Effects of Cationic Porphyrins as G-Quadruplex Interactive Agents in Human Tumor Cells


ABSTRACT

A series of cationic porphyrins has been identified as G-quadruplex interactive agents (QIAs) that stabilize telomeric G-quadruplex DNA and thereby inhibit human telomerase; 50% inhibition of telomerase activity was achieved in HeLa cell-free extract at porphyrin concentrations in the range \( \leq 50 \mu M \). Cytotoxicity of the porphyrins in \( \text{in vitro} \) was assessed in normal human cells (fibroblast and breast) and human tumor cells representing models selected for high telomerase activity and short telomeres (breast carcinoma, prostate, and lymphoma). In general, the cytotoxicity (\( EC_{50} \), effective concentration for 50% inhibition of cell proliferation) against normal and tumor cells was \( >50 \mu M \). The porphyrins were readily absorbed into tumor cell nuclei in culture. Inhibition of telomerase activity in MCF7 cells by subcytotoxic concentrations of TMPyP4 showed time and concentration dependence at 1–100 \( \mu M \) TMPyP4 over 15 days in culture (10 population doubling times). The inhibition of telomerase activity was paralleled by a cell growth arrest in G2-M. These results suggest that relevant biological effects of porphyrins can be achieved at concentrations that do not have general cytotoxic effects on cells. Moreover, the data support the concept that a rational, structure-based approach is possible to design novel telomere-interactive agents with application to a selective and specific anticancer therapy.

INTRODUCTION

Significant levels of telomerase activity have been detected in >85% of tumors (1). Telomerase is also present in stem and germ-line cells of normal tissues, albeit at much lower levels (2). Thus, telomerase presents a target with potentially good selectivity for tumor over healthy tissue, and telomerase inhibition has been proposed as a new approach to cancer therapy (2–4). The structure of the human telomerase protein remained elusive until recently and has been shown to be closely related to other reverse transcriptases (5–7). It has been possible to inhibit telomerase activity either by antisense strategies directed toward the telomerase RNA template, for example peptide nucleic acids (8) and phosphorothioate oligonucleotides (9), or by using inhibitors of reverse transcriptases [e.g., established agents such as, 3’-azido-3’-deoxythymidine (10) and other nucleosides (11)]. Inhibition by cisplatin, possibly due to cross-linking of the telomeric repeat sequences, has also been reported (12).

A novel approach toward achieving the net inhibition of telomerase is to target its substrate, the telomere. We used a rational, structure-based approach to the design of telomere interactive agents by considering unique nucleic acid secondary structures associated with the telomerase reaction cycle. One such structure is the G-quadruplex formed by folding of the single-stranded G-rich overhang produced by telomerase activity. The template region of the telomerase RNA has only 1.5 copies of the complementary sequence (3’-CAAUCCAAUCC-5’), so after each extension the end of the DNA must be translocated back to the beginning of the coding region before the next extension (13). Work by Zahler et al. (14) has shown that potassium ions stabilize the quadruplex and that high concentrations of potassium inhibit telomerase. Furthermore, we (15) have shown that there is an equilibrium between the DNA:RNA heteroduplex and the G-quadruplex that lies in favor of G-quadruplex formation. These two observations point to the involvement of G-quadruplex formation in dissociating the primer from the telomerase RNA template and possibly providing the driving force for the translocation reaction. This led us to hypothesize that the G-quadruplex would indeed be a viable target for drug design, as first suggested by Blackburn (13), and thus, we have undertaken a study of QIAs.4

The long-term goal of our studies is to identify an effective QIA (with significant concentration differences between telomerase inhibition and the cytotoxic effects), and bring it to clinical trial. Herein we report the inhibition of telomerase by TMPyP4, the related tetraquinoyl porphine QP3, and metal complexes thereof. The cytotoxicity and cellular uptake of this family of porphyrins have been examined in a series of human tumor and normal cell lines. We have demonstrated rapid repression of telomerase activity and cell growth arrest in intact tumor cells by subtoxic concentrations of TMPyP4. This finding suggests that the use of QIAs to directly target telomeres may be a possible therapeutic strategy. Three tumor models (breast, prostate, and lymphoma) are relevant to the future clinical development of telomerase inhibitors. The low cytotoxicity and inhibition of telomerase at low micromolar concentrations combine to make the cationic porphyrins attractive candidates for anticancer drug development.

MATERIALS AND METHODS

Molecular Modeling. Models were built using the Sybyl package (Tripos, Inc., St. Louis, MO). Coordinates for the DNA quadruplex (16) and TMPyP4 (15) were obtained from the Brookhaven Protein Data Bank. Hydrogen bonding constraints were added to the G-tetrads, and torsional constraints were set to maintain the planarity of the porphyrins. Porphyrins were inserted above and below the G-tetrads, and the complex was allowed to minimize using Kollman charges, Tripos force field, and conjugate gradient. After 100 iterations, the porphyrins were replaced, and the minimization was repeated for 500 iterations to a terminal gradient of 0.05 kcal/mol.

Chemicals and Cell Lines. All porphyrins were obtained from Midcentury (Posen, IL). The experimental work with porphyrins was performed under minimum exposure to light. All human tumor cell lines and normal human breast cells HS578Bst were purchased from the American Type Culture Collection. Normal human lung fibroblasts were obtained from Clonetics Corp. The cell lines were grown according to suppliers’ instructions.

Received 8/6/98; accepted 12/2/98.

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This work was supported by National Cooperative Drug Discovery Group Grant CA67760 from the National Cancer Institute. Preliminary reports of this work were presented at the 88th Annual Meeting of the American Association for Cancer Research, San Diego, CA, in Proceedings of The American Association for Cancer Research, 58: 637, 1997.

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3 The abbreviations used are: QIA, G-quadruplex interactive agent; TMPyP4, 5,10,15,20-tetra(-methyl-4-pyridyl)porphine chloride; QP3, 5,10,15,20-tetra(N-methyl-3-quinolyl)porphine chloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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Telomerase Inhibition Assay. Telomerase activity in human tumor cell lines was measured using the non-PCR-based telomerase assay (5) with 5'-biotinylated d(TTAGGG)2. Extracts were obtained from 1 × 10^6 cells. The cells were washed once in PBS (400 μl) and pelleted at 10,000 × g for 1 min at 4°C, resuspended in 1.5 ml tubes containing 400 μl of ice-cold washing buffer [10 mM HEPES-KOH (pH 7.5), 1.5 mM MgCl₂, 10 mM KCl, and 1 M DTT], then pelleted again at 10,000 × g for 1 min at 4°C. Washed cells were resuspended in 100–400 μl of ice-cold lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 1 mM DTT, 0.5% 3-[3-cholamidopropyl]dimethylamino]-1-propanesulfonate, 10% glycerol, and 40 μM RNase guard]. The suspension was incubated on ice for 30 min, and the lysate was then transferred to polyallomer tubes (Beckman) and spun at 100,000 g for 10 min. The resulting formazan precipitate was solubilized with 200 μl of ice-cold 37°C for 1 h. Reactions were terminated by adding 20 μl of streptavidin-coated Dynabead suspension containing 10 μl of Tris-HCl (pH 7.5) and 2 μl of NaCl. The beads were selectively complexed with the 5’-biotinylated DNA. The complex was separated from the suspension using a magnet (Dynal MPC) and washed several times with washing buffer (1 m NaCl) to eliminate [α-32P]dGTP background. Telomerase reaction products were separated from the magnetic beads by protein denaturation with 5.0 M guanidine-HCl at 90°C for 20 min. After ethanol precipitation, the reaction products were analyzed by 8% PAGE.

Telomerase activity in HeLa cells was used for reference and defined as 100% activity.

Cytotoxicity Assay (MTT). Exponentially growing cells (1–2 × 10^5 cells) in 0.1 ml of medium were seeded on day 0 in a 96-well microtiter plate. On day 1, 0.1-ml aliquots of medium containing graded concentrations of the investigational compound were added to the cell plates. On days 7–10, the cultures were incubated with 50 μl of MTT, 1 mg/ml in Dulbecco’s PBS for 4 h at 37°C. The resulting formazan precipitate was solubilized with 200 μl of 0.04 M HCl in isopropyl alcohol (17, 18).

Cell Cycle Analysis. The effects of TMPyP4 on the cell cycle were assessed by flow cytometry by measuring the percentage of cells in G₀-G₁, S, and G₂-M phases, with and without treatment with the porphyrin. Cells were stained with 50 μg/ml propidium iodide in a hypotonic sodium citrate solution with 0.3% NP40 and 1.0 mg/ml RNase A at 1.0 × 10⁶ cells/ml, vortexed, and stained for 30 min at room temperature in the dark. Before flow cytometric measurements, samples were filtered through a 37-μm nylon mesh into 12 × 75-mm tubes and stored at 4°C until analysis within 24 h. All samples were analyzed with an EPICS ELITE flow cytometer (Coulter Cytometry, Miami, FL).

Nuclear Localization of TMPyP4. MCF7 cells were seeded at 5 × 10⁵ cells per flask and cultured in the presence of 0 or 50 μM TMPyP4 for 7 days with one change of medium. The cells were harvested with trypsin-EDTA, washed with PBS, and counted using a Coulter counter. The cells were then pelleted by centrifugation at 700 × g and lysed in a hypotonic solution (0.1× PBS); the resulting lysate was sedimented at 700 × g for 10 min. The supernatant was removed, and the pellet was dissolved in 0.5 ml of 2% SDS. Absorptances of the samples were measured at 423 nm with subtracting the absorbances of the samples without porphyrin. TMPyP4 diluted in 2% SDS containing 1 mg/ml salmon sperm DNA was used for the standard curve.

Metaphase Spreads. MCF7 cells (at 5 × 10⁶ cells/flask) were grown for 3 days in the presence of 40 μM QP3-In³⁺ (the most fluorophorescent porphyrin). The cells were trypsinized, harvested by centrifugation, and incubated in Colcemide (Life Technologies, Inc.) at 100 ng/ml for 1 h. The cells were pelleted, and the medium was replaced with 75 mM KCl (hypotonic solution). After incubation for 20 min, the cells were spun at 700 × g for 2 min, all but 1 ml of the KCl solution was removed, and the cells were gently resuspended; 10 ml of freshly prepared methanol-glacial acetic acid (3:1) were added to the cells for fixation. The fluorescence microphotographs were taken with the filters for the excitation wavelength at 563–598 nm (maximum, 582 nm) and emission at 584–620 nm (maximum, 600 nm).

RESULTS

Modeling of Porphyrin Interactions with G-Quadruplex. The solution structure of human telomeric G-quadruplex DNA, d(A₂G₃T₂AG₃)₁, has been determined (16). It is an intramolecular foldover structure stabilized by three guanine tetrads, stacked at its center. Fig. 1 shows the structure of the cationic porphyrin TMPyP4 (Fig. 1A) and a G-tetrad (Fig. 1B). The X-ray crystal structure of this porphyrin with a short duplex has been solved (19). This structure has two notable features: the porphyrin only fits halfway into the duplex, and there is extensive disruption of the bases adjacent to the intercalation site. We have built a model consistent with preliminary biochemical data to assess how TMPyP4 molecules may complex with the human telomeric quadruplex. The model shows that TMPyP4 is a good fit for stacking with G-tetrads, where it can be oriented to place each of the cationic N-methylpyridine groups into each of the four grooves of the quadruplex. A section through the minimized model is shown in Fig. 1c, and further biochemical evaluation of the interaction of TMPyP4 with quadruplexes is under way.

Inhibition of Telomerase Activity in HeLa Cell-free Extract. The effects of porphyrins on telomerase activity have been examined in HeLa cells, which express high levels of processive telomerase activity. The HeLa cell-free system has been routinely used as a reference in evaluating the effects of standard chemotherapeutics and novel agents on telomerase activity in human tumor cell lines and primary tumors (3, 17, 18, 20, 21). In this experiment, solutions of TMPyP4, TMPyP4-In³⁺, TMPyP4-Cu²⁺, QP3, and QP3-Cu²⁺ at 2.5, 5, 10, and 25 μM were added to the telomerase reaction mixture containing extract from 2000 HeLa cells, and the assay was run. Fig. 2A illustrates the dependence of telomerase activity on porphyrin concentration. The 32P signal intensity, due to incorporation of [α-32P]dGTP into the ladder produced by the action of telomerase, was quantitated in each lane. An IC₅₀ (porphyrin concentration that inhibited 50% of the telomerase activity in the control) was determined from a plot of relative activity against porphyrin concentration (Fig. 2B). The interpolated IC₅₀ were in the range of ≤50 μM.

Cytotoxicity of Porphyrins against Tumor and Normal Cells. To identify tumor models most suitable for evaluation and development of novel agents that target telomerase and/or telomeres, a systematic study was undertaken to measure telomerase activity in a range of human tumor cell lines. HeLa telomerase activity and median telomere length were used as reference points. Telomerase activity has been examined in a total of 39 human tumor cell lines representing breast, prostate, leukemia, lymphoma, ovary, colon, and non-small and small cell lung carcinomas. Tumor cell lines such as breast, prostate, and lymphoma consistently showed high levels of telomerase in the range of 20–40% of the activity in HeLa cells (average values). These tumor types (breast, prostate, and lymphoma) were used as models to examine biological effects of the novel QIAs.

Within each tumor type, cell lines were selected to represent a spectrum of telomerase activities. In breast carcinoma cell lines, telomerase activity relative to HeLa cells was high in MCF7 (40%), intermediate in MDA-231 (30%), and very low in BT20 (<1% HeLa control). Some of these cell lines were estrogen sensitive (MDA-231), and the others were estrogen resistant (BT20, MCF7, and HS578T). Prostate carcinoma cells were androgen receptor positive (LNCaP) and negative (DU145, PC3), and both had intermediate telomerase activity (30%). Raji lymphoma cell line expressed intermediate telomerase activity (30%), and Daudi lymphoma cells had low telomerase activity (<20%). Most porphyrins showed low cytotoxicity.
against the tumor cell lines examined and against normal human fibroblasts (Table 1). No apparent correlation was observed between cytotoxic effects of porphyrins and the endogenous levels of telomerase activity or the steroid hormone receptor status in the tumor cell lines.

To determine whether the cationic porphyrins exhibit differential toxicity between tumor and normal cells [as shown by some other quaternary aromatic cations (22)], MTT assays using TMPyP4 or TMPyP4\textsuperscript{z}Pt\textsuperscript{II} were run in a matched pair of human breast cell lines. Hs578t cells represented a transformed phenotype and expressed intermediate telomerase activity; Hs578Bst, normal breast cells, did not express detectable telomerase activity (data not shown). The cationic porphyrins showed similar cytotoxic EC\textsubscript{50}s in normal and transformed breast cells (Table 1). Thus, the general toxicity of porphyrins appears not to be telomerase related because telomerase inhibition occurs at concentrations below the toxic threshold for the cells.

Effects of TMPyP4 in Intact MCF7 Human Breast Carcinoma Cells. To determine the effects of TMPyP4 on whole cells, MCF7 breast carcinoma cells were cultured in the continuous presence of 1, 10, and 100 μM TMPyP4. The porphyrin solution was freshly added to the medium from the concentrated stock at each passage of the cells (every 3–4 days). On days 4, 8, and 15, the cells were lysed, and telomerase activity was measured in the extracts. A clear concentration-dependent loss of telomerase activity in the presence of TMPyP4 was observed at days 4 and 8 (Fig. 3A). The results were quantitated and expressed as a percentage of control telomerase activity (Fig. 3B). Inhibition of telomerase activity was also time dependent (Fig. 3C). Even at 1 and 10 μM TMPyP4 (below the cytotoxic EC\textsubscript{50} for MCF7 cells), the inhibition of telomerase activity showed a concentration dependence. The inhibition of telomerase activity by 1, 10, and 100 μM TMPyP4\textsuperscript{Pt\textsuperscript{II}} was also concentration dependent at days 4 and 8, but TMPyP4\textsuperscript{Pt\textsuperscript{II}} was a less potent inhibitor than TMPyP4 (data not shown). The extent of inhibition of telomerase by the two porphyrins in whole MCF7 cells thus parallels the relative potency of telomerase inhibition in the HeLa cell-free system.

Recent reports addressed the link between the cell cycle, regulation...
of telomerase activity, and its possible repression during quiescence and cell differentiation. Diverse cell cycle blockers, including growth factors and cytotoxic agents that caused inhibition of telomerase activity, also arrested the cells in G2-M (20). Telomerase activity is repressed in quiescent cells that exit the cell cycle (21). We examined whether the treatment of MCF7 cells with TMPyP4 affected the cell cycle. A fraction of the MCF7 cells from the long-term treatment with 100 μM TMPyP4 (in which telomerase activity was measured) was also subjected to the analysis of the cell cycle. A time-dependent gradual increase in the G2-M phase and corresponding to 2, 4.9, 14.4, and 31.9% measured at days 0, 4, 8, and 15, respectively, was observed in cells cultured in the presence of 100 μM TMPyP4.

**Nuclear Localization of Porphyrins.** Several reports indicate that porphyrins localize specifically in tumor tissue, although sites of subcellular localization may vary widely with porphyrin structure and net charge (23). Porphyrin TMPyP4 has been shown to rapidly accumulate (within minutes) in the nuclei of cultured human dermal fibroblasts (23). To gain insight into the subcellular distribution of cationic porphyrins, we have cultured MCF7 cells with 0 or 50 μM TMPyP4 for 7 days. When MCF7 cells were grown in the presence of 50 μM TMPyP4, porphyrin concentration in the nuclear pellet was 1.39 nmol/10^6 cells, and the cytoplasm contained 0.047 nmol/10^6 cells (3.3% of the TMPyP4 concentration measured in the nuclear fraction). These measurements did not differentiate between free and bound porphyrin; consequently, the measured values are likely to be an underestimate of the local porphyrin concentration associated with the DNA fraction. Assuming that the volume of the nucleus is one-tenth of the cell and that the volume of 10^6 cells is 10 μl, the calculated concentration of TMPyP4 in the nucleus (1.4 mM) greatly exceeds that of the cytoplasm (4.7 μM) and can readily approach the telomerase IC50, seen in the cell-free system.

To examine whether porphyrin in the nucleus was associated with chromosomal DNA, we examined fluorescence in metaphase spreads prepared from cells cultured in the presence of the cationic porphyrin. From a preliminary assessment of intrinsic fluorescence in solution, porphyrin QP3·InIII was selected as the strongest fluorophore in the TMPyP4 and QP3 series. MCF7 cells were cultured in the continuous presence of 40 μM QP3·InIII for 3 days. After that time, the cells were washed, fixed, and used for preparation of metaphase spreads. Direct evidence for the chromosomal localization of QP3·InIII porphyrin is shown in Fig. 4. These findings are consistent with the known affinity of the cationic porphyrins for DNA and indicate that porphyrins can accumulate in the nuclei of intact tumor cells at concentrations possibly exceeding those in the surrounding medium.

**DISCUSSION**

This report demonstrates for the first time that targeting G-quadruplex is a sound strategy for the inhibition of telomerase activity in cancer cells. This finding is important because it demonstrates the validity of a rational, structure-based approach to the design of compounds that, albeit indirectly, yield net telomerase inhibition. The fact that DNA, not telomerase protein or RNA, can be a target for rational drug design has important implications for understanding the telomerase mechanism of action.

We have shown that cationic porphyrins inhibit telomerase and
cause G2-M arrest. These effects, happening in a time frame (<15 days) comparable with that of the cytotoxicity studies, might be expected from the G-tetraplex interactive agents interfering with the correct functioning of telomere structures.

The cationic porphyrins, particularly TMPyP4, are telomerase inhibitors at low micromolar concentrations. Furthermore, these porphyrins are relatively nontoxic to cells (both tumor and normal) at levels that can inhibit telomerase. Therefore, with this class of G-quadruplex interactive compounds, a selective inhibition of telomerase activity can be achieved at concentrations that do not have general toxic effects on cells. The uptake and accumulation data demonstrate that the cationic porphyrins can accumulate at useful levels (i.e., inhibitory to telomerase) in the nuclei of intact cells. Structure-activity relationships for the inhibition of telomerase by cationic porphyrins will be discussed elsewhere. Further development of cationic porphyrins as QIAs is under way.

Our results suggest that the short-term exposure of the human tumor cells to TMPyP4 leads to repression of telomerase activity. The repression was paralleled by a time-dependent increase in cell arrest at the G2-M phase. Telomerase activity may thus be different at various stages of the cell cycle, as proposed (20). However, with no more than 32% of the porphyrin-treated MCF7 cells arrested in G2-M, only residual telomerase activity was measured. We did not see an apparent correlation between G2-M arrest and telomerase activity because in separate control experiments, using untreated cells with varying percentages of the cells in G2-M phase, we still measured high telomerase activity. These results would suggest that although TMPyP4 causes G2-M arrest, G2-M arrest alone does not necessarily lead to inhibition of telomerase. Therefore, a variation of telomerase activity in the cell cycle is not a plausible explanation for the observed decrease of telomerase activity. Another possibility is an indirect effect of TMPyP4 on down-regulation of telomerase expression, recently postulated as a part of the mechanism of cisplatin action against human testicular cancer cells (12).

The first indication that TMPyP4 induces chromosomal abnormalities has been found in sea urchin embryos. Recently, we found that TMPyP4 significantly decreased proliferative rates in the developing embryos, increased the percentage of the cell in mitosis, and led to formation of largely elongated mitotic chromosomes. A similar phenotype has been described in Tetrahymena with telomerase RNA template mutation (24) and in cells expressing dominant-negative TRF2 allele (25). In HeLa cells cultured in the presence of 3 μM TMPyP4 for 2–4 days, the number of fused chromosome was increased in comparison with the untreated controls. These chromosomal aberrations may be sufficient to inhibit cellular proliferation even in the absence of repression of telomerase activity.

Although our results indicated that TMPyP4 may induce chromosome destabilization, telomere shortening was not likely to occur under the experimental conditions. Telomeres shorten by ~65–100 bp/cell division (26), and 3'-azido-3'-deoxythymidine effects on telomere length have been demonstrated in long-term human cell line cultures (10). At this rate of telomere loss, even with relatively short telomeres in MCF7 cells, a long time in culture (>20 population doubling time) in the presence of the porphyrins may be required to achieve significant effects on telomere length.

Telomeres have been shown to play a direct role in mitosis: a physical block in anaphase chromosome separation is caused by telomerase template mutation (25). One implication of this finding is that porphyrins (as telomere-interactive agents) may arrest the cells in mitosis. We have already observed an increase of the G2-M fraction in MCF7 cells treated with TMPyP4. A thorough investigation of the antiproliferative effects of porphyrins in tumor cells is in progress.

Telomere shortening may not be a paradigm for all telomerase inhibitors. Highly variable telomere lengths in two immortalized cell lines treated with reverse transcriptase inhibitors were supposedly the result of telomerase-dependent and telomerase-independent mechanisms for telomere maintenance (10). Therefore, agents like TMPyP4 that repress telomerase activity and that can directly interact with telomeres may be effective in situations where telomere length in tumor cells is maintained by telomerase-independent mechanisms (27). In the present work, we show that the QIAs directly interact with telomeres and rapidly evoke antiproliferative effects in tumor cells. Telomerase repression may be a secondary event to tumor growth arrest. Thus, the QIAs that represent a different mechanism of action than other telomerase inhibitors may be most effective in tumors with low telomerase activity. Ongoing studies address the issue of the best clinical models for QIAs.

In summary, our work provides proof of principle for the development of small-molecule telomerase inhibitors targeting telomeric DNA rather than telomerase per se. The rapid cell response to QIAs indicates greater significance and multiple roles for G-quadruplex in the whole telomere system. Recent reports describing telomere shortening and tumor formation by mouse cells lacking telomerase RNA (28, 29) raised multiple questions regarding the validity of telomerase as a target for anticancer agents. In the light of these findings, our alternative strategy focusing on telomere interactive agents is strongly justified.

ACKNOWLEDGMENTS

We thank Gina Mangold, Maria Chapa, and Jeff Chavez for the MTT assays; Dr. John McGill and Vicki Mattern for the mitotic spreads; Richard Salinas for the flow cytometry assays; and Margaret Durack and Dr. Meredith Sterling for excellent assistance in preparation of the manuscript.

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