[Frontiers in Bioscience 11, 2759-2766, September 1, 2006]

Uridine phosphorylase in breast cancer: a new prognostic factor?

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1. ABSTRACT

Uridine phosphorylase (UPase) is an enzyme that converts the pyrimidine nucleoside uridine into uracil. Upon availability of ribose-1-phosphate, UPase can also catalyze the formation of nucleosides from uracil as well as from 5-fluorouracil, therefore involved in fluoropyrimidine metabolism. UPase gene expression is strictly controlled at the promoter level by oncogenes, tumor suppressor genes, and cytokines. UPase activity is usually elevated in various tumor tissues, including breast cancer, compared to matched normal tissues and this induction appears to contribute to the therapeutic efficacy of fluoropyrimidines in cancer patients. In this review, we will discuss in detail the role of UPase in the activation of fluoropyrimidines and its effect on the prognosis of breast cancer patients.

2. INTRODUCTION

Uridine phosphorylase (UPase) is an enzyme identified from liver more than 50 years ago (1). This protein enzyme utilizes mainly uridine, a precursor of pyrimidine nucleotides, as a substrate, conferring UPase a specific function in regulating the homeostasis of uridine in plasma and tissues (1-5). Given that 5-fluorouracil (5-FU) and 5'-deoxy-5-fluorouridine (5'DFUR) are identified as substrates for this enzyme, the expression of UPase in human and rodent tumors and its effect on fluoropyrimidine activation and tumor selectivity have attracted the attention of investigators in cancer research (see refs. 6-8 for reviews). Here we review the recent advancements in UPase research, in terms of its expression regulation and role in tumor development and prognosis, with focus on its effect on breast cancer therapy and prognosis.

3. TRANSCRIPTIONAL REGULATION OF URIDINE PHOSPHORYLASE EXPRESSION

UPase is ubiquitously distributed in various cells and tissues in human and animals. Its basic phosphorolytic activity is conserved through all the evolutional hierarchy of living organisms, from bacteria to human, although the

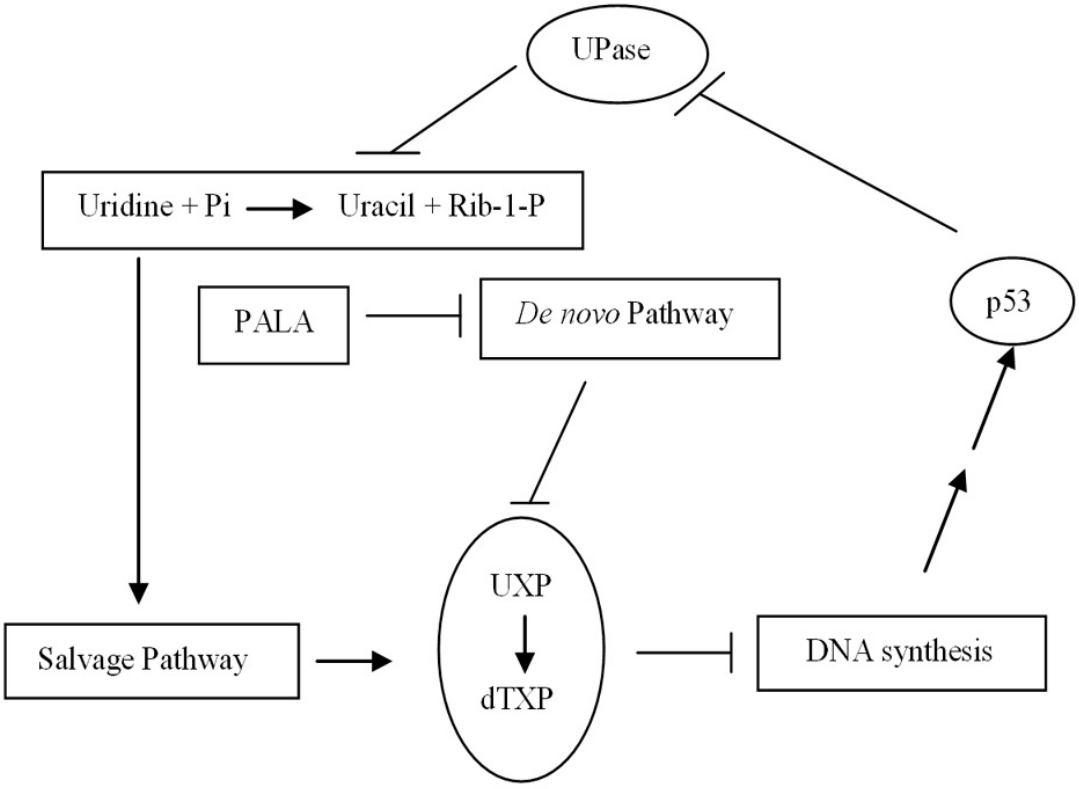

Figure 1. p53-dependent control of UPase expression and the regulation of pyrimidine salvage pathway activity following inhibition of *de novo* pathway by inhibitors, such as N-(phosphonacetyl)-L-aspartate (PALA). UPase, uridine phosphate; Rib-1-p, ribose-1-phosphate; Pi, phosphate UXP, ribonucleotides; and dUXT, deoxyribonucleotides.

gene structure and protein sizes vary (9-14). Expression levels of UPase in mammalian tissues also vary with tissue types and differentiation status. In general, UPase activity is higher in multipotential and immature cells than in well differentiated adult cells. In murine embryonic stem (ES) cells, UPase activity, measured as the formation of uracil from uridine, reaches up to 6000 nmol/mg protein/hour while the activity in most mature tissues ranges from 20 to 100 nmol/mg protein/hour, except for the small intestine (2, 15). In chick, UPase activity in various embryonic organs, including brain, liver, and heart, declines with the development of the embryos (16). The studies on postnatal development of rat heart also demonstrated that UPase activity decreases with the maturation of heart cells (17). In human tumor tissues, the UPase activity is usually elevated compared to surrounding normal tissues and may be correlated with the differentiation and the stage of tumors (18).

However, UPase gene expression is strictly controlled at the promoter level by oncogenes, tumor suppressor genes, and cytokines. This is a rare phenomenon seen only in a few important genes such as p21. Cytokines such as tumor necrosis factor-alpha (TNF-alpha), interleukin-1 alpha (IL-1 alpha) and interferon-alpha and gamma (IFN-alpha and gamma) as well as vitamin D_3 can induce UPase gene expression alone or synergistically (12, 19-21). Our recent study indicates that TNF-alpha induces UPase gene expression through a NF-kappa B subunit p65-dependent pathway (22). In addition, UPase induction has also been reported in NIH/3T3 cells transformed by the *c-H-ras* oncogene (23). On the contrary, the tumor suppressor gene, p53, has been shown to repress UPase expression by DNA sequence-specific binding (24). In p53 null cells, UPase protein is significantly enriched and the enzyme activity is 30 fold higher than that in wild type cells (24).

The p53 repressive regulation on UPase gene may have significance in the regulation of tissue pyrimidine ribonucleotide pools, by activating the pyrimidine salvage pathway. p53 acts as a sensor of intracellular nucleotide pools (25). The inhibition of UPase expression by p53 may represent a negative feedback regulation on the UPase-catalyzed uridine catabolism when depletion of nucleotide pools occurs, accumulating the precursor uridine to be utilized in the pyrimidine salvage pathway (Figure 1). During exposure to antimetabolites affecting pyrimidine synthesis, this feedback regulation may neutralize the action of the anti-neoplastic agents, such as 5-FU and methotrexate (MTX), by overcoming the inhibition of pyrimidine *de novo* synthesis (6, 14). In addition, p53 mutation occurs frequently in various types of human tumors, and mutant p53 usually lacks DNA and protein binding functions. Therefore, this regulatory mechanism of p53 on UPase expression may also suggest that p53

Table 1. Kinetic constants of UPase extracted from different tissues

Tissues	Substrates	Co-substrate	K_m (μM)
Rat liver	5-FU	Rib-1-P	36
Rat RPMI colon tumor	5-FU	Rib-1-P	60
Mouse colon tumor 38	5-FU	Rib-1-P	46
Rat liver	Rib-1-P	5-FU	20
Rat RPMI colon tumor	Rib-1-P	5-FU	40
Mouse colon tumor 38	Rib-1-P	5-FU	38
Rat liver	Uracil	Rib-1-P	485
Mouse colon tumor 38	Uracil	Rib-1-P	367
Rat liver	Rib-1-P	Uracil	30
Rat RPMI colon tumor	Rib-1-P	Uracil	87

Reference: 32

mutation could play a role in the induction of UPase in tumor cells as noted in p53 null cells (24).

4. FLUOROPYRIMIDINE ACTIVATION BY URIDINE PHOSPHORYLASE

Fluoropyrimidines exert their antitumor effects through two main mechanisms. Via inhibition of thymidylate synthase (TS) by its active anabolite 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), fluoropyrimidines block the synthesis of deoxythymidine monophosphate (dTMP). Alternatively, the incorporation of 5-fluorouridine-5'-triphosphate (FUTP) and 5-fluoro-2'-deoxyuridine-5'-triphosphate (FdUTP) into nucleic acids impairs RNA and DNA functions, respectively (6, 26-28).

Three pathways are involved in the activation of 5-FU. In the presence of 5-phospho-D-ribosyl-1(alpha)-pyrophosphate (PRPP), orotate phosphoribosyltransferase (OPRT) directly converts 5-FU to its nucleotide form, FUMP (6, 29, 30). Alternatively, 5-FU is converted to 5-fluorouridine by UPase with ribose-1-phosphate (Rib-1-P) as a co-substrate and then to FUMP by UK in the presence of ATP (15, 30-32). The common product, FUMP, of these two pathways is then converted to its active products FUTP, FdUTP and FdUMP (these last two metabolites obtained after reduction of FUDP by ribonucleotide reductase). The contribution of these two pathways to 5-FU activation depends on the relative enzyme activity and the intracellular bioavailability of their co-substrates (30, 31). The third pathway indicates that 5-FU is directly anabolized to its active anabolite, FdUMP, sequentially by TPase with deoxyribose-1-phosphate (dRib-1-P) as a co-substrate and thymidine kinase in the presence of ATP. However, this pathway has not been experimentally demonstrated (30, 33, 34).

4.1. Activation of 5-FU and 5'DFUR by uridine phosphorylase

The role of UPase in the activation of fluoropyrimidines includes the phosphorolysis of 5'-deoxy-5-fluorouridine (5'DFUR) to 5-FU and the phosphorylation of 5-FU to FUMP, through the pyrimidine salvage pathway (30, 31, 33, 35-38). In the majority of the studies conducted with intact cells and cell free extracts, it has been shown that the pattern of enzyme activity correlates with the initial metabolism of 5-FU (30, 31, 33, 39, 40) and that the reduced UPase activity is one of the mechanisms of the cell resistance to 5-FU (40-42). Peters *et al.* (30) investigated 5-FU metabolism in human and mouse cancer cell lines and found that the activity of fluorouridine synthesis in IGR3 and M5 cells with elevated UPase activity was 5 to 10 times higher than that in B16 cells with low UPase activity. On the contrary, a PRPP decrease was found in B16 and WiDr cells with high OPRT activity. These results suggested that the initial metabolism of 5-FU in intact cells could be deduced from the enzyme activity determined in cell free extracts. This was proven by Maehara's work (33) that the cell free extracts from lung carcinoma tissues or cancer cells could efficiently convert 5-FU to FUMP in the presence of Rib-1-P and ATP.

Using our UPase knockout murine ES cell model, we have recently demonstrated that UPase activity directly correlates with the cell sensitivity to fluoropyrimidines (15). In these cells, the abrogation of UPase has resulted in an 8- and 16-fold increase in the IC50's to 5-FU and 5'DFUR, respectively, while wild type cells maintain their sensitivity to fluoropyrimidines without any extra additions of Rib-1-P or its donors. Kinectic analyses with 5-FU as substrate have revealed that UPase actually shows a better affinity and anabolic activity for 5-FU even if uracil is the natural substrate. Ikenaka *et al*. (43) reported that the presence of 2500μM uracil had no effect in Yoshida sarcoma cells on the phosphorylation of 5-FU at 2.5μM (1000 folds lower than uracil) although the degradation of 5-FU by dihydropyrimidine dehydrogenase (DPD) was inhibited by 70%. A similar inhibition pattern was also present in cell free extract, i.e., in presence of Rib-1-P and ATP, uracil had very limited inhibition on the phosphorylation of 5-FU. Schwartz *et al.* (32) measured the K_m values of both uracil and 5-FU with Rib-1-P as a co-substrate, using UPase crude extracts from rat liver, RPMI colon tumor, and mouse colon tumor 38. They found that the K_m value of 5-FU (averaged at 47μM) was 9 folds lower than that of uracil (averaged at 426μM) (Table 1). More interestingly, the K_m value of Rib-1-P was almost 2 folds lower when determined with 5-FU (33μM) as a co-substrate than that in the presence of uracil (59μM).

The intracellular availability of Rib-1-P has been questioned in the evaluation of the role of UPase in 5-FU activation. Some investigators have proposed that the intracellular Rib-1-P is a rate-limiting factor in 5-FU anabolism through UPase-uridine kinase (UPase-UK) pathway (30, 35). However, Schwartz *et al*. (32) measured the Rib-1-P concentration in mouse and rat liver and kidney, rat RPMI colon tumor, and mouse colon tumor 38, and found that the concentration of Rib-1-P in these tissues was close to or even higher than its K_m value in the presence of 5-FU as a co-substrate. Houghton *et al.* reported a similar result in human colon adenocarcinoma xenografts (31). More recently, we have also examined the Rib-1-P concentration in UPase knockout murine ES cells and our results indicated that the intracellular Rib-1-P concentration was not affected by the abrogation of uridine phosphorolytic activity and that the 5-FU antiproliferative activity appeared not related to the measured intracellular Rib-1-P level (15). Considering the universal existence of intracellular donors of the ribose moiety such as inosine, adenosine, or guanosine (44, 45), we believe that Rib-1-P is

unlikely a rate-limiting factor in 5-FU phosphorylation through UPase-UK pathway.

Compared to 5-FU, a clearer correlation between UPase activity and growth inhibition by 5'DFUR has been established (15, 30, 36-38, 46). In order to improve the oral bioavailability of 5-FU, prodrugs of 5-FU have been developed to mimic the constant plasma concentration achievable with a continuous infusion of 5-FU (see (47) & (48) for reviews). 5'DFUR was the first oral prodrug of 5-FU introduced into clinical trials. Different from 2'-deoxy-5-fluorouridine (FdUrd) that is directly converted to FdUMP by uridine kinase (UK), 5'DFUR is not a substrate for UK. To exert its antiproliferative effect on cells, 5'DFUR must be converted to 5-FU through phosphorolysis catalyzed by UPase and/or TPase, serving as a continuing source of 5-FU. This activation process provides a rationale for the tumor-selective accumulation of 5-FU or a tumor-specific high ratio of 5-FU to intact 5'DFUR in tumor tissues that usually possess high nucleoside phosphorylases (49, 50). However, Cory *et al.* (51) thought that a high UPase activity alone appears insufficient for the antiproliferative activity of 5'DFUR because of the competition of 5'-deoxyribose-1-phosphate derived from 5'DFUR with Rib-1-P, affecting the activation of 5-FU released from 5'DFUR via the UPase-UK pathway.

4.2. Activation of capecitabine, a novel prodrug of 5-FU

Compared to 5-FU, the clinical advantage of 5'DFUR has not been able to be documented because of its neurotoxicity, cerebellopathy and encephalopathy. After oral delivery, 5'DFUR is converted to 5-FU in high concentrations by TPase/UPase present in the gastrointestinal mucosa before it enters the blood circulation and distributes into tumor tissues. Therefore, 5'DFUR clinical trials have been discontinued in Europe and Northern America. However, its pharmacological interest still remains because of the important intermediate role of 5'DFUR in the activation process of capecitabine, a new prodrug of 5-FU recently approved for the treatment of advanced breast and colon cancers in USA and Europe (see 52 for review).

Capecitabine (Xeloda, N^4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine) is a triple prodrug of 5-FU, characterized by tumor-selective activation. The carbamate modification facilitates its passage through the gastrointestinal mucosa without activation to its active metabolites, leading to higher bioavailability, close to 100% (52) and reduced gastrointestinal toxicity (52-54). In liver, capecitabine is converted to 5'-deoxy-5-fluorocytidine (5'DFCR) by carboxylesterase, and then to 5'DFUR by cytidine deaminase (see 53 & 54 for reviews). In tumor tissues, 5'DFUR is activated to 5-FU by TPase and UPase that are frequently elevated in various tumor tissues (53-56), leading to the selective accumulation of 5-FU and its active metabolites in neoplastic tissues.

The tumor selectivity of capecitabine has been confirmed in both tumor xenografts (55) and human colon carcinoma tissues (56). Schüller *et al.* (56) analyzed 5-FU concentration in plasma and biopsies of tumor and healthy tissues. They found that 5-FU concentration in tumor biopsies was 2.9 times higher than that in surrounding healthy tissues and 16.6 folds higher than in plasma. Using microdialysis technique, Mader *et al.* (57) found that capecitabine and its metabolites DFCR and DFUR distributed extensively into the interstitium of malignant and healthy tissues with very little discrimination before the final activation to 5-FU. This study displayed the intratumoral pharmacological basis of the tumor selectivity of capecitabine mediated by nucleoside phosphorylases.

The role of TPase in the selective activation of capecitabine in tumor tissues appears more attractive due to its high expression in human tissues and its potential angiogenic activity (58-62). However, the elevated expression of UPase in tumor tissues not only contributes to the tumor selective activation of 5'DFUR into 5-FU, but also serves the phosphorylation of 5-FU through the UPase-UK pathway. Compared to TPase, UPase has low activity levels in various normal human tissues, but its activity or its mRNA level arose up to 10 to 1000 fold in human tumors (11, 63). This significant difference of UPase activity between normal and cancerous tissues contributes to the tumor-selectivity of fluoropyrimidine-based therapy. More importantly, UPase has no identified angiogenic activity because of its inability to catalyze the phosphorolysis of deoxy-nucleosides, where formed 2-deoxyribose-1-phosphate acts as an endothelial-cell chemoattractant and angiogenesis-inducing factor (64, 65). Therefore, modulation of UPase activity in tumors by UPase gene transfer or delivery of inducers of UPase gene expression, such as cytokines, will not cause concerns of potential stimulation of tumor growth due to increased angiogenesis, as is in the case of TPase (66). For instance, combination of IFN-alpha with 5-FU has resulted in a significant improvement in response rate and patient survival, compared with 5-FU alone (67). In colon 26 tumor cells, a mixture of TNF-alpha, IL-1 alpha, and IFN-gamma efficiently enhanced 5-FU and 5'DFUR cytotoxicity by a 2.7- and 12.4-fold, respectively, due to the induction of the UPase (21), indicating the importance in the development of UPase-specific bio-modulation strategies or 5-FU prodrugs to avoid the angiogenic effect of TPase.

5. URIDINE PHOSPHORYLASE INDUCTION IN TUMOR TISSUES

Since the clinical introduction of 5-FU more than 40 years ago, its metabolic enzymes in normal and tumor tissues have been extensively investigated (27, 29, 31). In spite of lack of systematic studies, UPase induction in tumor tissues has been reported in human colorectal carcinomas (18, 68, 69), human melanoma (70, 71), human breast carcinomas (63), human oral squamous cell carcinomas (72), human lung adenocarcinomas and squamous cell carcinomas (33), and mouse sarcomas, hepatoma and Ehrlich ascites carcinomas (38). Finan *et al.* (68) evaluated the UPase activity in 12 human colon carcinoma tissues and found that 9 of them showed elevated UPase activity with a maximum up to a 8-fold.

Katsumata *et al.* (18) further reported a positive correlation between UPase activity level and the tumor stages in colorectal carcinomas.

Heterogeneity of UPase expression in tumor tissues was also reported. In Denton's studies (73), only one of eight human colon carcinomas showed an elevation of UPase activity, and on the contrary, 6 of them actually revealed a decreased UPase activity, indicating a need of more comprehensive investigation of UPase expression in tumor tissues. Interestingly, studies with xenografts of colon carcinomas have demonstrated that UPase activity in tumor tissues remained same or increased with the passages of xenografts while TPase activity declined, indicating that UPase is elevated in cancerous cells while TPase is mainly expressed in stromal cells that are eliminated in xenografting (69, 74). This finding may be a key element for tumor cell-specific activation of fluoropyrimidines and its overall therapeutic efficiency.

6. URIDINE PHOSPHORYLASE IN BREAST CANCER

As a most common type of cancer in women, our group has been interested in the expression and role of UPase in this disease, and for the first time reported a 2- to 3-fold increase of UPase activity in the tumor tissues, compared to the adjacent normal tissues, and unraveled the presence of a phosphorolytic activity insensitive to 5-benzylacyclouridine (BAU), a potent inhibitor of UPase (11). This BAU-insensitive phosphorolytic activity in selected tumors, coupled with the potent inhibitory activity of BAU against the "classical" uridine phosphorylase in normal human tissues, provides the rationale for combining BAU with fluoropyrimidines in the treatment of the breast cancer, to achieve high intratumoral 5-FU levels and low adverse effects on non-tumorous tissue. UPase induction in breast cancerous tissues was further confirmed by Kanzaki's work (63). Using real-time PCR, they examined 43 human breast carcinomas and found that the UPase mRNA levels increased up to 1000 folds in some tumor tissues, even in the presence of tumor heterogeneity.

The contribution of increased UPase activity to breast cancer chemotherapy has been confirmed by a study by Hiraga, *et al.* (75). They evaluated the effect of phosphorylase activity on the effectiveness of 5'-DFUR and capecitabine in the inhibition of orthotopic tumor formation and distant metastases to bone, lung and liver, using an animal model transplanted with 4T1/luciferase mouse breast cancer cells. In this study, they found that oral 5'-DFUR and capecitabine are effective for the treatment of primary and secondary breast tumors, and also for preventing bone metastases, a common complication of breast cancer. At the lowest dose of 5'-DFUR (31 mg/kg) and capecitabine (90 mg/kg), the bone metastases were significantly prevented, although these doses were not high enough to inhibit orthotopic tumor development as well as lung and liver metastases.

Cuq, et al (76) reported a controversial result on the role of UPase in fluoropyrimidine activity. Their observation indicated that the overexpression of UPase by gene transfer did not increase the fluorouracil sensitivity of MCF-7 human breast cancer cells, although 5-fluorouridine synthesis was increased. Nevertheless, we believe that it is important to take into account other related metabolic enzymes, such as uridine kinase, a rate-limiting enzyme in the pyrimidine salvage pathway, when one evaluates the role of UPase in the activation of 5-FU (6, 39). Combined with its efficiency in the conversion of 5-DFUR to 5-FU, a high UPase activity in breast cancer tissues would positively affect the use of capecitabine in this disease.

Kanzaki, *et al*. has studied the potential of UPase as a prognostic marker and reported a negative correlation between UPase expression and the clinical outcome of breast cancer patients (63). They found that a higher UPase expression was detected in tumors of patients who relapsed. Furthermore, a worse survival rate was also observed in the patients with the higher UPase gene expression. Therefore, they proposed the expression level of UPase gene in breast cancer as a negative prognostic marker of this disease. A limitation of this observation, however, is the small patient population investigated. Thus, to draw a clear conclusion, obviously, a more systematic investigation is required.

7. SUMMARY AND PERSPECTIVE

UPase plays a critical role in the activation of fluoropyrimidines, such as 5-FU and its oral prodrug capecitabine recently approved for the treatment of advanced breast and colorectal carcinoma. The upregulation of this protein in various human tumors, including breast cancer, would provide a higher level of tumor-selectivity of fluoropyrimidine-based treatment of cancer patients, especially for 5'DFUR and/or capecitabine-based regimens. However, the controversial data currently available indicates a need for a more comprehensive investigation of UPase expression in breast cancer and the relationship with the type, stage, and differentiation status of the disease. Understanding of the cause and effect relationship between UPase expression and the development of breast cancer will aid in the evaluation of UPase as a prognostic marker.

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Key Words: Uridine Phosphorylase, Thymidine Phosphorylase, Promoter, Pyrimidines, Uridine, Uracil, R-bose-1-phosphate, Neoplasm, Breast Cancer, Prognostic Factor, Fluoropyrimidines, 5-Fluorouracil, 5'-Deoxy-5-fluorouridine, 5'DFUR, Capecitabine, Gene Expression, and Gene Knockout, Review

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