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Uptake of ^{99m}Tc- and ^{32}P-Labelled Oligodeoxynucleotides in an Osteosarcoma (OHS) Cell Line

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SUMMARY

The concept of using oligonucleotides in imaging relies on the passage of a sufficient number of molecules through the cell membrane to reach the intracellular mRNA target. We have investigated the uptake in OHS osteosarcoma cells of phosphodiester and phosphorothioate oligodeoxynucleotides (ODNs) modified with the bifunctional chelating agent mercaptoacetyldiglycine (MAG2) and labelled with ^{99m}Tc. In comparison to the results of corresponding experiments with ^{32}P-labelled ODNs, the presence of the MAG2 chelating moiety and technetium-99m radiolabel was found to be of minor importance in the uptake process. The overall uptake in OHS cells was in the range 4.9-9.1 pmol/10^6 cells after 3 hours for the ^{99m}Tc-MAG2-ODNs irrespective of backbone. The corresponding uptake of ^{32}P-ODNs was 10.3 and 25.9 pmol/10^6 cells after 3 hours for the phosphodiester and phosphorothioate backbones, respectively. No differences in uptake related to the sequences of the

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oligonucleotides were observed. In view of the demonstration that the ^{99m}Tc-MAG2-ODNs are taken up in OHS cells in vitro, this study brings promise to the possible utilisation of oligonucleotides in nuclear medicine.

INTRODUCTION

The design of antisense oligodeoxynucleotide (ODN) drugs for use in human medicine appears facile at first glance. If the DNA or mRNA sense sequence is known, the antisense can easily be composed by simply matching the cytidine nucleotides with guanosines and adenosines with thymidines or uridines or vice versa. However, an exact nucleotide match with a mRNA target is not the only element involved in antiser.se drug targeting; accessibility to the sense site is important, and the ability for the antisense probe to cross cell membranes and actually reach the target is essential.

The cell membrane provides the cell with an efficient selection mechanism to uptake of extracellular material. The uptake of phosphodiester (PO) and phosphorothioate (PS) oligonucleotides with various fluorescence or radioactive labels has in recent years been studied in a number of transformed and normal cell types (1- 6). While the uptake of these polyanions in general is modest and variable from one cell type to another, two concentration dependent mechanisms of uptake have been described, i.e. receptor-mediated endocytosis and fluid-phase pinocytosis (1, 7-11). The existence of a 80-kD membrane protein in HL-60 cells that is a possible mediator of the active ODN uptake has been reported (12).

To be useful in diagnostic nuclear medicine, an antisense ODN labelled with an imageable radionuclide has to be able to survive the journey through the blood stream and to efficiently cross the cell membrane to reach the mRNA target. Cell uptake of a ^{111}In-labelled, and thus suitable for imaging, 15-nucleotide (15-nt) ODN conjugated to the metal binding diethylene triaminepentaacetic acid (DTPA) has been reported in P388 cells. The cell uptake was reported as the radioactivity associated with a cell suspension after washing, and a high uptake was observed (13).

The radionuclide of choice for imaging is ^{99m}Tc, and in the present study, the uptake of ^{99m}Tc-labelled antisense ODNs with PO or PS backbones in OHS osteosarcoma cells was evaluated. The OHS cell line expresses high levels of the CAPL gene, which in turn is associated with the metastatic phenotype of tumour types like human mammary carcinomas, osteosarcomas and colon carcinomas (14). One of the ODNs investigated in this study is antisense to a sequence on the CAPL gene.

To investigate the influence of the ^{99m}Tc radiolabel and the chelating moiety on the cell uptake, ^{32}P-labelled antisense ODNs with the same sequence and backbones were included in the study. The cell uptake of ^{99m}Tc-labelled ODNs has not previously been reported, but since several reports have dealt with the level of uptake of various ^{32}P-ODNs, these analogues were chosen for comparative purposes (15, 16).

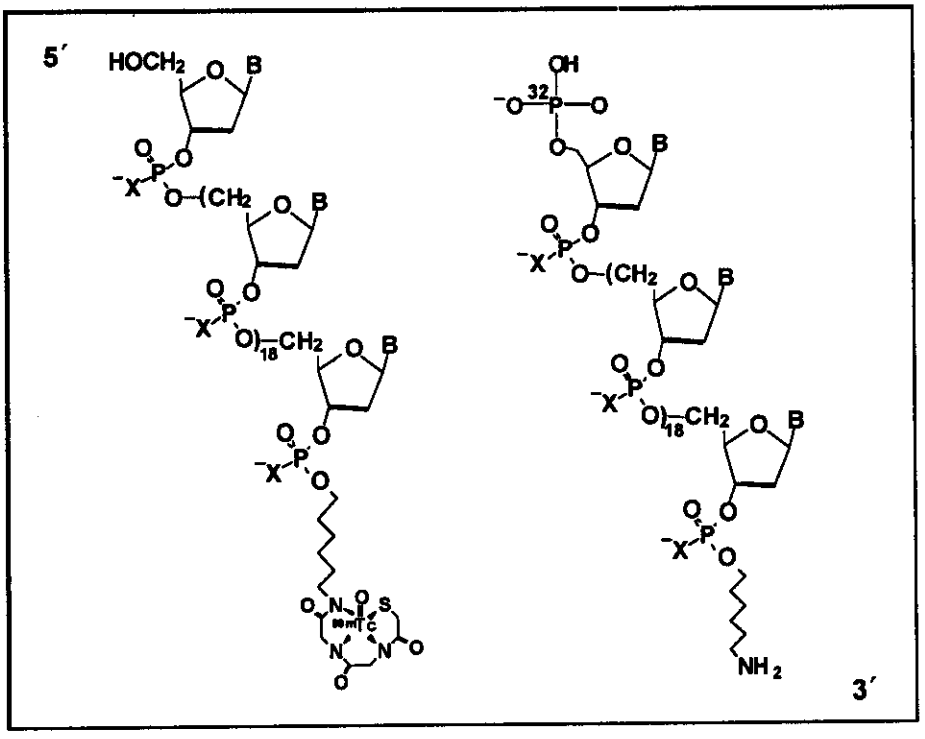

Figure 1. Proposed structures of a 20-nt 3'-labelled ^{99m}Tc-MAG2-ODN and 5'-labelled ^{32}P-ODN. B = base (adenine, guanine, cytosine, thymine). X = O (phosphodiester) or S (phosphorothioate).

MATERIALS AND METHODS

Oligodeoxynucleotides

GX-1 is a 20-nucleotide antisense oligodeoxynucleotide with proven access to a site on exon 2 of the mRNA of the CAPL gene (17). The sequence of GX-1 is 5'-GGA AGG TGG ACA CCA TCA CA-3'. CTRL2 is a scrambled control ODN that contains the same 20 nucleotides as GX-1, but in a random order. The sequence of CTRL2 is 5'-AGT GAC CGA GTA GGC ACC AA-3'. Phosphodiester GX-1 and CTRL2 with a 1-aminohexyl linker on the 3'-end were synthesised by Eurogentec (Ougree, Belgium). The corresponding phosphorothioate GX-1 and CTRL2 ODNs were synthesised by Oligon AS (Oslo, Norway).

Labelling with ^{99m}Tc

Conjugation of the bifunctional chelating agent S-benzoylmercaptoacetyldiglycine-N-hydroxysuccinimide (S-benzoyl-MAG2-NHS) to the 3'-end of each of the ODNs, purification of the conjugates and their labelling with ^{99m}Tc was performed as described earlier. The labelling yields were in all cases determined using reversed phase HPLC (18). If necessary, a calculated volume of a 200 pmol/μl unconjugated ODN solution was added in order to obtain a final ODN concentration of 10 pmol/μl. The proposed structure of a ^{99m}Tc-MAG2-ODN is presented in Figure 1.

Labelling with ^{32}P

Phosphodiester or phosphorothioate GX-1 was 5'-labelled with ^{32}P by an enzymatic labelling reaction (19)(Figure 1). Lyophilised ODN (0.02-0.03 μmol) was dissolved in 2000 μl sterile water, heated in a boiling water bath for 2 min and snap cooled. One μl of this solution was mixed with 3 μl of a γ-^{32}P-ATP solution (370 MBq/ml, 110 GBq/μmole, Amersham, England), 1 μl of 10x kinase buffer (Promega, Madison, WI), 5.5 μl water and 10 units T4 polynucleotide kinase (Promega). The reaction mixture was incubated at 37°C for 30 min. Finally, 1 μl 100x TE (1 M TRIS/0.1 M EDTA) buffer pH 7.5 was added to stop the kinase reaction. The ^{32}P-ODNs were separated from unbound ^{32}P-ATP on a NAP-25 column (Amersham Pharmacia Biotech, Uppsala, Sweden). The concentrations of the ^{32}P-ODNs were

measured and the solutions were diluted with Hepes buffer to a suitable radioactivity concentration of 1-4 kBq/μl. To obtain a final ODN concentration of 10 pmol/μl, a calculated volume of a 200 pmol/μl unconjugated ODN solution was added.

Cell uptake studies

OHS cells (5-10x10^3) were plated in sterile 96-well plates and grown in 200 μl fresh growth medium, comprising RPMI 1640 medium (GibcoBRL, Life Technologies, Rockville, Maryland), 10 % heat-inactivated foetal calf serum and 200 mM L-alanyl-L-glutamine. The cells were incubated for 24 hours at 37°C in 5 % CO_2/95 % air. Then the confluence of the cells was controlled under the microscope and the cell number determined. The medium was replaced with 90 μl growth medium without serum (preheated to 37°C) and then 10 μl of the radiolabelled ODN was added to a final concentration of 1 μM radiolabelled ODN. The amount of radioactivity used varied to a certain extent according to the labelling yield and, for ^{99m}Tc-labelled ODNs, the activity in the ^{99m}Tc-eluate. Normally, the activity range was 10-40 kBq ^{32}P-ODNs or 1-5 MBq ^{99m}Tc-MAG2-ODN. The cells were incubated at 37°C for 30 min or 3 hours, or in some cases up to 24 hours. After removal of the incubation medium containing the excess radiolabelled ODN, a four-step wash procedure was applied to remove non-internalised ODN and dead cells, which can accumulate radiolabelled ODNs and thereby skew any uptake measurement (4). The cells were washed successively with 100 μl fresh growth medium (37°C), 100 μl PBS buffer, 100 μl high salt/low pH buffer (1 M NaCl, 0.4 M acetate pH 2.6), and 100 μl PBS buffer (20). To quantify the uptake of the radiolabelled ODNs, the cells were lysed with 100 μl 1% sodium dodecyl sulphate (SDS) and the lysates transferred to 96-well counting plates (CulturPlate®, Packard Instruments, Meriden, CT)(^{32}P) or polypropylene tubes (^{99m}Tc). Subsequently, the wells were washed with 200 μl PBS that was mixed with the lysates. The ^{32}P-ODNs present in the plates were quantified after addition of MicroScint-20 scintillation liquid (Packard Instruments) using a TopCount sample changer (Packard Instruments) equipped with six phototubes. The ^{99m}Tc present in the polypropylene

tubes was counted in a Wizard 1480 sample changer (Wallac Oy, Turku, Finland) with a 3-in. NaI(Tl) scintillation detector.

As a control, radiolabelled ODNs were incubated in 100 µl serum-free growth medium in the absence of cells. Other control experiments were the incubation of cells together with the potential impurities ^{99m}Tc-MAG2, $^{99m}TcO_4^-$ and γ-^{32}P-ATP in the same manner as for the radiolabelled ODNs. For all controls, the same washing and quantification procedures as described above were applied. Uptake was calculated as the remaining radioactivity in the lysate as a function of the total radioactivity in all samples.

Analysis of the wash solutions

The incubation medium and each of the solutions used in the wash procedure were transferred to polyethylene tubes and samples were removed for analysis in the TopCount or Wallac detectors.

RESULTS

^{99m}Tc-MAG2-ODNs

The internalisation of ^{99m}Tc-MAG2-ODNs in OHS cells was initially studied over a time period of 3 hours due to reports demonstrating rapid cell uptake up to 3 hours followed by a more gradual increase over the next 21 hours (20). The observed cell uptake of the ^{99m}Tc-MAG2-ODNs after 30 min was in the range 1.6-4.3 pmol/10^6 cells, increasing to 4.9-9.1 pmol/10^6 cells after 3 hours (Figure 2). The uptake of the CTRL2 sequences mainly followed the same patterns as the GX-1, irrespective of the backbone modification.

In a separate control study, in which the uptake of ^{99m}Tc-MAG2-PS-GX-1 was compared with the uptake of ^{99m}Tc-MAG2 and $^{99m}TcO_4^-$, a 24 hours time point was included. ^{99m}Tc-MAG2 could be present in the preparations as a consequence of contamination of the conjugate after the purification process, while $^{99m}TcO_4^-$ was a potential impurity as a starting material in the labelling reaction. Pertechnetate did not cross the cell membrane to any extent. The percentage uptake of ^{99m}Tc-MAG2 was similar to that of the ^{99m}Tc-MAG2-ODN at the early time points, but almost twice as high at 24-hours (Figure 3).

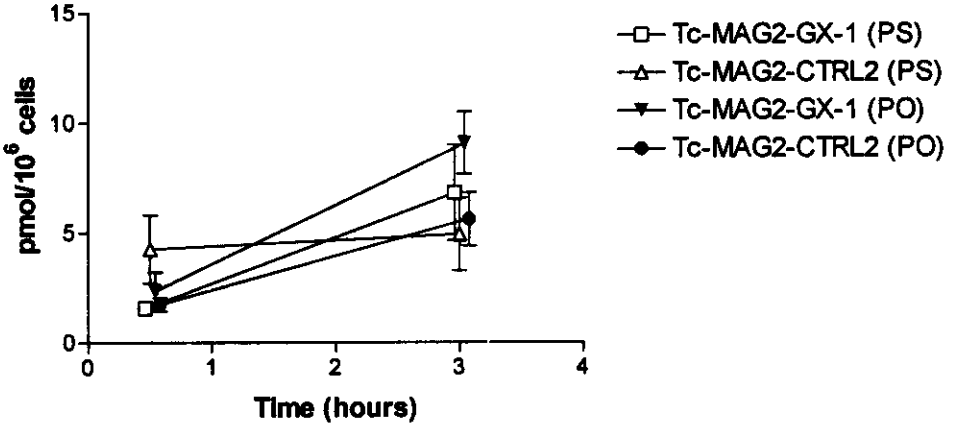

Figure 2. Uptake of ^{99m}Tc-MAG2-PS-ODNs (n=6) and ^{99m}Tc-MAG2-PO-ODNs (n=3) in the OHS cell line 30 min and 3 hours after addition of radiolabelled ODN. Standard errors of the means are indicated.

The distribution of radioactivity in the waste solutions was analysed, showing that, on average, 99.8 % of the radioactivity was removed with the incubation solution and the solutions of the washing procedure. Less than 0.01 % of the radioactivity could be removed with the last wash fraction.

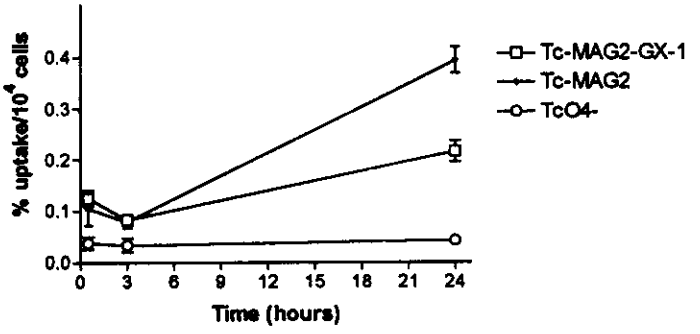

Figure 3. Percentage of the radioactivity of the ^{99m}Tc-MAG2-PS-GX-1 (n=8), ^{99m}Tc-MAG2 (n=4) and $^{99m}TcO_4^-$ (n=4) in the OHS cell line up to 24 hours after addition of radioactivity. Standard errors of the means are indicated.

As a further control on the effect of dilution on the residual activity, ^{99m}Tc-MAG2-PO-GX-1 and ^{99m}Tc-MAG2-PS-GX-1 were incubated without cells. The remaining activity in the wells

that was removed with the lysis buffer and subsequent washing with PBS was corresponding to 0.002 % and 0.007 % of the dose, respectively.

^{32}P-ODNs

The cell uptake of 0.10 % (10.3 pmol/10^6 cells) of the ^{32}P-labelled PO-ODN after 3 hours was similar to the uptake observed with ^{99m}Tc-ODNs. However, the uptake of the ^{32}P-labelled PS-ODN was more than twice as high (0.26 % of the dose, 25.9 pmol/10^6 cells) at this time point. After 24 hours, there were no significant differences in uptake between the PO and PS ^{32}P-ODNs (Figure 4).

The percentage uptake of the single nucleotide ^{32}P-adenosine triphosphate was a factor of 5 higher than the radiolabelled oligonucleotides after 24 hours. To ensure a negligible contribution from ^{32}P-ATP in the cell uptake experiments, the ^{32}P-ODNs used in this study were separated from unbound ^{32}P-ATP by purification on NAP-25 columns prior to use in the cell incubation experiments. By counting the radioactivity in the fractions eluting off the columns, the labelling yield for each labelling reaction could be determined. For the PO-ODNs, the labelling efficiency was 85-90 %. Surprisingly, the kinase reaction for the PS ODNs was much less efficient; a yield of only 30-35 % ^{32}P-PS-ODNs was reproducibly obtained.

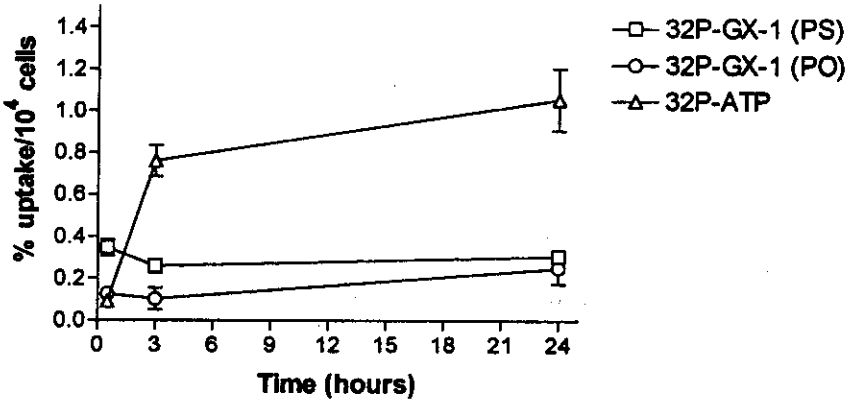

Figure 4. Percentage of the radioactivity of ^{32}P-PS-GX-1, ^{32}P-PO-GX-1 and ^{32}P-ATP in the OHS cell line up to 24 hours after addition of the radiolabelled substances to the medium. Standard errors of the means are indicated (n=12).

Analysis of the wash solutions obtained from the ^{32}P-ODN uptake experiments gave the same pattern of radioactivity distribution as described above for the ^{99m}Tc-ODNs.

DISCUSSION

The important findings of the present study are that ^{99m}Tc-MAG2-ODNs can be internalised by OHS cells and that the cellular uptake is within the same order of magnitude as ^{32}P-ODNs. The range of uptake of 1.6-9.1 pmol/10^6 cells of ^{99m}Tc-MAG2-ODNs after 30 min and 3 hours was similar to the uptake previously reported for fluorescein-labelled 18-nt ODNs in CD34+ immature hematopoietic cells (21) and fluorescein-labelled CAPL-specific ribozymes in THX cells (22). The influence of the 3'-modification on the ODN with a MAG2 chelating agent and a technetium radionuclide was less on the PO ODNs than on the PS ODNs. The cellular uptake of the phosphodiester ^{99m}Tc-MAG2-ODNs in OHS cells was found to be slightly lower, but comparable to the uptake of the 5'-labelled ^{32}P-PO-ODN after 3 hours. The uptake of the phosphorothioate ^{32}P-ODN was three times higher than that observed for the ^{99m}Tc-MAG2-ODNs after 3 hours. The size, charge and lipophilicity of the ^{99m}Tc-MAG2 moiety has possibly altered the overall properties of the oligonucleotide moderately, as previously reported for cell uptake in 293 cells of PS ODNs with various modifications on the 3'-end (23). This means that further investigations on bifunctional chelating agents with different size, lipophilicity and charge after labelling, may result in ^{99m}Tc-labelled ODNs with higher cell uptake.

The higher initial uptake of phophorothioate over phosphodiester ^{32}P-ODNs at 30 min and 3 hours was almost balanced at 24 hours (30.3 and 25.2 pmol/10^6 cells for PO and PS ODNs, respectively). While the PS ^{32}P-ODN uptake was relatively constant from 30 min to 24 hours, the PO ^{32}P-ODN uptake gradually increased up to 24 hours. This could possibly be due to dephosphorylation of the PO ^{32}P-ODN, with liberation of the ^{32}P label for more efficient uptake as a result, as has previously been described for PO and PS ^{32}P-ODNs in Caco-2 monolayers (16). The uptake of ^{99m}Tc-MAG2-PS-GX-1 also increased to 21.7 pmol/10^6 cells after 24 hours, possibly due to slower uptake kinetics of the 3'-modified ODN. The stability

of the ^{99m}Tc-MAG2-PS-GX-1 for 24 hours in the presence of cells is not known, but a good stability of the complex has previously been demonstrated in fresh growth medium up to 3 hours (18).

Natural (PO) and backbone modified (PS) ODNs were included in this study for comparative evaluation. The uptake was independent of oligonucleotide backbone modification, since the uptake followed the same patterns for the phosphodiester and phosphorothioate ^{99m}Tc-MAG2-ODNs, as well as the PO and PS ^{32}P-ODNs. Previously, there has been studies showing higher cell association of PS ODNs than the equivalent PO ODNs in various cell types (4, 16, 20). The PS ODNs were found to have higher affinity for the cell surface receptors than the PO ODNs. In the present study, an extensive wash procedure was applied in order to remove cell surface bound radioactivity and dead cells to be able to measure only internalised radiolabelled ODNs.

The sequence of the oligonucleotide is important for sense sequence recognition and hybridisation of the antisense oligodeoxynucleotides to the mRNA target. In the uptake process, however, the polyanionic backbones of PO and PS ODNs appear to be most important, there is a relatively minor expectancy to the importance of the sequence of the ODN in the cell uptake process. This is particularly so in the case of two ODNs as similar as GX-1 and CTRL2. Even though some reports have indicated a sequence dependency for some ODNs (13, 24), there was no evidence of any difference in the uptake of the two different sequences GX-1 and CTRL2 in this study.

The uptake of the pertechnetate anion in OHS cells was negligible, while the uptake of the free chelate, ^{99m}Tc-MAG2, was equally efficient to that of the corresponding ^{99m}Tc-MAG2-ODN conjugate complex after 30 min and 3 hours. However, after 24 hours, the percentage uptake of ^{99m}Tc-MAG2 was almost twice as high as that of the ^{99m}Tc-MAG2-ODN. Theoretically, the presence of ^{99m}Tc-MAG2 as an impurity in the ^{99m}Tc-MAG2-ODN preparation could skew the uptake measurements if present in substantial amounts. In the preparations used for these studies, the ^{99m}Tc-MAG2 impurity was less than 3 % of the total radioactivity and should not be the cause of a major contribution to the uptake values.

In this study, it has been demonstrated that the uptake of ^{32}P-ATP was more efficient than the uptake of ^{32}P-ODN in OHS cells.

The extensive washing procedure and the non-existent uptake in wells without cells, ensure that the uptake values observed really reflect cell uptake and not merely residual activity.

As a consequence of the data observed in this study, the focus for further research on oligonucleotides for use in nuclear imaging should be on the membrane penetration properties and measures to increase the cellular uptake. This could mean the development of carrier systems for oligonucleotides, the synthesis of oligonucleotides with modifications in the backbone, ribose or bases, or the development of other chelating agents for technetium-99m to assist in the membrane penetration.

CONCLUSIONS

The uptake of ^{99m}Tc-MAG2-ODNs in OHS cells has been demonstrated. The uptake was slightly less efficient as the uptake of ^{32}P-ODNs, which means that the ^{99m}Tc-MAG2 moiety exerts a minor influence on the uptake of oligodeoxynucleotides on the cellular level.

There was no difference in the uptake in OHS cells with respect to the phosphodiester or phosphorothioate backbones of the ^{99m}Tc-MAG2-ODNs.

There was not observed any difference in the uptake in OHS cells with respect to the sequences of the nucleotides in the two ODNs included in this study.

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