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Intracellular uptake and inhibitory activity of aromatic fluorinated amino acids in human breast cancer cells

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Nonproteinogenic amino acids that either occur naturally or are synthesized chemically are becoming important tools in modern drug discovery. In this context, fluorinated amino acids have great potential in the development of novel pharmaceuticals and drugs. To assess whether different fluorinated aromatic amino acid analogues of phenylalanine, tyrosine, and tryptophan are potentially interesting as therapeutic drugs, we examined their cytostatic and cytotoxic effects on the growth of the human breast cancer cell line MCF-7. Of all the tested analogues L-4-fluorotryptophan, L-6-fluorotryptophan and L-p-fluorophenylalanine effectively and irreversibly inhibited cell growth with IC_{50} values in the low micromolar range (3–15 μM). Additionally, using L-4-[^{14}C]fluorotryptophan, and L-6-[^{14}C]fluorotryptophan, we discovered that the cellular uptake of these fluorinated amino acids occurs through active transport with a 70-fold excess of intracellular over extracellular concentrations. We identified system L as the responsible amino acid transporter. Our findings fully support the idea that fluorinated aromatic amino acid analogues are promising chemotherapeutics with the potential for use in combination with classical cancer therapy, and as new cytotoxic drugs for certain tumor types such as melanoma.

Introduction

A number of different micro-organisms, fungi and plants produce many unusual amino acid-like substances that are toxic to other species.[1] For example, some marine organisms developed chemical defenses against predators or as preying tools; these are derived from post-translational conversion of amino acids into pharmacologically active substances by halogenation.[2] In fact, natural organohalogens occur in a wide variety of both marine and terrestrial species.[3] The number of known, naturally occurring, halogenated compounds is steadily increasing, and new chemotherapeutics including antibiotics are expected to arise from these novel compounds.[4] The first well-characterized, naturally occurring, post-translational halogenated amino acids to be identified were iodo, bromo and chloro derivatives of tyrosine in vertebrate tyreoglobulins and invertebrate scleroproteins.[5] Unsurprisingly, halogenated compounds have been extensively investigated for more than half a century.[6]

Curiously, among the thousands of halogenated substances present in nature, only a few organofluorine compounds participate in the chemistry of living cells, despite fluorine being one of the most abundant elements.[7] Conversely, a wide variety of synthetic fluorinated compounds, including fluorine-containing amino acids, have been available for a long time.[8] Of all halogen-containing amino acids, the fluorine derivatives possess the most interesting properties.[9] For example, fluorine substitution causes minimal structural perturbations since CH→CF modifications can be easily accommodated in natural compounds such as proteins.[10] In material sciences as well as in synthetic biology, fluorinated amino acids are often used for the design of biomaterials,[11] proteins and even cellular structures with superior properties compared with the parent forms.[12] Monofluorinated analogues of amino acids, in particular those with aromatic side chains, have been used to study the physiology, metabolism and enzymology of living cells as well as in experimental animal studies for many years.[13] Of particular medicinal interest is the possible cytostatic or cytotoxic effects of halogenated amino acids on tissue.[6] The biological activity of fluorinated amino acids was first studied decades ago; the cytotoxicity in rats and mice was determined for *o*-FPhe, *m*-FPhe, *p*-FPhe and *m*-FTyr, with LD_{50} values of ~10 mg kg^{-1} for both *m*-FTyr and *m*-FPhe.[15–17] Additionally, many examples of the cytostatic effect of fluorinated amino acids on the growth of micro-organisms and human cancer cell lines can be found in the literature. For instance, L-isomers of *o*-FPhe, *m*-FPhe, *p*-FPhe, *m*-FTyr, (4-F)Trp, (5-F)Trp and (6-F)Trp all inhibited the growth of the respective auxotrophic

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E. coli strain.[10,18,19] The incubation of HeLa cells with *p*-FPhe (200 μM) in phenylalanine-free medium led to cell cycle arrest.[20] Similarly, the growth of several human cancer cell lines was suppressed by D,L-β-*threo*-fluoroasparagine with IC_{50} values of ~40 μM.[21]

Possible reasons for the cytostatic or cytotoxic effect of these compounds include: i) the inhibition of certain enzymes by the amino acid analogue or an active metabolite, ii) interference with amino acid biosynthesis and amino acid transport, or iii) the incorporation of the amino acid analogue into the proteome.[14,22] Indeed, the in vivo incorporation of fluorinated and other non-canonical amino acids into proteins is now well established.[23] For example, it was possible to almost quantitatively replacement aromatic amino acid side chains with fluorinated analogues in human recombinant annexin A5, azurin and green fluorescent protein, among others.[10,18,19,24,25] These global substitutions did not introduce large structural changes in any of the proteins studied. Other amino acids such as selenomethionine or α-aminobutyrate can be incorporated into whole proteomes of micro-organisms.[26] Similarly, proteome-wide substitutions in mammalian cells have also been reported, for example L-β-*threo*-fluoroaspartate has been incorporated into the proteome of rat hepatoma 7800C1 cells.[27]

Literature data regarding the uptake of halogenated amino acids into cells, and their influence on growth and metabolism are scarce.[14] In order to gain more precise and quantitative data about the potential of fluorinated amino acids in cancer therapies, we studied their effect on the growth of tumor cells. A systematic study of the bioactivity of L-4-fluorotryptophan (L-(4-F)Trp), L-5-fluorotryptophan (L-(5-F)Trp), L-6-fluorotryptophan (L-(6-F)Trp), L-*ortho*-fluorophenylalanine (L-*o*-FPhe), L-*meta*-fluorophenylalanine (L-*m*-FPhe), L-*para*-fluorophenylalanine (L-*p*-FPhe) and and D,L-*meta*-fluorotyrosine (D,L-*m*-FTyr) (Figure 1) against the human breast cancer cell line MCF-7 is described. Furthermore, using [^{14}C]-labeling, we determined the intracellular concentrations of both L-[^{14}C](4-F)Trp and L-[^{14}C](6-F)Trp, and identified the amino acid transport system responsible for the active uptake of the fluorinated aromatic amino acids into the MCF-7 cells.

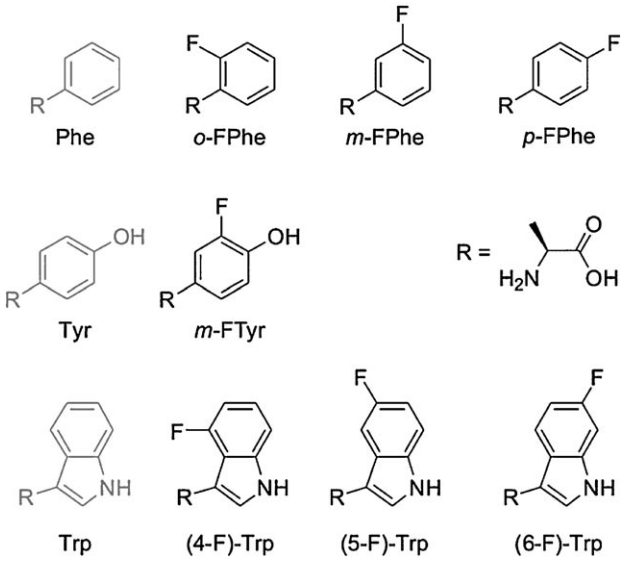

Figure 1. Proteinogenic aromatic amino acids (grey) and their fluorinated derivatives (black). Abbreviations: ***o*-FPhe**: L-*ortho*-fluorophenylalanine or L-*o*-fluorophenylalanine; ***m*-FPhe**: L-*meta*-fluorophenyl-alanine or L-*m*-fluorophenylalanine; ***p*-FPhe**: L-*para*-fluorophenylalanine or L-*p*-fluorophenylalanine; ***m*-FTyr**: L-*meta*-fluorotyrosine or L-*m*-fluorotyrosine; **(4-F)Trp**: L-4-fluorotryptophan; **(5-F)Trp**: L-5-fluorotryptophan; **(6-F)Trp**: L-6-fluorotryptophan.

Results and Discussion

Fluorinated aromatic amino acids inhibit the growth of MCF-7 cells

In order to assess the growth inhibitory effect of the fluorinated amino acids shown in Figure 1, MCF-7 cells were cultured in complete medium, medium lacking Trp, Phe or Tyr, or the depleted medium containing the fluorinated analogue of the deficient amino acid (200 or 400 μM) (Figure 2A). As expected, the withdrawal of any of the essential aromatic amino acids Trp, Phe or Tyr, led to a reduced growth rate of MCF-7. However, slow growth was still observed over a period of at least 58 h in the case of Trp-free medium (Figure 2B). Presumably, protein turnover and recycling in the cells could provide a sufficient amount of Trp to facilitate growth for a short period of time even in the absence of this essential amino acid. In contrast, the presence of L-(4-F)Trp inhibited cell growth completely (Figure 2A and B). Similar effects could be observed for fluorinated Phe (L-*p*-FPhe) and Tyr (D,L-*m*-FTyr), respectively (Figure 2A).

The observed effects of the fluorinated amino acids on cell growth strongly suggest cell cycle arrest (Figure 2B). To further support these findings, we used FACS (Fluorescence-activated cell sorting) to determine the DNA content of cells that were grown for 48 h in complete medium, or in the presence of 5 or 50 μM of L-(4-F)Trp (Figure 2C). Cells grown in complete medium displayed a bimodal distribution of DNA typical of a nonsynchronized cell culture. The majority of the cells were in the G1 phase, and the other significant populations were in the G2 or M phase. In the presence of 5 μM of L-(4-F)Trp the G1 phase population decreased, whereas the G2 or M phase population increased. Incubation of the cells in 50 μM of L-(4-F)Trp resulted in a further decrease in the number of cells in the G1 phase. In addition, cells arrested in the S phase could be discerned. The shift in fluorescence of propidium-stained cells at high concentrations of L-(4-F)Trp might originate from cell damage caused by the inhibitory effect of L-(4-F)Trp. Cells incubated with a higher concentration of L-(4-F)Trp showed a dramatic change in morphology; MCF-7 cells are typically flat and extended, however, high concentrations of L-(4-F)Trp induced a rounded sphere-like cell form (data not shown). Even though typical cytotoxic characteristics of cells such as an increase in membrane permeability was not observed, the growth inhibitory effect induced by L-(4-F)Trp was not reversible after transferring the cells back into complete medium (data not shown). Thus, fluorinated L-Trp, L-Phe, and L-Tyr act as cytostatic compounds, irreversibly suppressing cell proliferation on an experimental time scale of several weeks.

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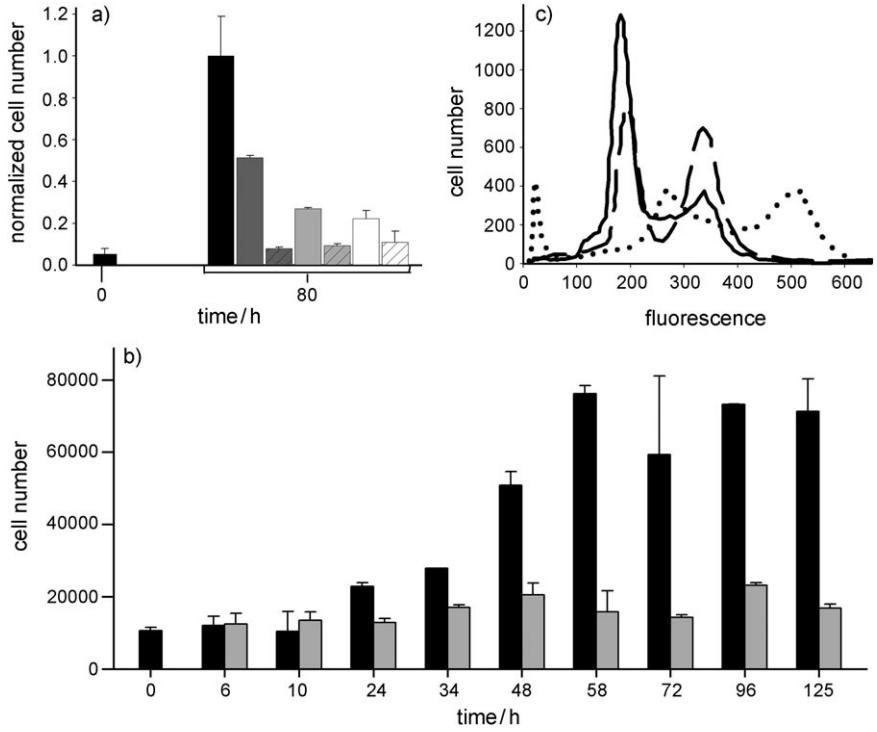

Figure 2. The growth inhibition of MCF-7 cells by fluorinated aromatic amino acids: a) The incubation (80 h) of MCF-7 cells in complete medium (■), medium devoid of Trp (■), Phe (■) or Tyr (□), or medium substituted with 200 μM L-(4-F)Trp (■, hashed), 200 μM L-*p*-FPhe (■, hashed), and 400 μM D,L-*m*-FTyr (□, hashed); b) Time course study on the cytostatic effect of L-(4-F)Trp (■) compared with an absence of Trp (■); c) The DNA content of MCF-7 cells after incubation (48 h) in complete medium (——), Trp-free medium supplemented with 5 μM (– – –) or 50 μM (•••••) of L-(4-F)Trp, as monitored by propidium iodide staining and FACS analysis.

All other fluorinated aromatic amino acids shown in Figure 1 exhibited IC_{50} values of 50–100 μM (Figure 3 and data not shown). The L-isomer and the D,L-racemate of (4-F)Trp displayed identical concentration-dependent growth inhibitory effects, calculated on the basis of the L-isomer concentration in each compound, indicating that only the L-isomer was biologically active. Surprisingly, the inhibitory activity of L-(4-F)Trp was only slightly affected by the addition of unmodified L-Trp. In the presence of complete medium containing 25 μM of L-Trp, the IC_{50} value of L-(4-F)Trp was only twofold higher compared with that measured in Trp-free medium ($IC_{50} = 6.0 \pm 2.5$ μM, Figure 3) suggesting that the biological effect of L-(4-F)Trp cannot be easily remedied. Presumably, once L-(4-F)Trp has entered metabolic pathways, it blocks them irreversibly and without competition from L-Trp.

Efficacy of the growth inhibitory effect

All analyzed fluorinated aromatic amino acids exhibited a growth inhibitory effect. Titration showed that the most potent compounds were L-(4-F)Trp ($IC_{50} = 2.9 \pm 0.6$ μM), L-(6-F)Trp ($IC_{50} = 15.3 \pm 3.5$ μM), and L-*p*-FPhe ($IC_{50} = 11.8 \pm 2.7$ μM).

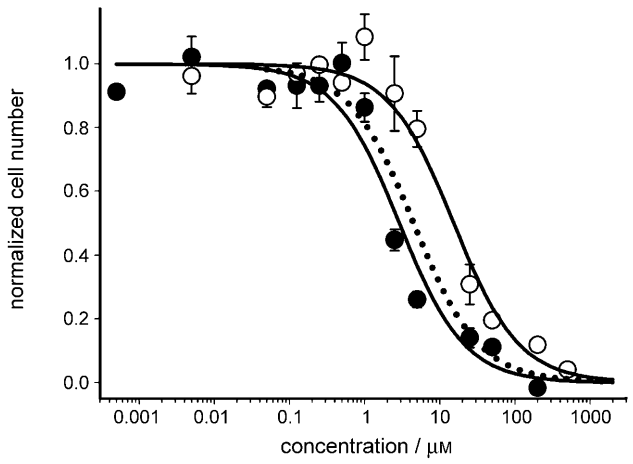

Figure 3. Biological activity of L-(4-F)Trp and L-(6-F)Trp: MCF-7 cells were incubated (80 h) in Trp-free medium with varying concentrations of L-(4-F)Trp (●) and L-(6-F)Trp (○). In addition, the effect of L-(4-F)Trp was quantified in the presence of 25 μM of L-Trp (•••••). Data are means ± S.E. ($n = 3$).

Intracellular concentration of L-(4-F)Trp and L-(6-F)Trp

Fluorinated amino acids effectively inhibited the growth of MCF-7 cells with IC_{50} values in the low micromolar range. Yet, these concentrations only referred to the extracellular concentration of the amino acid in the culture medium. Thus, these data did not distinguish whether the different biological activities of the fluorinated amino acids were due to different efficacies in blocking essential metabolic pathways, or just reflected different uptake efficiencies into the cells.

Therefore, we decided to determine actual intracellular concentrations of L-(4-F)Trp and L-(6-F)Trp by tracking the radio-labeled amino acids. L-Trp can be enzymatically synthesized through a condensation reaction between L-serine and indole, catalyzed by tryptophan synthase (EC 4.2.1.20/Trp-indole-lyase EC 4.1.99.1).[31] In this way, we were able to obtain L-[^{14}C](4-F)Trp and L-[^{14}C](6-F)Trp by enzymatically condensing 4-, and 6-fluoroindole with L-[^{14}C]Ser (Experimental Section).

Using the radio-labeled amino acids, the intracellular concentrations of L-(4-F)Trp and L-(6-F)Trp in MCF-7 cells were monitored and determined. The time course study of L-[^{14}C](6-F)Trp (1.8 μM) showed that a maximum of 530 ± 63 pmol per mg of protein ($139 \pm 16 \times 10^6$ molecules per cell) was reached after ~10 min (Figure 4). Since the cell-associated radioactive signal was affected by the addition of other compounds (see below), the active transport of L-[^{14}C](6-F)Trp is strongly indicated rather than an unspecific association with the cells. The

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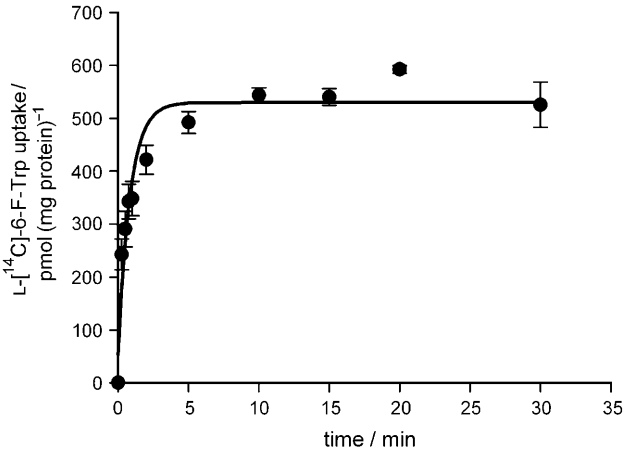

Figure 4. The time course study of L-[^{14}C](6-F)Trp uptake by MCF-7 cells. Data are means ±S.E. ($n=4$).

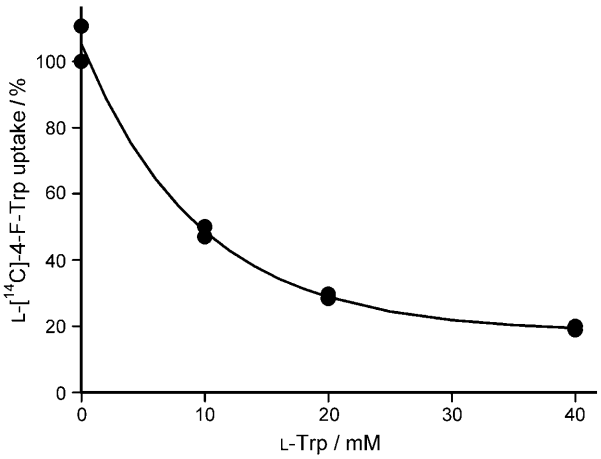

Figure 5. The inhibitory effect of L-Trp on the uptake of L-[^{14}C](4-F)Trp by MCF-7 cells: Cell-associated radioactivity was measured after incubation (2 min) of MCF-7 cells with L-[^{14}C](4-F)Trp (1 μM) in the absence of unlabeled L-Trp (set to 100%), and in the presence of unlabeled L-Trp (10, 20 and 40 mM). Data of two independent measurements are shown.

high concentration of L-[^{14}C](6-F)Trp found in a single cell further supports active uptake over a mechanism involving the binding and blocking of specific carrier systems. Therefore, the radioactive signal could be used to calculate the intracellular concentration of L-(6-F)Trp. The intracellular volume of the cells was determined using radioactively labeled 3-*O*-[^{14}C]methyl-D-glucose; this compound is taken up by cells via alleviated diffusion and thus simply equilibrates between the extracellular and intracellular space. Since 3-*O*-[^{14}C]methyl-D-glucose is not metabolized, its cell-associated radioactive signal can be used to determine the cell volume.[32] For MCF-7 cells this was calculated as 4.4±0.5 μL per mg of protein, corresponding to ~2 pl per single cell (data not shown). Consequently, when starting with an initial extracellular concentration of 1.8 μM of L-(6-F)Trp, the intracellular concentration was calculated as 120±20 μM. Thus, the intracellular concentration exceeded the extracellular one 70-fold, clearly indicating an active transport of L-(6-F)Trp into the cells.

The enzymatic synthesis of L-[^{14}C](4-F)Trp did not reach completion, and the product was used without purification and so contained residual L-[^{14}C]Ser (Experimental Section). To distinguish the uptake of L-[^{14}C](4-F)Trp from that of L-[^{14}C]Ser, the uptake was analyzed in the presence of various concentrations of unlabeled L-Trp. The addition of 40 mM of unlabeled L-Trp to the buffer inhibited the uptake of L-[^{14}C](4-F)Trp completely. The residual cell-associated radioactive signal (20%) can be attributed to the passage of L-[^{14}C]-serine through the cell membrane (Figure 5). The time-dependent uptake of 1 μM of L-[^{14}C]-(4-F)Trp was then examined both in the absence and presence of 40 mM of unlabeled L-Trp (Figure 6A). After correction for the residual L-[^{14}C]Ser signal, an intracellular maximum of 316±30 pmol per mg of protein was reached after ~20 min for L-(4-F)Trp (Figure 6B), corresponding to an intracellular concentration of 72±11 μM, 70-fold greater than the extracellular concentration. Furthermore, starting with an extracellular concentration of 10 μM of L-[^{14}C](4-F)Trp, the intracellular concentration was found to be 788±90 μM (data not shown). Thus, higher extracellular concentrations drive higher intracellular

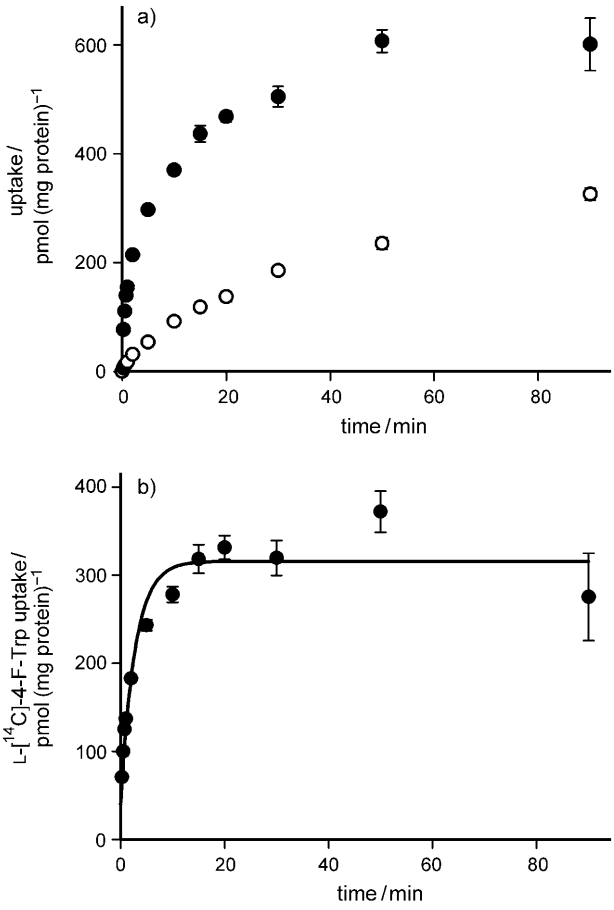

Figure 6. Time-dependent uptake of L-[^{14}C](4-F)Trp by MCF-7 cells: a) The cells were incubated with L-[^{14}C]-L-(4-F)Trp (1 μM) in the presence (○) and absence (●) of unlabeled L-Trp (40 mM); b) Corrected values for the uptake of L-[^{14}C](4-F)Trp by MCF-7 cells, taking into account the residual L-[^{14}C]Ser signal. Data are means ±S.E. ($n=4$).

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ones, generating a growth inhibitory effect as soon as a critical intracellular concentration is reached.

Taken together, the determination of the intracellular concentrations of both L-(6-F)Trp and L-(4-F)Trp indicated that both amino acid analogues were transported with equal efficiency into MCF-7 cells. Both amino acids accumulated in a 70-fold excess within the cells. Thus, the different biological activities of L-(6-F)Trp and L-(4-F)Trp do not arise through a difference in their uptake efficiencies, but presumably due to different metabolic fates upon their intracellular accumulation.

The uptake of fluorinated Trp is mediated by the amino acid transport system L

Many transport systems are capable of transporting amino acids across the cell membrane.[33] To gain insight into which transport system is responsible for the uptake of the fluorinated tryptophan derivatives, we investigated the dependence of L-[^{14}C](6-F)Trp uptake on the energy source, and the substrate specificity of the process (Figure 7). There are three mechanisms by which active transport of amino acids occur. In most cases the active transport of amino acids is driven by either a Na^+ or H^+ gradient. However, neither Na^+ nor H^+ ions stimulated the uptake of L-[^{14}C](6-F)Trp (Figure 7 A). *Trans*-activation of a transport system is a process by which an amino acid is secreted for every amino acid taken up by a cell, and is the final mechanism by which active transport of amino acids occurs. The amino acid transport systems L (y^+L, x^-_c and $b^{0,+}$) are known to be *trans*-activated,[34–38] and are classified by their substrate specificities. In order to analyze this specificity, the uptake of L-[^{14}C](6-F)Trp was quantified in the presence of various unlabeled compounds (Figure 7 B). The most prominent inhibition of L-[^{14}C](6-F)Trp uptake was observed in the presence of the known system L-specific amino acid analogue 2-amino-2-norbornane carboxylic acid (BCH, 93.1 ± 0.5 %). Other amino acids like D,L-(4-F)Trp, D,L-(5-F)Trp, D,L-(6-F)Trp, L-Trp, D,-Trp, and L-Leu also reduced the uptake of L-[^{14}C](6-F)Trp, whereas L-Pro, L-Lys and L-Ser showed no or only little inhibitory effects on cellular uptake of the radio-labeled amino acid. This substrate specificity, as well as the postulated *trans*-activation of the transport process, is reminiscent of the amino acid transport system L. Furthermore, since all fluorinated L-Trp derivatives and unmodified L-Trp reduced the uptake of L-[^{14}C](6-F)Trp, we conclude that all of these derivatives are transported by amino acid transport system L.

Two system L subtypes, LAT1 and LAT2, function as obligatory amino acid exchangers with a 1:1 stoichiometry.[38] The driving force of this transport is presumed to be an amino acid gradient towards the extracellular matrix. This gradient, in turn, would have to be created by a transport system that is co-expressed with, and displays overlapping substrate specificity with, system L, such as, system A.[39]

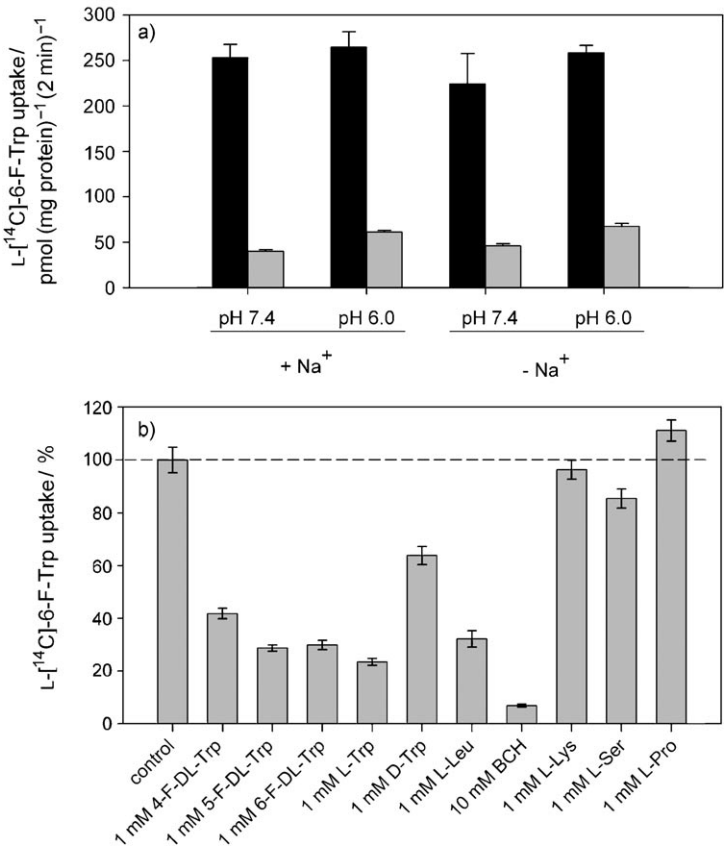

Figure 7. Na^+/H^+-independence and substrate specificity of L-(6-F)Trp uptake by MCF-7 cells: a) MCF-7 cells were incubated (0 min, ■; 2 min, ■) with L-[^{14}C](6-F)Trp (1.8 μM) in the presence or absence of NaCl at pH 6.0 and pH 7.4; b) MCF-7 cells were incubated (2 min) with L-[^{14}C](6-F)Trp (1.8 μM) at pH 7.4 with different, unlabeled amino acids to determine the substrate specificity. The control experiment, where no amino acid was added, was set as 100 %. Data are means ± S.E. ($n = 3$).

Conclusions

A large number of plant nonproteinogenic amino acids, which are structurally similar their proteinogenic counterparts, adversely affect the metabolism in other organisms.[1] Application of this strategy in medicine is highly promising for the development of new therapeutics against different diseases. In our study, we have demonstrated that fluorinated tryptophans, tyrosines, and phenylalanines possess cytostatic activities, which make them potential chemotherapeutics. The in vitro IC_{50} values of the most potent derivatives (3–15 μM) are similar to those of known anticancer agents, for example cisplatin or doxorubicin.[40–42] To the best of our knowledge, L-(4-F)Trp has the highest antitumor efficacy of any amino acid-derived drug, known to date.

The mechanism of action that gives rise to the cytostatic activity of L-(4-F)Trp is not yet known. One hypothesis is based on the incorporation of L-(4-F)Trp into proteins, thus affecting their activity. In order to incorporate an amino acid into a protein it has to be charged onto its cognate tRNA in the aminoacylation reaction catalyzed by aminoacyl-tRNA synthetases. Indeed, it was shown that D,L-(4-F)Trp, D,L-(5-F)Trp, and D,L-(6-

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F)Trp as well as D,L-*o*-FPhe, D,L-*m*-FPhe, and D,L-*p*-FPhe can be charged onto their respective tRNA (figure S2, Supporting Information).[28–30] Yet, fluorotryptophans showed different affinities towards tryptophanyl-tRNA synthetase from both beef pancreas[28] and *Bacillus subtilis*.[29] In both cases D,L-(4-F)Trp showed the highest affinity, followed by D,L-(6-F)Trp and D,L-(5-F)Trp. Experiments addressing the substrate specificity of phenylalanyl-tRNA synthetase from *E. coli* revealed decreasing affinities in the order L-Phe > D,L-*p*-FPhe > D,L-*m*-FPhe > D,L-*o*-FPhe.[30] Interestingly, L-(4-F)Trp and L-*p*-FPhe showed the highest biological activity among the fluorotryptophans and fluorophenylalanines studied, suggesting that the biological effect of the analogue might correlate with its affinity for the respective aminoacyl-tRNA synthetase.

Recent genome-wide RNAi screens of HeLa[47] and U2OS[48] cells identified 1351 and 1152 genes to be essential for proper cell cycle progression. A total of 96 genes were identified in both screens (table 1, Supporting Information), of which 60 caused an accumulation of DNA as observed in our study (Figure 2C), that is, an arrest in the S, G2 or M phase of the cell cycle. Of these 60 genes, 54 encode proteins containing at least one tryptophan, consequently, any of these proteins could potentially have been affected by incorporation of fluorinated tryptophan in our study.

The active uptake of L-(4-F)Trp and L-(6-F)Trp into the cells leading to intracellular concentrations 70-fold greater than extracellular concentrations was demonstrated. Additionally, we were able to identify the amino acid transport system L as the main route for the intracellular supply and accumulation of the fluorinated aromatic amino acids. System L is expressed by most human cell types, which could limit the potential of L-(4-F)Trp as a therapeutic agent. A systemic application of L-(4-F)Trp would probably lead to severe side effects, for example, the uptake and accumulation of the compound in epithelial blood vessel cells. However, a local application such as ectopic administration in cutaneous diseases like melanoma, which possess the transport system L,[43] is conceivable. Currently approved melanoma therapies include IFN 2αb or dacarbazine, however, only a small fraction of patients with advanced melanoma respond to dacarbazine, and only for a moderate duration.[44,45] Ectopic application of L-(4-F)Trp, or combinations of different fluorinated amino acid-derived drugs, might lead to a new chemotherapy against melanoma. Importantly, cells should not recognize the fluorinated aromatic amino acids as xenobiotic compounds, consequently, cellular defense mechanisms should not be activated including the induction of phase I and II drug metabolizing enzymes (e.g. cytochrome P450 enzymes, sulfotransferases, glutathione-S-transferases) and phase III transporters such as P-glycoprotein. A down regulation of the amino acid transport system L could be possible; however, cells would suffer from a shortage of all amino acids transported by this system. Thus, fluorinated aromatic amino acids, especially the potent analogue L-(4-F)Trp, might be interesting lead compounds for development as cytostatic and antitumor agents.

Experimental Section

General information

L-4-fluorotryptophan, L-6-fluorotryptophan, L-[^{14}C]4-fluorotryptophan and L-[^{14}C]-6-fluorotryptophan were synthesized from fluorinated indole derivatives and serine, and the products were verified by mass spectrometry and ^{19}F NMR (Supporting Information). 3-*O*-[^{14}C]methyl-D-glucose and L-[^{14}C]Ser were purchased from GE Healthcare, all other chemicals were obtained from Sigma–Aldrich unless otherwise indicated. Complete medium used in this study refers to RPMI 1640 medium purchased from PAA. Fetal bovine serum (FBS), GlutaMAX I, trypsin–EDTA, and gentamicin were obtained from Invitrogen. RPMI 1640 powder medium, purchased from Applichem, has a similar composition to RPMI 1640 medium purchased from PAA though lacks the amino acids. The appropriate amino acids were added to give the Trp-, Phe- and Tyr-free media with final amino acid concentrations similar to those found in the complete medium. All media were supplemented with 10% (*v*/*v*) heat-inactivated FBS, 2 mM GlutaMAX I and 50 μg mL^{-1} gentamicin.

Fluorescence intensities were determined using a FACS Calibur cytometer (Becton Dickinson). Radioactivity was determined by liquid scintillation spectrometry using a Tri-Carb 2100 TR scintillation analyzer (Canberra-Packard). Centrifugation was performed on a Centricon-10 (Amicon, Beverly, MA, USA).

Culture of MCF-7 cells and biological activity of fluorinated analogues

MCF-7 cells were cultured in complete medium and grown at 37 °C, 5% CO_2, and 95% humidity. To assay the cytostatic effect of L-*p*-FPhe, D,L-*m*-FTyr, L-(4-F)Trp, and L-(6-F)Trp, MCF-7 cells (3×10^4 cells in 200 μL medium) were seeded in 24-well cell culture plates containing 500 μL complete medium and incubated overnight. After removal of the culture medium, the cells were washed with 500 μL of phosphate-buffered saline (PBS) (3×) and incubated for different periods of time with 500 μL of complete or modified RPMI 1640 medium. Subsequently, cells were trypsinized using 0.05% (*w*/*v*) trypsin, 0.53 mM EDTA in Hanks balanced salt solution (HBSS), centrifuged (10 min, 400 *g*, 4 °C), washed with 800 μL of a buffer containing 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 5 mM $CaCl_2$ and finally resuspended in 200 μL of the same buffer. The cell number was then determined by FACS analysis. IC_{50} values were calculated using equation (1), where y_{max} corresponds to the cell number at the highest concentration of fluorinated amino acid, y_{min} to the cell number at the lowest concentration of fluorinated amino acid, x is the concentration of fluorinated amino acid, and IC_{50} represents the concentration of fluorinated amino acid that inhibits cell growth by 50%.

$$y = y_{min} + (y_{max} - y_{min})/[1 + (x/IC_{50})] \quad (1)$$

DNA content of MCF-7 cells

MCF-7 cells were seeded in 35 mm culture dishes at a density of 0.8×10^6 cells per dish and incubated overnight. After removal of the culture medium, the cells were washed with PBS (3×) before 2 mL of complete or Trp-free medium supplemented with 5 and 50 μM of L-(4-F)Trp, respectively was added. After incubation (48 h), the cells were harvested by trypsination and centrifugation (10 min, 400 *g*, RT), and resuspended in 200 μL 0.1% (*w*/*v*) tri-

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sodium citrate and 0.1% (*v*/*v*) Triton X-100. To stain the nuclear DNA, 100 μL of staining solution (2.5 μg mL^{-1} propidium iodide dissolved in 0.1% sodium citrate and 0.1% Triton X-100) were added and the cells were incubated in the dark for 15 min at RT. Subsequently, the fluorescence intensity of 10^5 cells was determined by FACS.

Amino acid uptake by MCF-7 cells

MCF-7 cells were seeded in 35 mm culture dishes at a density of 0.8×10^6 cells per dish. After incubation (24 h), the culture medium was replaced with fresh RPMI 1640 medium and the cells were incubated for a further 24 h. The culture medium was removed and the cells were briefly washed with uptake buffer containing 25 mM HEPES/Tris (pH 7.4), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM D-glucose. Uptake experiments were initiated by aspirating the uptake buffer and replacing it with 500 μL of the same buffer containing L-[^{14}C](4-F)Trp or L-[^{14}C](6-F)Trp and the appropriate unlabeled amino acids. After incubating the cells at RT for the desired time, the uptake buffer was removed and the cells were quickly washed with ice-cold uptake buffer (4×). Subsequently, cells were lysed by adding 1 mL of a buffer containing 50 mM Tris/HCl (pH 9.0), 140 mM NaCl, 1.5 mM $MgSO_4$ and 0.5% (*v*/*v*) igepal Ca-630, and transferred to 5 mL scintillation vials. The cell-associated radioactivity was determined by liquid scintillation spectrometry. Experimental data were corrected for the protein content as described below. The intracellular concentration of L-(4-F)Trp or L-(6-F)Trp was calculated using equation (2): where c is the intracellular concentration of L-(4-F)Trp or L-(6-F)Trp in μM, y the maximum uptake of the radiolabeled analogue in pmol per mg of protein, and V is the accessible intracellular volume of MCF-7 cells in μL per mg of protein.

$$c = y/V \quad (2)$$

To determine whether the transport of L-(6-F)Trp is Na^+- or H^+-dependent, MCF-7 cells were incubated for 2 min with either Na^+-containing uptake buffer or Na^+-free uptake buffer (containing 140 mM choline chloride instead of NaCl) at pH 7.4 (25 mM MES/Tris) or pH 6.0.

Determination of the cellular protein content

The protein content of MCF-7 cells was determined using cells that were cultured identical to those used for the respective uptake experiment. After removal of the culture medium the cells were washed with uptake buffer (2×) and ice-cold uptake buffer (4×). Finally, 1 mL of distilled water was added, the cells were frozen at −20 °C, thawed at RT, resuspended and centrifuged (15 min, 4 °C, 4000 *g*). The protein content of the supernatant was determined according to the method of Bradford using bovine serum albumin as a reference.[46]

Determination of the intracellular accessible volume

The intracellular accessible volume of MCF-7 cells was determined using 3-*O*-[^{14}C]methyl-D-glucose as a tracer. MCF-7 cells were seeded in 35 mm culture dishes and incubated as described before. After removing the culture medium, the cells were briefly washed with uptake buffer and incubated at RT for different periods of time with 500 μL of the same buffer supplemented with 3.3 μM of 3-*O*-[^{14}C]methyl-D-glucose. After removing this buffer, the cells were quickly washed with ice-cold uptake buffer supplemented with 200 μM phloretin (4×). The cell-associated radioactivity was determined by liquid scintillation spectrometry as described before. The accessible intracellular volume was calculated according to equation (3): where V corresponds to the accessible intracellular volume in μL per mg of protein, y to the uptake of 3-*O*-[^{14}C]methyl-D-glucose in pmol per mg of protein, and c_T to the concentration of 3-*O*-[^{14}C]methyl-D-glucose in the uptake buffer.

$$V = y/c_T \quad (3)$$

Synthesis of L-(4-F)Trp and L-(6-F)Trp

L-(4-F)Trp or L-(6-F)Trp were enzymatically synthesized from serine and fluorinated indole derivatives by tryptophan synthase (*E. coli*). 4-Fluoroindole or 6-fluoroindole (200 mg) was incubated overnight with L-serine (200 mg) in Tris/HCl (pH 7.8, 50 mL, 100 mM), pyridoxal phosphate (0.02 mM) in the presence of 200 units of the $\alpha_2\beta_2$-complex of tryptophan synthase under an argon atmosphere at RT. Subsequently, the enzyme was removed by centrifugation. Non-reacted indole derivatives were selectively extracted using ethyl acetate. The purity was checked by thin layer chromatography and the amount of the product was estimated by UV-spectroscopy: Trp; ε_M at $\lambda_{max}=5695.9\ M^{-1}cm^{-1}$ ($\lambda_{max}=279$ nm), (4-F)Trp; ε_M at $\lambda_{max}=4824.2\ M^{-1}cm^{-1}$ ($\lambda_{max}=264$ nm), (6-F)Trp; ε_M at $\lambda_{max}=4843.2\ M^{-1}cm^{-1}$ ($\lambda_{max}=280$ nm).

Enzymatic synthesis of L-[^{14}C](4-F)Trp and L-[^{14}C](6-F)Trp from 4-fluoroindole and 6-fluoroindole, respectively, and L-[^{14}C]Ser by the method described above. While the synthesis of L-[^{14}C](6-F)Trp resulted in full consumption of L-[^{14}C]Ser, residual L-[^{14}C]Ser was detected (108 μM) in L-[^{14}C](4-F)Trp indicating incomplete reaction. Uptake experiments were performed using L-[^{14}C](4-F)Trp in the presence of 40 mM unlabeled L-Trp giving the cell-associated radioactivity attributed to L-[^{14}C]Ser only. This value was then subtracted from the cell-associated radioactivity determined for L-[^{14}C](4-F)Trp in the absence of unlabeled L-Trp, to give the true cell-associated radioactivity arising from L-[^{14}C](4-F)Trp.

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