source were also assayed at pH values 7.0 and 7.3. This was done to see if physiological pH could improve transport. The uptakes were again poor (Figure 8).

Cells grown in Vogel's-NH₄NO₃ were also tested for transport. It was thought a richer nitrogen source could improve transport. The assays were done at pH values seen in Figure 9. Fluoroorotic acid was not taken up by these cells.

Enzyme Assays

Orotic acid is a precursor in the de novo pyrimidine biosynthetic pathway (31). The two enzymes responsible for converting OA to OMP then to UMP are called orotate phosphoribosyl transferase (PRTase) and orotidylate decarboxylase (ODCase). The enzymes catalyze the reactions (1) and (2) respectively:



The activity of these two enzymes was tested for in a cell extract of Candida. Orotidine-5-monophosphoric acid production versus time is plotted in Figure 10. Orotic

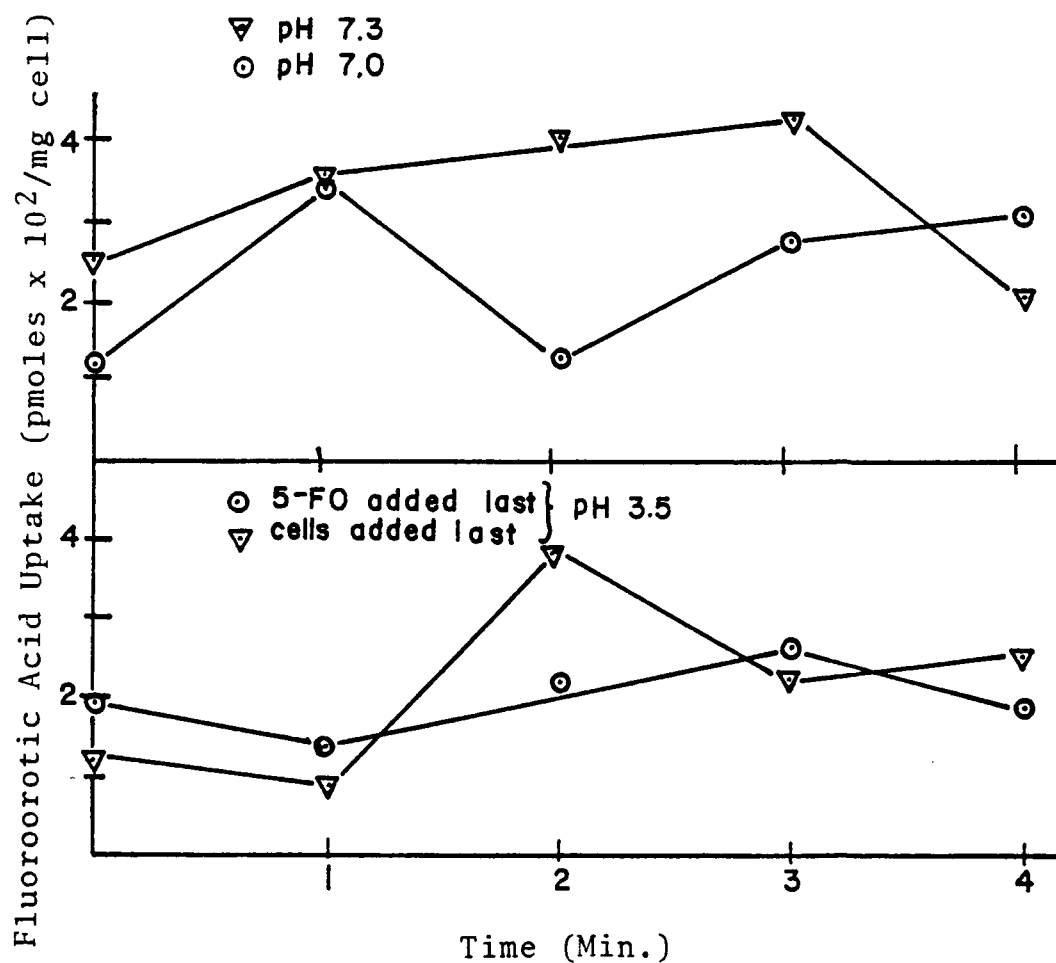


Figure 8. Fluoroorotic acid transport assays-- cells grown in yeast-N-base with isoleucine as the nitrogen source were assayed for 5-FO uptake. The final concentration of 5-FO was 5×10^{-5} M and uptakes were done at the pH values shown. FO = Fluoroorotic acid.

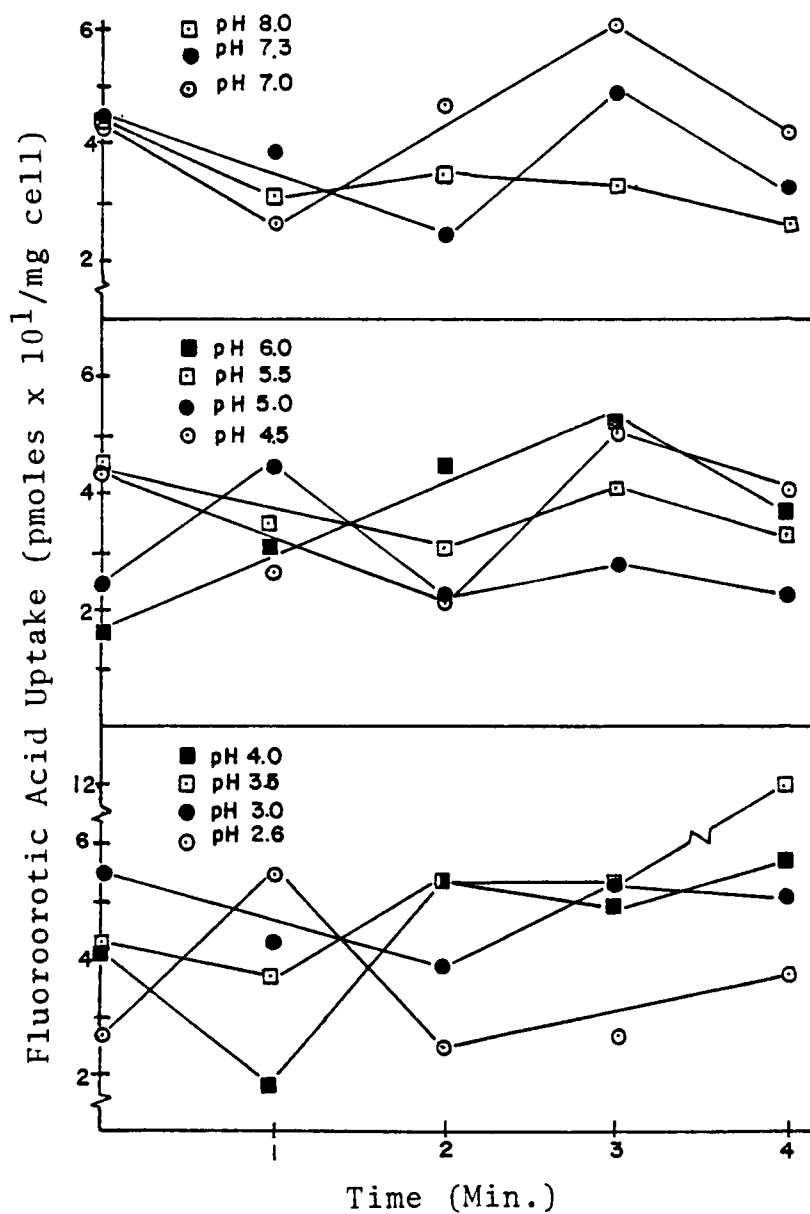


Figure 9. Fluoroorotic acid transport assays-- cells grown in Vogel's- NH_4NO_3 were assayed for 5-FO uptake at a final concentration of 1×10^{-5} M. A range of pH values were used. FO = Fluoroorotic acid.

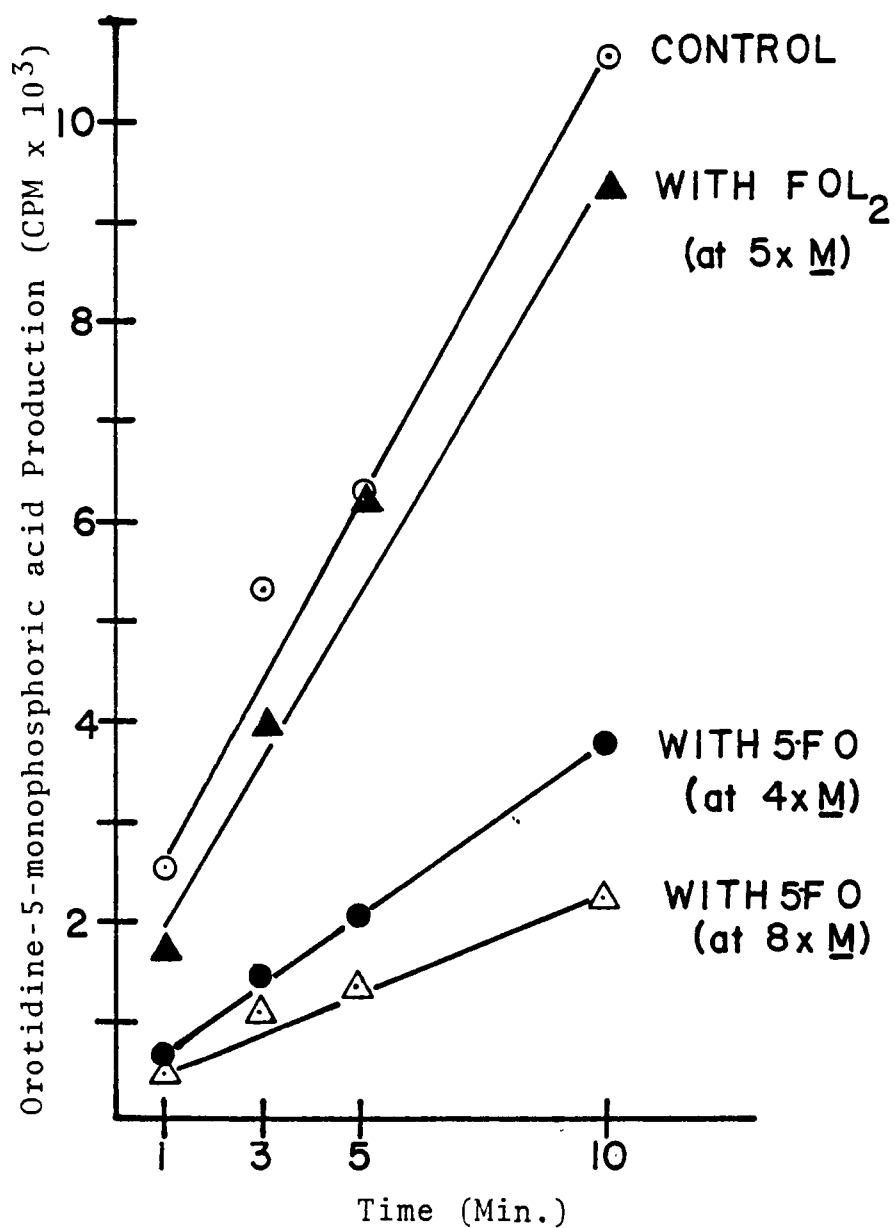


Figure 10. Time course of orotidine-5-monophosphoric acid production. Competitors, at designated concentrations as compared with orotic acid, are shown. FOL₂ = Fluoroorotyl-leucyl-leucine; FO = Fluoroorotic acid.

acid was the substrate in the control assay. In the competition experiments, FOL₂, at five-fold higher concentration, did not compete with OA for the production of OMP. Fluoroorotic acid, which was at four- and eight-fold higher concentration than OA, was an effective and dose dependent inhibitor of OMP production. Similar results were obtained with orotidylate decarboxylase (Figure 11). Fluoroorotyl-leucyl-leucine did not compete at all while 5-FO inhibited the conversion of OMP to UMP.

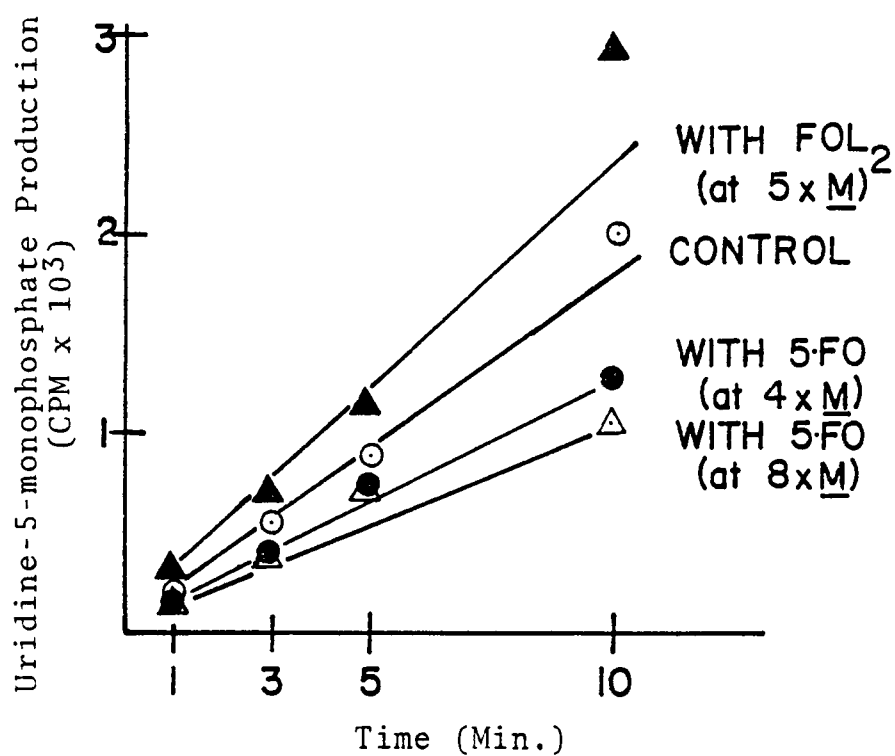


Figure 11. Time course of uridine-5-monophosphate production. Competitors, at designated concentrations as compared with orotic acid, are shown. FOL₂ = Fluoroorotyl-leucyl-leucine; FO = Fluoroorotic acid.

CHAPTER IV

DISCUSSION

The increasing use of immunosuppressants, cytotoxic drugs, antibiotics, and corticosteroids has resulted in an increase in opportunistic infections. A group of organisms frequently encountered in compromised hosts are the fungi and Candida is the most reported species causing infection (22). With the problems inherent in the most widely used anti-mycotic drugs (5, 29), it was decided to explore the use of drug-peptide conjugates to help in the treatment of fungal infections.

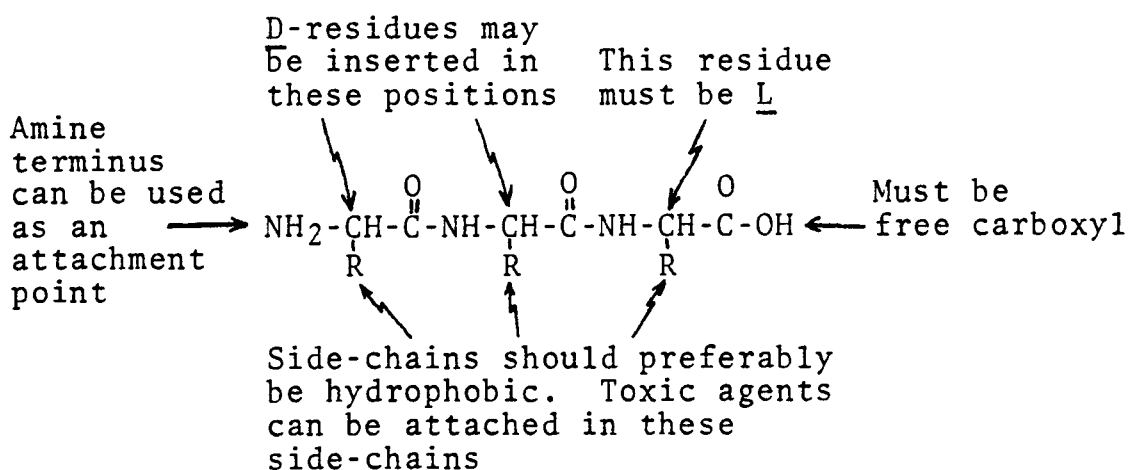
The approach of using peptides as carriers of a drug has several advantages. It can result in a 100-1000 fold concentration of the toxic agent in the cell (2). The peptide transport systems of microorganisms tolerate changes in the side-chains and end-groups of the peptides (16, 18). In addition, a peptide is easily modified chemically (18). The latter two could be exploited to target drugs to the pathogen.

To design drug-peptides specific for the pathogen and to be safely used in the compromised host, information on the transport of peptides in the biological systems involved was required. The transport systems of the host's gut (12, 16), microbial flora, particularly of

E. coli (16), and Candida (27, 28) have been studied. Structural specificities of the oligopeptide transport in the three systems are summarized below:

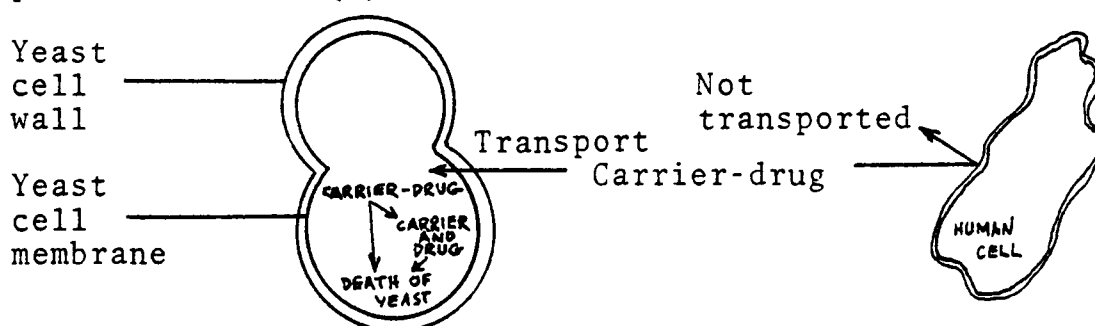
	Side chain specificity	N-terminal amino	C-terminal carboxyl
Mammalian gut	broad	free group required	may require free group
<u>E. coli</u>	broad	protonatable group required	no requirement for a free group
<u>Candida</u>	broad	no requirement for a free group	free group required

The results of the studies on the structural specificities of transport have led to this hypothetical model for an anti-candidal drug-peptide conjugate (28):



The idea of a "magic bullet" was first proposed by Ehrlich (9). He envisioned the bullet to be a chemical

compound that was not toxic to the host but specifically searched for and killed the invading organism. This coincides with the approach proposed here. A model is presented below (4):



The use of carriers for "smuggling" (30) poorly transported substances into cells is not a new one. Attachment to macromolecules (20) and the use of liposomes (17) have been attempted to overcome permeability problems in mammalian systems. Several investigators (1, 18, 27) have shown that peptides can carry toxic and poorly transported moieties into microbes. Lichliter (27) showed that methionine peptides containing ethionine and norleucine could serve as the methionine source for a methionine auxotroph of Candida. Fickel and Gilvarg (18) saw that homoserine phosphate could enter E. coli as dilysyl-homoserine phosphate. Ethionine, norleucine and norvaline were more toxic to Salmonella typhimurium when presented as part of peptides than as free amino acids (1). In addition, Ames and co-workers (1) have shown that histidinol phosphate (HOLP) could be transported in as

diglycyl-HOLP via the peptide transport system. Peptides have also been shown to carry toxic amino acid anticapsin into Candida (25).

Fluorocytosine is a known and effective drug against a variety of systemic fungal diseases (5, 6). For the working hypothesis presented here this would be the logical toxic agent to be used. By putting 5-FC on a specifically designed peptide, targeting of the drug could be realized. This has been achieved (33). However, it was discovered that the drug peptides were unstable under test conditions. It appeared the peptides were hydrolyzed extracellularly and 5-FC was subsequently transported into Candida. This, of course, defeated the purpose of the peptide carrier.

To overcome the instability problems of the 5-FC peptides, 5-FO peptides were synthesized. Fluoroorotic acid was selected because it could easily be linked to the α or ϵ amino group of a peptide and the bond used in conjugation was expected to be stable. The list of orotyl and fluoroorotyl-peptides appears in the chapter on Materials and Methods.

In order to delineate the toxicity, to decide whether the peptides were transported into the cells and to look at the metabolism of the peptides, a three-pronged approach was utilized. Minimum inhibitory concentration assays would give information on the toxicity of the peptides,

competition of transport assays would answer questions about the transport of the peptides and in vitro enzyme assays in the presence of the peptides would tell us about the metabolism of the drug-peptide conjugates. Toxicity assays by the method of Fisher and Armstrong (19) showed that the peptides tested and 5-FO were not toxic to the yeast. Possible reasons for the non-toxicity were (1) the non-transport of the peptides and 5-FO, (2) transport and non-toxicity of the peptides and 5-FO, and (3) transport and the failure of the peptides to be cleaved thereby not releasing the drug.

The poor transport of 5-FO has been reported (7). Evidence supporting this was the poor competition of 5-FO with OA (Figure 6, page 25) and the direct assays of radioactive 5-FO uptake (Figures 6, 7, and 8, pages 25, 28 and 30, respectively). Although different conditions were employed to optimize 5-FO transport, these efforts were unsuccessful. A pH profile for uptakes could not be constructed, use of a poor (Ile) as well as a richer (NH_4NO_3) nitrogen source for growth showed no improvement and "energization" of the cells was not helpful. It was concluded 5-FO is not toxic to this strain of Candida probably because of its poor uptake.

Transport of the peptides was assayed indirectly by competition of transport assays and the data were analyzed by use of a Lineweaver-Burk plot. Among the peptides

tested OL₂ was the best inhibitor of Met₃ (Table 2, page 21). A possible explanation of this could be that OL₂ was recognized by the oligopeptide transport system as a peptide similar to Leu₃. This compound competes especially well with Met₃ (Table 2) apparently because of its hydrophobic nature. Fluoroorotyl-leucyl-leucine was the only fluorinated peptide to compete with Met₃, though the competition was not as good as with OL₂. The high electronegativity of fluorine may play a role in altering the configuration of the peptides and the chemistry of neighboring atoms thus affecting the recognition of the peptide by the carrier protein. None of the peptides blocked at the carboxy-terminus competed with Met₃ transport. This was expected as a free carboxy terminus is necessary for transport of a peptide by Candida albicans WD 18-4 (27, 28). An interesting point was the inhibition of Met₃ uptake by DMF at 0.9 percent final concentration. At this level DMF inhibited Met₃ while at 0.7 percent it did not (Figures 2 and 3, pages 19 and 20, respectively). Although the difference between the two concentrations was slight, there was a pronounced difference in inhibition. This result served to emphasize the omnipresent need for the proper controls for uptakes.

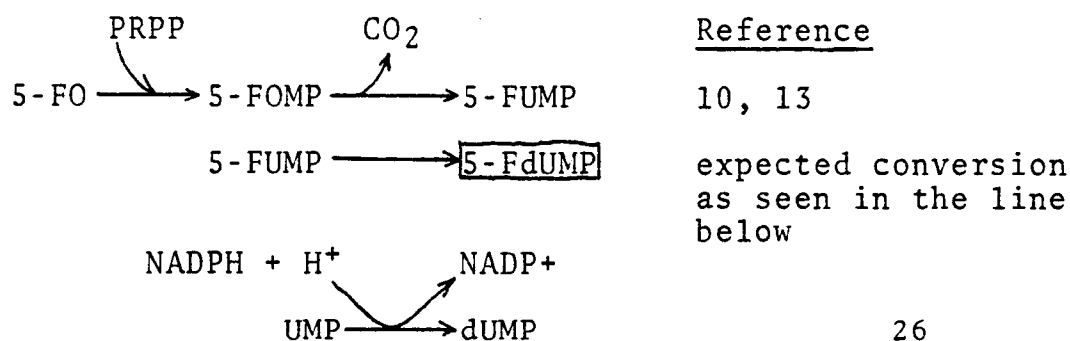
Lineweaver-Burk plot analysis showed competitive inhibition of Met₃ by FOL₂. Although this does not prove actual transport of the drug-peptide into the cells, it

does show an affinity of FOL₂ for the Met₃ carrier. Since Met₃ is transported via a carrier mediated, saturable system (28), it appears that FOL₂ is likewise getting through this particular system.

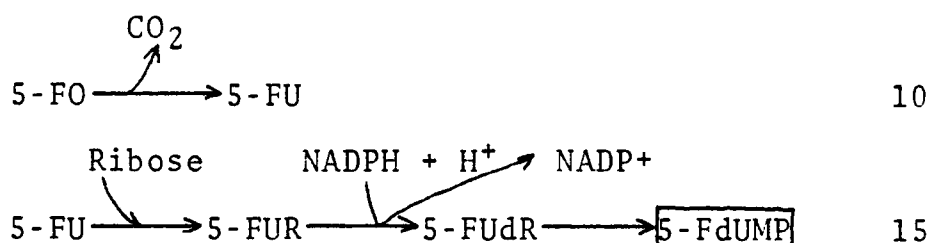
Competition assays between OA and the peptides were also done. None inhibited OA uptake indicating that they were not transported through this system. This was not surprising as it seemed unlikely that the entry of such dissimilar compounds occurred by a common system. There was no inhibition of OA uptake by DMF (Table 3, page 27).

Fluoroorotyl-leucyl-leucine was not toxic to Candida but appeared to be transported into the cells. Assuming the drug-peptide was transported intact and once inside was not cleaved to release 5-FO, it was still possible that the intact peptide could be toxic to the cells. Accordingly, FOL₂ was tested to determine if it inhibited the conversion of OA to OMP and UMP. The intact peptide, under the conditions employed, was not inhibitory (Figures 10 and 11, pages 32 and 34, respectively). This evidence pointed to the fact that even if the peptide was accumulated, it would not be toxic via a mechanism involving the major de novo synthesis of pyrimidines. Whether the peptide could inhibit enzymes other than the ones tested is not known.

It is known that once 5-FO gets into cells, it is a competitive substrate for PRTase and ODCase (7, 13). This was shown to be also true in Candida (Figures 10 and 11, pages 32 and 34, respectively). One point worth noting is that in mammalian tissues PRTase and ODCase exist as a bifunctional complex (31) which means that OMP does not accumulate and is quantitatively converted to UMP. However, in Saccharomyces the enzymes are not found as a complex and could be resolved by conventional purification techniques (34). Thus, OMP would be expected to accumulate and not be converted quantitatively to UMP (31). This was also the case with Candida (compare Figures 10 and 11) where more OMP was produced. In addition, 5-FO, once inside the cells, could be metabolized as outlined below:

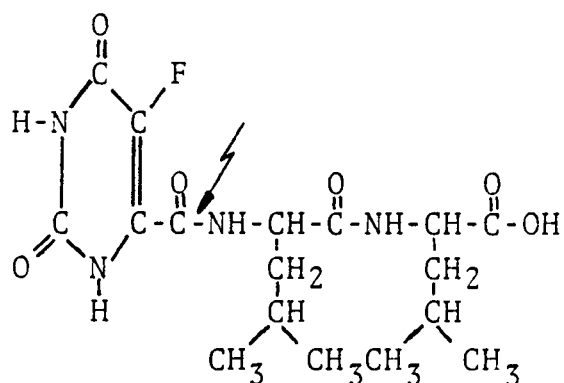


and/or



In both outlines it can be seen that 5-FO is converted to 5-FdUMP. This compound is now thought to be involved in the mode of action of 5-FC (15); it is a potent inhibitor of thymidylate synthetase. This enzyme is essential in DNA biosynthesis because it converts dUMP to dTMP (26). Since 5-FO is a substrate for enzymes of Candida, the non-toxicity of 5-FO appears to be due to its lack of transport. So, if 5-FO is carried in and released from the drug-peptide, it should be toxic to Candida.

It was already mentioned that FOL₂ did not inhibit PRTase and ODCase. It has been observed in our lab that when FOL₂ was incubated (90 minutes) with a cell extract of C. albicans WD 18-4, the breakdown of FOL₂ to 5-FO and dileucine was not observed. However, leucine and an unidentified fluorescent compound (most probably FOL) were detected. In addition, CHOP cell extract, serum or pure hog acylase I, even after extended incubation, failed to release 5-FO from FOL₂. These results point to the fact that 5-FO is not cleaved off of FOL₂. The amide bond formed during conjugation (see arrow below) was expected to be stable to hydrolysis. However, it is reasonable to assume that the inability of the 5-FO to be enzymatically cleaved off of the peptide is the reason for its non-toxicity.



The results could be summarized:

1. 5-FU is not toxic, FOL₂ is not toxic.
2. 5-FU is not transported, FOL₂ apparently gets in.
3. 5-FU is a substrate for and competes for enzymes of pyrimidine biosynthesis.
4. FOL₂ does not inhibit these enzymes.
 - a. It is not toxic as an intact peptide.
 - b. 5-FU is not released from the peptide conjugate by the cells' enzymatic contents.

Although the peptides that were synthesized and tested for biological activity were not toxic, it was shown that the working hypothesis could be realized. That is, it was shown that 5-FU can be carried into cells through the peptide transport system.

Future work on this subject will involve drug-peptide conjugates with different bond characteristics so that the drug could be cleaved off more readily. Another approach will make use of the drug polyoxin D. This compound is an inhibitor of the synthesis of chitin. Chitin

is a structural macromolecule found in the walls of yeast and not in mammalian cells. Polyoxin D-peptide conjugates that will be synthesized are expected to be specific for the pathogen because of the differences in the cell envelopes of yeast and mammalian cells.

REFERENCES

REFERENCES

1. Ames, B. N., Ames, G. F-L., Young, J. D., Tsuchiya, D., and Lecocq, J. (1973) Proc. Natl. Acad. Sci. U.S.A. 70:456.
2. Atherton, F. R., Hall, M. J., Hassall, C. H., Lambert, R. W., and Ringrose, P. S. (1979) Antimicrob. Ag. Chemother. 15:677.
3. Becker, J. M. and Naider, F. (1977) Arch. Biochem. Biophys. 178:245.
4. Becker, J. M., Steinfeld, A., and Naider, F. (1977) Proc. Fourth Intl. Conf. Mycoses, PAHO, Scientific Publication No. 356, pp. 303-308.
5. Bennett, J. E. (1974) N. Engl. J. Med. 290:320.
6. Bennett, J. E. (1977) Ann. Internal Med. 86:319.
7. Bono, V. H., Jr., Cheng, C. C., Frei, E. III, and Kelly, M. G. (1964) Cancer Rsch. 24:513.
8. Bray, G. A. (1960) Anal. Biochem. 1:279.
9. Burrows, W. (1973) Textbook of Microbiology. W. B. Saunders, Philadelphia, PA. pp. 9, 158.
10. Chaudhuri, N. K., Montag, B. J., and Heidelberger, C. (1958) Cancer Rsch. 18:318.
11. Cohen, S. S., Flaks, J. G., Barner, H. D., Loeb, M. R., and Lichtenstein, J. (1958) Proc. Natl. Acad. Sci. U.S.A. 44:1004.
12. Csaky, T. Z. (Ed.). (1975) Intestinal Absorption and Malabsorption. Raven Press Publishers, New York City. pp. 95-111.
13. Dahl, J. L., Way, J. L., and Parks, R. E., Jr. (1959) J. Biol. Chem. 234:2998.
14. Danneberg, P. B., Montag, B. J., and Heidelberger, C. (1958) Cancer Rsch. 18:329.
15. Diasio, R. B., Bennett, J. E., and Myers, C. E. (1978) Biochem. Pharmacol. 27:703.

16. Eisenberg, H., Katchalski-Katzir, E., and Manson, L. A. (Eds.). (1975) Biomembranes, Vol. 7, Chap. 7.
17. Fendler, J. H. and Romero, A. (1977) Life Sciences 20:1109.
18. Fickel, T. E., and Gilvarg, C. (1973) Nature New Biol. 241:161.
19. Fisher, B. D. and Armstrong, D. (1977) Antimicrob. Ag. Chemother. 12:614.
20. Ghose, T. and Blair, A. H. (1978) J. Natl. Cancer Inst. 61:657.
21. Golovinsky, E., Maneva, L., Markov, K. I., and Karamanov, G. (1973) Comptes rendus de L'Academie bulgare des Sciences 26:1399.
22. Hart, P. D., Russell, E., Jr., and Remington, J. S. (1969) J. Inf. Dis. 120:169.
23. Heidelberger, C., Chaudhuri, N. K., Danneberg, P., Mooren, D., Greisbach, L., Duschinsky, R., Schnitzer, R. J., Plevin, E., and Scheiner, J. (1957) Nature 179:663.
24. Heidelberger, C., Greisbach, L., Montag, B. J., Mooren, D., and Cruz, O. (1958) Cancer Rsch. 18:305.
25. Kenig, M. and Abraham, E. P. (1976) J. Gen. Micro. 94:37.
26. Lehninger, A. L. (1975) Biochemistry. Worth Publishers, Inc., New York City.
27. Lichliter, W. D., Naider, F., and Becker, J. M. (1976) Antimicrob. Ag. Chemother. 10:483.
28. Logan, D. A., Becker, J. M., and Naider, F. (1979) J. Gen. Micro. 114:179.
29. Miller, R. P. and Bates, J. H. (1969) Ann. Internal Med. 71:1089.
30. Payne, J. W. (1976) Adv. Microb. Physiol. 13:55-113.
31. Reyes, P. (1977) Anal. Biochem. 77:362.
32. Reyes, P. and Gunganig, M. E. (1975) J. Biol. Chem. 250:5097.

33. Steinfeld, A. S., Becker, J. M., and Naider, F.
(1979) J. Med. Chem. 22:1104.
34. Umezu, K., Amaya, T., Yoshimoto, A., and Tomita, K.
(1971) J. Biochem. 70:249.
35. Vogel, H. J. (1956) Microb. Gen. Bull. 13:42.

VITA

Ali Gulumoglu was born September 21, 1954 in Tekirdağ, Turkey. He attended elementary school in Turkey until the fifth grade. November 5, 1964, he and his family emigrated to New York City, where he finished elementary school, junior high school, and graduated with merit from Erasmus Hall High School in June 1972. The following September he matriculated at The City College of C.U.N.Y., graduating with a B.S. in Biology in 1976. In the Fall of 1977 he started Graduate School at The University of Tennessee, Knoxville, and received a M.S. in Microbiology in March of 1980. During his graduate studies he was awarded graduate teaching and research assistantships. The author was the winner of the graduate student competition, physiology section, for presenting his work at the meetings of the Kentucky-Tennessee Branch of the American Society for Microbiology, October 1979.

Ali has been accepted to dental school and will be attending the S.U.N.Y. at Stony Brook beginning the Fall of 1980.

January 17, 1980 was a happy occasion for the author as he became a citizen of the United States.