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# The Effects of DL-*p*-Fluorophenylalanine and L-3-Nitrotyrosine on the Growth and Biochemistry of the Taper Liver Tumor[1]

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## SUMMARY

Two amino acid analogs, DL-*p*-fluorophenylalanine and L-3-nitrotyrosine, retarded the growth of Taper liver tumor in mice and increased mouse survival time. Both of the analogs were taken into the tumor cells *in vitro* and *in vivo*, and DL-*p*-fluorophenylalanine inhibited the uptake of L-phenylalanine *in vitro*.

tRNA in the "pH 5 fraction" from tumor cells could be acylated with DL-*p*-fluorophenylalanine-$^{14}$C, and DL-*p*-fluorophenylalanine-$^{14}$C was incorporated into protein of tumor cells *in vivo*. DL-*p*-Fluorophenylalanine also caused inhibition of L-phenylalanine tRNA formation.

Pyrophosphate formation could not be detected after incubation of L-3-nitrotyrosine with the pH 5.0 fraction from tumor cells, and there was no evidence of L-3-nitrotyrosine incorporation into tumor protein *in vivo*. Very high concentrations of L-3-nitrotyrosine were required to inhibit L-tyrosine tRNA formation *in vitro*.

The studies, therefore, showed that there was a relationship between the biochemical effects of DL-*p*-fluorophenylalanine and its retardation of growth, while this relationship did not seem quite as significant with L-3-nitrotyrosine.

## INTRODUCTION

The growth-inhibitory properties of the numerous amino acid analogs which have been studied may result from several different effects (4). Several studies have been carried out on the effects of amino acid analogs on tumor growth (1, 2, 6, 8, 11, 15, 16). Other studies have reported some biochemical effects of analogs with tumor systems (12, 13, 17, 19, 20). This study is an attempt to correlate the effects of amino acid analogs on tumor growth with the biochemical effects of the amino acid analogs. The Taper liver tumor was chosen for study since it is nonregressive and was readily available. Preliminary studies with 18 different amino acid analogs of 12 natural amino acids indicated that FPhe[2] and $NO_2$Tyr were consistently effective in retarding the growth of the Taper liver tumor. For this reason and because an earlier study indicated that phenylalanine and tyrosine are important to the growth of some tumors (9), these 2 analogs were chosen for study.

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[2] The abbreviations used are: FPhe, DL-*p*-fluorophenylalanine; $NO_2$Tyr, L-3-nitrotyrosine.

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## MATERIALS AND METHODS

**Maintenance of the Tumor.** Taper liver tumor cells (21) were maintained and studied in their ascitic form in Swiss-Webster mice and were administered i.p. (0.1 ml, approximately $5 \times 10^5$ cells) for both maintenance and study.

**Analogs and Amino Acids.** FPhe and L-phenylalanine were purchased from Sigma Chemical Company (St. Louis, Mo.), and $NO_2$Tyr and L-tyrosine were purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio). FPhe-$^{14}$C-3 was obtained from Calatomic (Los Angeles, Calif.), and uniformly labeled L-phenylalanine-$^{14}$C and uniformly labeled L-tyrosine-$^{14}$C were obtained from Amersham/Searle Corporation (Toronto, Ontario, Canada). All of the analogs and amino acids were examined chromatographically to ensure that they were not broken down by the autoclaving process used to sterilize the solutions.

**Growth Retardation Studies.** Each study was composed of the following 4 groups: Group 1, the control group (no analog, no tumor); Group 2, tumor group (0.1 ml of tumor cells on Day 1); Group 3, amino acid analog group (1 injection each day for 5 days); and Group 4, tumor-amino acid analog group (0.1 ml of tumor cells on Day 1 and analog injected each day for 5 days). Each group contained 5 female mice (20 to 25 g). Separate studies were carried out at each of several different dosages of analog (FPhe or $NO_2$Tyr), and a control study with sterilized water instead of analog was run (composed of 4 groups as above). The average change in weight, following treatment, was used to estimate tumor growth. The significances of differences of growth and of mouse survival time between Groups 2 and 4 of each study were determined with the *t* test.

**Uptake of Amino Acids and Analogs.** Forty-eight hr after inoculation, tumor cells were collected on ice, and 0.5-ml samples of the cells were suspended in 3.0-ml portions of Krebs-Ringer bicarbonate buffer, pH 7.4. FPhe-$^{14}$C (0.06 mmole) or $NO_2$Tyr (0.06 mmole) was added to the solution. The mixture was incubated for 60 min at 37°, and the reaction was stopped by rapid chilling. The extracellular and intracellular concentrations were determined (14). Zero time and lysed cell controls were run. FPhe-$^{14}$C contents were estimated by the cpm (dioxane-base scintillation fluid: 1 liter of dioxane, 70 g of naphthalene, 7 g of PPO, and 0.05 g of POPOP). For $NO_2$Tyr, $A_{420}$ was used to estimate the concentrations.

A study was carried out to determine inhibition of phenylalanine-$^{14}$C (0.01 mM) uptake by various

concentrations of FPhe. (Uptake of phenylalanine-$^{14}$C was determined as described for FPhe-$^{14}$C.)

*In Vivo* **Loss of Analog and Amino Acid.** Groups of mice with tumors (48 hr) were given injections of 7 μmoles of either FPhe-$^{14}$C, $NO_2$ Tyr, Phe-$^{14}$C, or Tyr-$^{14}$C and were sacrificed at various times. The peritoneal fluids (serum and tumor cells) were collected, and the radioactivity of the fluids (except in the case of $NO_2$ Tyr) was determined in a toluene-base scintillation fluid (1 liter of toluene, 4 g of PPO, and 0.05 g of POPOP). For $NO_2$ Tyr estimation, the fluids were placed into a boiling water bath for 5 min (to lyse the cells), centrifuged at 4000 × *g* for 15 min, and the supernatant was spectrophotometrically analyzed ($A_{420}$). The extracellular and intracellular concentrations of $NO_2$ Tyr and FPhe-$^{14}$C remaining at various times after administration were also studied. The cells were collected over ice and studied as in the uptake studies.

**Aminoacyl tRNA Formation.** Ascitic fluid (72 hr after tumor injection) was collected over ice. A pH 5 fraction (10) was prepared from the tumor cells and from liver cells. Aminoacyl-tRNA formation was estimated in a reaction mixture containing 20 to 30 mg ($A_{280}/A_{260}$) of pH 5 fraction protein (10) in 5 ml of buffer containing the following compounds: 0.05 M Tris (pH 7.4), 0.01 M ATP·$MgCl_2$, and $2 \times 10^{-7}$ M labeled amino acid. Incubation was at 37° for 10 min. After the pH of the mixture was adjusted to 5.2 with 1.0 M acetic acid, the aminoacyl-tRNA was isolated by the method of Hoagland *et al.* (72) and collected on a Millipore filter (1.2 μ). Filters were dried before counting (toluene-base scintillation fluid). Controls without ATP were routinely run.

Since radioactive $NO_2$ Tyr was not available, the pyrophosphate formed in assay mixtures with $NO_2$ Tyr which were incubated as above was determined. Activated charcoal (2.5 g) was added to the pH 5.2 supernatants (15 ml) to absorb ATP and AMP. The slurry was filtered, and the cake was washed with 15 ml of $H_2O$. Pyrophosphate in a 1-ml aliquot was converted to inorganic phosphate with 0.5 ml of 3 N HCl (100°, 20 min). The inorganic phosphate was then measured by the method of Chen *et al.* (3).

**Analog Incorporation into Tumor Proteins.** One group of mice, with 72-hr tumor cells, was given injections of 7 μmoles of FPhe-$^{14}$C (specific activity, 1.5 mCi/μmole). Another group was given injections of 7 μmoles of $NO_2$ Tyr. One hr later, the tumor cells were collected and then lysed by the addition of 5 volumes of distilled water. The solution was brought to 10% trichloroacetic acid. Precipitates which were collected by centrifugation at 4000 × *g* for 10 min were resuspended and homogenized in water and reprecipitated. The process was repeated 3 times. The final precipitate was dialyzed extensively against several changes of distilled water, and a portion was counted. Some protein which was hydrolyzed in 6 N HCl for 24 hr was examined for $NO_2$ Tyr by $A_{420}$ (after adjustment to pH 9.0). Other aliquots of protein (native and hydrolyzed in 6 N HCl) were spotted directly onto paper for high-voltage electrophoresis (Whatman No. 3, pH 2.1, 2500 V). FPhe-$^{14}$C was detected with a Packard Model 385 radioactive scanner, and $NO_2$ Tyr was detected by the appearance of yellow spots after exposure to ammonia fumes.

## RESULTS

**Effects of Analogs on Tumor Growth.** Various dosages of FPhe and $NO_2$ Tyr significantly decreased the rate of tumor growth in ascitic mice (Table 1). The weights of mice without tumors were not affected by the analogs. When survival times of mice were compared, significant increases for mice in groups treated with analog were obvious (Table 1). Sterilized water did not affect the growth rate of the tumors or the survival time.

**Uptake Studies.** The intracellular concentration of FPhe tripled in 60 min as compared to zero time (Table 2). Timed studies showed that the increase in intracellular concentration of FPhe was linear with time up to 2 hr. The high intracellular concentration of radioactive material at zero time was probably FPhe-$^{14}$C that was rapidly bound to the cell membrane and was washed off by the lysing process (boiling water) and so tended to give a high value. This is shown by the high intracellular reading after 60 min of incubation with lysed

Table 1

*Mouse growth rates and survival times*

Average growth rates and survival times (days after tumor initiation) of groups of mice with tumors treated with various dosages of analog. Tumor growth was estimated by increases in weight of mice per day, calculated at 5 days. The *t* test[a] was carried out between mice with no analog (tumor groups) compared to groups given various doses of analog (tumor analog groups).

| Total dose of analog | Growth (g/day) | Significant level of growth by *t* test (%) | Survival time | Significant level of survival time by *t* test (%) |
|---|---|---|---|---|
| No analog | 7.0 ± 0.7 | | 6.7 ± 0.6 | |
| FPhe, 35 μmoles | 3.4 ± 0.5 | 99 | 13.4 ± 0.7 | 97.5 |
| FPhe, 70 μmoles | 4.2 ± 0.3 | 97.5 | 11.4 ± 0.9 | 95 |
| FPhe, 140 μmoles | 4.6 ± 0.5 | 97.5 | 10.7 ± 1.1 | 95 |
| $NO_2$ Tyr, 35 μmoles | 3.6 ± 0.1 | 99.5 | 12.7 ± 0.8 | 97.5 |
| $NO_2$ Tyr, 105 μmoles | 3.3 ± 0.7 | 99.5 | 8.7 ± 1.0 | 75 |
| $NO_2$ Tyr, 140 μmoles | 2.0 ± 0.1 | 99.95 | 10.8 ± 0.7 | 97.5 |
| $NO_2$ Tyr, 175 μmoles | 2.2 ± 0.6 | 99 | 9.8 ± 1.0 | 90 |
| Sterilized $H_2O$ | 7.1 ± 0.6 | | 6.7 ± 0.4 | |

[a] Ref. 5.

Table 2

*Uptake studies*

The location of the counts after *in vitro* uptake studies of FPhe-$^{14}$C, $NO_2$Tyr, and phenylalanine-$^{14}$C in the presence of various concentrations of FPhe. For FPhe-$^{14}$C and $NO_2$Tyr, 0.06 mmole was added to 3.0 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) with 0.5 ml of tumor cells. For the inhibition studies, 0.01 mmole of Phe-$^{14}$C was added to each mixture, and the incubations were for 60 min.

| | Intracellular (μmoles/ml) | Extracellular (μmoles/ml) |
|---|---|---|
| FPhe | | |
| 0 min | 0.27 ± 0.02 | 0.18 ± 0.02 |
| 60 min | 0.80 ± 0.08 | 0.16 ± 0.01 |
| 60 min lysed | 0.40 ± 0.04 | 0.20 ± 0.01 |
| $NO_2$Tyr | | |
| 0 min | 0.8 ± 0.1 | 35.0 ± 1.0 |
| 30 min | 3.5 ± 0.1 | 35.0 ± 1.0 |
| FPhe/Phe-$^{14}$C ratio | | |
| 0 | 1.09 ± 0.10 | 0.032 ± 0.003 |
| 1 | 0.80 ± 0.06 | 0.046 ± 0.003 |
| 10 | 0.50 ± 0.05 | 0.058 ± 0.005 |
| 100 | 0.45 ± 0.03 | 0.061 ± 0.005 |
| Zero time (control) | 0.11 ± 0.01 | 0.075 ± 0.006 |

cells. Table 2 also indicates that $NO_2$Tyr was taken into the cell and that FPhe inhibited phenylalanine-$^{14}$C uptake.

***In Vivo* Loss of Analogs and Amino Acids.** Injected analog and amino acid were both rapidly lost from the i.p. cavity, but about 5 to 10% of the initial amount injected remained after 24 hr. There was little difference in the rate of loss between the analogs and the amino acids. The intracellular and extracellular concentrations of FPhe and $NO_2$Tyr with time after injection showed that both analogs were rapidly taken into the intracellular region and only slowly lost from this region with time. The loss of extracellular FPhe-$^{14}$C and $NO_2$Tyr was much more rapid. The intracellular FPhe-$^{14}$C concentration 24 hr after injection was still 20% as great as shortly after injection while the extracellular concentration was only about 2% as great.

**Aminoacyl-tRNA Formation.** After a 30-min incubation, a significant amount of FPhe-$^{14}$C-tRNA was formed by the tumor preparation (0.037 ± 0.002 μmole). Studies showed that the formation was linear with time only up to 30 min. The formation of a significant amount of FPhe-$^{14}$C-tRNA by a liver preparation was not observed even after longer times.

Pyrophosphate assays showed that although the assay with tyrosine formed pyrophosphate (0.63 ± 0.05 μmole/30 min), none was formed with $NO_2$Tyr in tumor systems (even after longer periods of incubation). $NO_2$Tyr also did not form pyrophosphate in liver systems.

Chart 1 shows the inhibitory effect of FPhe on the formation of Phe-$^{14}$C-tRNA in tumor and liver systems. The drop in rate of formation of Phe-$^{14}$C-tRNA at low ratios of FPhe to Phe-$^{14}$C was greater with the liver preparation than with the tumor preparation. With higher ratios, the inhibitory effect in both tissues was not as great, and the formation of Phe-$^{14}$C-tRNA began to be inhibited more in tumor preparations than in liver preparations. At a ratio of FPhe to Phe-$^{14}$C of 100, there was almost complete inhibition of the tumor system while the rate was still about 30% with the liver system. Further similar experiments confirmed the complete

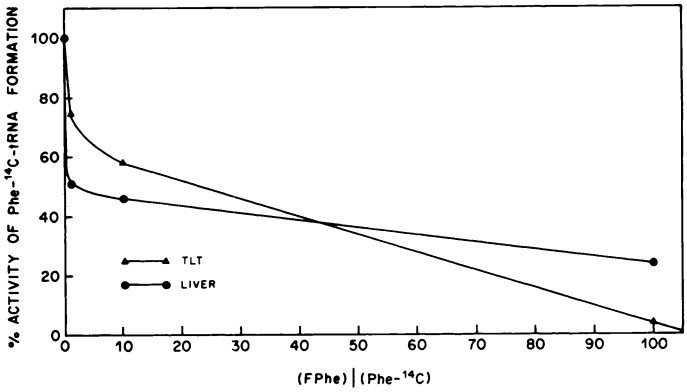


Chart 1. Percentage of Phe-$^{14}$C-tRNA formed as a function of the ratio of concentrations of FPhe to Phe-$^{14}$C. The concentration of Phe-$^{14}$C was kept constant at $2 \times 10^{-7}$ M. The amount of Phe-$^{14}$C-tRNA formed in the absence of FPhe is considered 100% activity. The results are based on 10-min assays. •, liver pH 5 fraction; ▲, Taper liver tumor (*TLT*) pH 5 fraction.

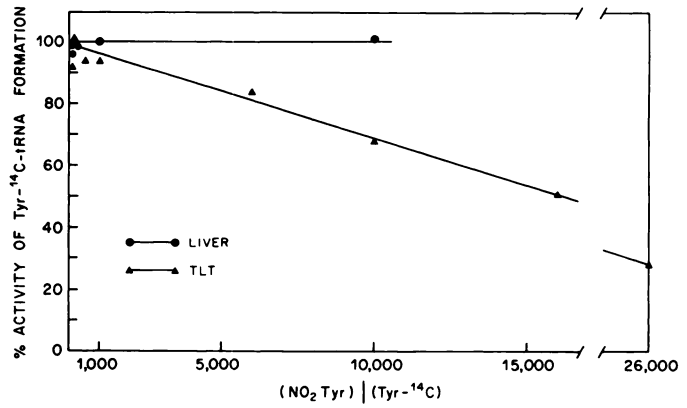


Chart 2. Percentage of Tyr-$^{14}$C-tRNA formed as a function of the ratios of the concentrations of $NO_2$Tyr to Tyr-$^{14}$C. The concentration of Tyr-$^{14}$C was held constant at $2 \times 10^{-7}$ M. The amount of Tyr-$^{14}$C-tRNA formed in the absence of $NO_2$Tyr is considered 100% activity. The results are based on 10-min assays. •, liver pH 5 fraction; ▲, Taper liver tumor (*TLT*) pH 5 fraction.

inhibition of the tumor fraction at a ratio of about 100 while about one-third of the liver rate remained.

Chart 2 shows the inhibition of Tyr-$^{14}$C-tRNA formation in tumor and liver systems by $NO_2$Tyr. The liver preparation was resistant to inhibition at the ratios studied. The tumor system was inhibited, but a very large ratio of $NO_2$Tyr to Tyr-$^{14}$C (16,000) was required to achieve 50% inhibition.

**Incorporation of Analog into Tumor Protein.** The hydrolyzed protein sample from mice given injections of FPhe-$^{14}$C resulted in activity at the area where authentic FPhe-$^{14}$C migrated. For the unhydrolyzed sample, all of the activity remained near the origin. In no case was $NO_2$Tyr observed in the tumor protein preparations. The amount of FPhe incorporated per mg of protein was about 0.21 ± 0.01 μmole. If even 10% as much $NO_2$Tyr would have been present, it could have been detected by its absorption at 420 nm.

## DISCUSSION

The results indicate that there is a definite relationship between the effect of FPhe on retarding the growth and increasing the survival time of the Taper liver tumor and its effect on the biochemical processes studied. The biochemical effects of $NO_2$ Tyr were lesser than those of FPhe. The actual amount of retardation of tumor growth probably depends partially on the size of the natural amino acid pools. Free tyrosine, if normally present in very small pool concentrations, might allow the tumor to be affected by $NO_2$ Tyr even if the biochemical effects of $NO_2$ Tyr are not dramatic. L-Nitrotyrosine may also have other effects on biochemical or physiological processes which were not studied.

Other significant results were the distinctly different patterns of inhibition observed for analogs in tumor cells as compared to liver cells. At a ratio of FPhe to phenylalanine of about 100, the tumor Phe-tRNA synthesis was almost completely inhibited while the liver system was still partially active. Concentrations of both FPhe and $NO_2$ Tyr should be possible so that these functions can be greatly inhibited in tumors while they would remain normal in other tissue. The 2-phase inhibition curves seen for FPhe (Chart 1) suggest the occurrence of 2 Phe-tRNA synthesis systems, one of which is easily inhibited by FPhe and the other which is not. Two $tRNA^{Phe}$'s are known to exist in *Escherichia coli* (17).

Very little work has been previously reported of the biochemical effects of FPhe or $NO_2$ Tyr in conjunction with a study of their tumor growth-retarding effects. Tamemasa *et al.* (19, 20) reported that FPhe inhibited the activation of phenylalanine and stimulated the accumulation of free phenylalanine in Ehrlich ascites tumor. FPhe was found by Rabinovitz *et al.* (12) to inhibit activation of phenylalanine in Ehrlich ascites tumor. Ryan and Elliott (15) showed that growth of 2 types of tumor in mice were inhibited by p.o. doses of FPhe when administered in conjunction with a low-phenylalanine diet. Biesele and Jacquez (2) earlier had shown that growth of mouse sarcoma T241 and 180 tumors were inhibited by FPhe. Each of these studies reported either only the growth-retarding effects of the analogs or only some of the biochemical effects. This study was an effort to study the relationship of these effects.

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