Brief Articles

Structural Analogues of Smoothened Intracellular Loops as Potent Inhibitors of Hedgehog Pathway and Cancer Cell Growth

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Received May 15, 2007

Smoothened is a critical component of the Hedgehog pathway that is essential for stem cell renewal and is dysregulated in many cancer types. We have found synthetic analogues of the second and third intracellular loops of smoothened to be potent inhibitors of the Hedgehog pathway. Palmitoylated peptides as short as 10 residues inhibited melanoma cells growth with IC_{50} in the low nanomolar range. The compounds are promising drug candidates and convenient tools for solving mechanisms of Hedgehog signaling.

Introduction

The Hedgehog (HH[a]) pathway is essential for embryonic and stem cell growth and differentiation.[1] It is also known to be dysregulated in breast,[2] prostate,[3] stomach,[4] colon,[5] liver,[6] pancreatic,[7] and lung[8] cancers, melanomas,[9] multiple myelomas,[10] basal cell carcinomas,[11] and medulloblastomas.[12,13] Hedgehog is a soluble protein secreted by cells. It binds to a membrane transporter called Patched (Ptch), which in turn regulates the function of another integral membrane protein smooothened (SMO). It was found recently that vitamin D3 is the molecule that potentiates the effects of Ptch on SMO.[14] SMO signals further to the nucleus to activate a family of transcription factors called Gli (from glioma-associated oncogene). Most of the dysregulations in Hedgehog pathway in cancer are caused by mutations in Ptch and SMO. Since SMO signals downstream from Ptch, SMO antagonists could be effective in inhibiting the pathway that was activated by both types of mutations. Steroidal alkaloid cyclopamine isolated from corn lily (*Veratrum californicum*) and its derivatives are well characterized SMO antagonists.[15−17] They were shown to suppress the growth of cancer cells in vitro[3,18] and in vivo.[19−22] Although SMO is a seven-transmembrane domain protein and resembles G-protein-coupled receptors (GPCR) in general topology (Figure 1), its signaling mechanisms appear to differ significantly from GPCRs. Unlike GPCRs, direct interaction of SMO with G-proteins was not detected, although Gi proteins have been suggested to be involved in the pathway.[23] In general, downstream events of SMO signaling in mammals are poorly understood and immediate signaling partners have not been identified.[24]

Synthetic analogues of third and second intracellular loops of GPCRs have been shown to inhibit or in several instances activate signaling of the target receptor.[25−28] However, the resulting inhibitors turned out in some cases to be insufficiently specific.[29] The nonspecific inhibition may be due to the fact that different GPCRs have significant sequence homology in intracellular loops, which is not surprising because they interact with the same G-proteins and kinases. Primary structures of SMO intracellular loops are unique and very much conserved among the species (Figure 2), which is indicative of a significant and unique role in intermolecular or intramolecular interactions. We have undertaken the evaluation of synthetic analogues of SMO intracellular loops as possible effectors of HH signaling and have found several compounds to be potent and specific SMO antagonists with remarkable antitumor activity.

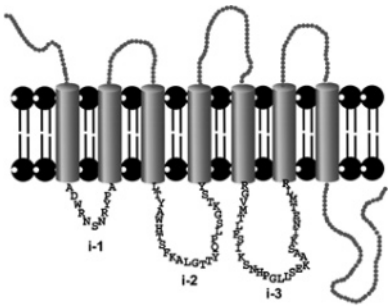

Figure 1. Predicted topology of smoothened (SMO) with seven-transmembrane helixes shown as cylinders and three intracellular loops shown in one-letter amino acid residues codes.

Results

For initial evaluation, we have synthesized peptides corresponding to full lengths of all three intracellular loops and equipped them with N-terminal palmitoyl residues for easier membrane penetration. All three peptides inhibited growth of breast cancer MCF-7 cells (Figure 3). The peptide corresponding to the third loop (SMOi3-1) had the most significant effect on cell growth, followed by the second loop derivative SMOi2-1, while the first loop-derived peptide (SMOi1-1) was the least active of the three.

For determination of the minimal effective peptide length, gradual truncation from both C-terminal and N-terminal ends

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[a] Abbreviations: HH, hedgehog; SMO, smoothened; Ptch, patched; GPCR, G-protein-coupled receptor; Hcy, homocysteine.

10.1021/jm0705657
Published on Web 08/08/2007

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                i1
SMO_HUMAN    LFTLATFVADWRNSNRYPAVILFYVNACFFVGSIGWLAQFMDGARREIVC
SMO_MOUSE    LFTLATFVADWRNSNRLPAVILFYVNACFFVGSIGWLAQFMDGARREIVC
SMO_RAT      LFTLATFVADWRNSNRYPAVILFYVNACFFVGSIGWLAQFMDGARREIVC
SMO_CHICK    FFTLATFVADWRNSNRYPAVILFYVNACFFVGSIGCVAQFMDGARDEIVC
SMO_XENLA    FFTLATFLADWKNSNRYPAVILFYVNACFFVGSIGWLAQFMDGARDEIVC
SMO_BRARE    FFTLATFLADWKNSNRYPAVILFYVNACFFIGSIGWLAQFMDGARNEIVC
              ****** *** **** ****************** *
                                                 i2
SMO_HUMAN     VIIFVIVYYALMAGVVWFVVLTYAWHTSFKALGTTYQPLSGKTSYFHLL
SMO_MOUSE     VIIFVIVYYALMAGVVWFVVLTYAWHTSFKALGTTYQPLSGKTSYFHLL
SMO_RAT       VIIFVIVYYALMAGVVWFVVLTYAWHTSFKALGTTYQPLSGKTSYFHLL
SMO_CHICK     VIIFVIVYYSLMSGVIWFVMLTYAWHTSFKALGTTYQPLLGKTSYFHLI
SMO XENOPUS   VIIFIIVYYSMMSGVIWFVMLTYAWHTSFKALGTTHQPLSGKTSYFHLI
SMO BRARE     VIIFVIVYYSLMSGVIWFVMLTYAWHTSFKALGTTHQPLSGKTSYFHLV
              *********  * ** *** *************** *** ****
                                 i3
SMO_HUMAN      VGGYFLIRGVMTLFSIKSNHPGLLSEKAASKINETMLRLGIFGFLA
SMO_MOUSE      VGGYFLIRGVMTLFSIKSNHPGLLSEKAASKINETMLRLGIFGFLA
SMO_RAT        VGGYFLIRGVMTLFSIKSNHPGLLSEKAASKINETMLRLGIFGFLA
SMO_CHICK      VGGYFLIRGVMTLFSIKSNHPGLLSEKAASKINETMLRLGIFGFLA
SMO XENLA      GGGYFLIRGVMTLFSIKSNHPGLLSEKAASKINETMLRLGIFGFLA
SMO BRARE      IGGYFLIRGVMTLFSIKSNHPGLLSEKAASKINETMLRLGIFGFLA
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Figure 2. Sequence alignment of SMO from different species shows significant conservation in the portions corresponding to intracellular loops (i1, i2, and i3, underlined).

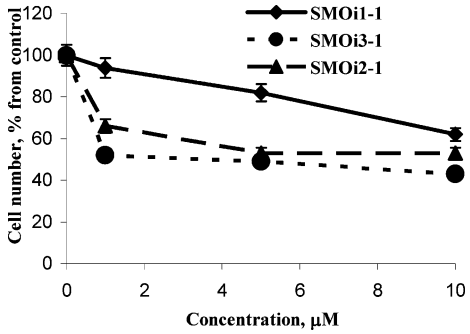

Figure 3. Growth inhibition of breast cancer MCF-7 cells by N-palmitoylated peptides spanning entire intracellular loops of SMO: SMOi1-1, Pal-TFVADWRNSNRY; SMOi2-1, Pal-TYAWHTSFKAL-GTTYQPLSGKTS; SMOi3-1, Pal-RGVMTLFSIKSNHPGLLSEKAA-SKINETMLR.

was performed on i2 and i3 peptides. Interestingly, both N-terminal and C-terminal halves of the loops had some antiproliferation activity. C-Terminal halves were derivatized with palmitic acid residue by addition of ϵ-palmitoyl-Lys to the C-terminus. Initial activity testing was performed on breast cancer cell line MCF-7, while subsequent SAR studies were conducted on melanoma cell line SK-Mel2, which was chosen because it was found to be one of the most sensitive to an established SMO antagonist, cyclopamine in the NCI 60 cell line screen. Palmitoylation of the peptides was necessary for the activity and substitution of palmitoyl residue with an acetyl (SMOi2-9) and resulted in significant loss of potency. Palmitoyl group had to be placed at the end of the loop adjacent to the membrane. Positioning it inside the loop generated a significantly less active peptide (SMOi3-4) (Table 2). Peptides corresponding to parts of i3 loop had activities comparable to or lower than that of the full-length loop (Table 2).

In contrast, C-terminal truncation of the second intracellular loop allowed the obtaining of peptides that were significantly more toxic to cancer cells than the derivatives of the full-length loop (Table 1). Both halves of the loop provided with palmitic acid residue at the end adjacent to the membrane were active. However, C-terminal extension of the N-terminal half (SMOi2-8) lowered the activity of the 12-residue peptide (peptides SMOi2-6 and SMOi2-3). The most potent peptide (SMOi2-12) was 10 residues long. Further truncation of the 12-mer on the N-terminus (SMOi2-10) resulted in less active compounds. Palmitic acid derivatization appeared to be essential for activity,

Table 1. Structure−Activity Relationships in Derivatives of the Second Intracellular Loop of SMO[a]

Compound	Structure	IC_{50}, μM
SMO i2-1	Pal-LTYAWHTSFKALGTTYQPLSGKYSY	0.45±0.05
SMO i2-2	Pal-LTYAWHTSFKALGTTYQPLSGKTSY	0.45±0.05
SMO i2-3	Pal-LTYAWHTSFKALGTTYQPLSG	1.4±0.4
SMO i2-4	Ac-LTYAWHTSFKALGTTYQPLSGKTSYK-ε-Pal	1.0±0.1
SMO i2-5	Ac-YAWHTSFKALGTTYQPLSGKTSYK-ε-Pal	1.0±0.1
SMO i2-6	Pal-LTYAWHTSFKALGTTYQP	0.3±0.05
SMO i2-7	Ac-GTTYQPLSGKTSYK-ε-Pal	2.7±0.4
SMO i2-8	Pal-LTYAWHTSFKAL	0.08±0.02
SMO i2-9	Ac-LTYAWHTSFKAL	>10
SMO i2-10	Pal-TYAWHTSFKAL	0.7±0.1
SMO i2-11	Pal-LTYAWHTSFKA	0.09±0.007
SMO i2-12	Pal-LTYAWHTSFK	0.06±0.007
SMO i2-13	Ac-TYAWHTSFKA	2.8±0.3
SMO i2-14	VWFVVLTYAWHTSFKAL	>5
SMO i2-15	WFVVLTYAWHTSFKAL	>5
SMO i2-26	Myr-LTYAWHTSFKAL	0.2±0.05
SMO i2-18	Pal-LTYABpaHTSFKAL	0.1±0.05
SMO i2-21	Pal-LTYABpaHTSFKAL-Hcy-Biotin	>15
SMO i2-23	Pal-LTYAWHTSFKALGTTYQPLSGKTSYK-ε-Pal	0.05±0.02
SMO i2-29	Ac-LTYAWHTSFKAL-Penetratin	>15
SMO i2-30	Penetratin-LTYAWHTSFKAL	>15
SMO i2-16	Ac-LAKFSTHWATYLK-ε-Pal (all D-)	0.006±0.0005
SMO i2-17	Ac-AKFSTHWATYLK-ε-Pal (all D-)	0.0004±0.0001
SMO i2-20	Ac-KFSTHWATYLK-ε-Pal (all D-)	0.0003±0.0001

[a] IC_{50} was determined by MTT assay in SK-Mel2 melanoma cells after 48 h of exposure to the compounds. Penetratin = RQIKIWFQNRR-Nle-KWKK.

Table 2. Cell Growth Inhibition Activity of Palmitoylated Fragments of the Third Intracellular Loop of Human SMO[a]

Compound	Structure	IC_{50}, μM
SMO i3−1	Pal−RGVMTLFSIKSNHPGLLSEKAASKINETML	0.64±0.1
SMO i3−2	Pal−RGVMTLFSIKSNHPGLLSEKA	0.50±0.1
SMO i3-4	Pal−LFSIKSNHPGLLSEKAASKINETMLR	1.5±0.2
SMO i3−5	AcRGVMTLFSIKSNHPGLLSEKAASKINETMLRK-ε-Pal	0.9±0.2
SMO i3−6	Ac-LLSEKAASKINETMLRK- ε-Pal	0.8±0.1
SMO i3−7	Ac-LFSIKSNHPGLLSEKAASKINETMLRK- ε-Pal	0.95±0.2
SMO i3−8	Pal−RGVMTLFSIKSNHPGLLS	0.5±0.1
SMO i3−10	Pal−RGVMTLFSIKSNH	0.95±0.2
SMO i3−12	Ac-LLSEKAASKINETMLRK- ε-Pal	1.33±0.2

[a] Activity was measured against SK-Mel-2 cells with the help of MTT assay after 48 h of exposure to compounds as described in Experimental Section.

since substitution of a palmitoyl residue with an acetyl significantly reduced activity probably because of poor cell penetration of the peptide (SMOi2-9 and SMOi2-13). As an alternative delivery of the peptides inside the cells, we fused the SMOi2-9 sequence to penetratin, a 16-amino acid long *Antennapedia* peptide (RQIKIWFPNRR-Nle-KWKK), which has been used to introduce a variety of biologically active molecules such as DNA, peptides, or proteins into cells.[30] Neither C-terminal (SMOi2-29) nor N-terminal (SMOi2-30) fusion helped to restore the activity, suggesting that palmitoylation provides more than just cell permeability. Replacement of the palmitoyl residue with partial sequences of the transmembrane domain (SMOi2-14 and SMOi2-15) also did not help to regain the activity. The resulting peptides were poorly soluble, and the apparent lack of activity could be due to precipitation. Substitution of palmitoyl residue with slightly shorter myristoyl resulted in a 2.5-fold less potent compound (SMOi2-26). For the study of peptide localization inside the cells and characterization of the interacting protein molecules, we attempted the synthesis of a cross-linkable derivative labeled with biotin. Substitution of Trp residue of SMOi2-8 with *p*-benzoylphenylalanine that can be UV-cross-linked to a protein ligand produced a fairly active compound (SMOi2-18, Table 1). However, addition of maleimide−biotin that was coupled through the SH-

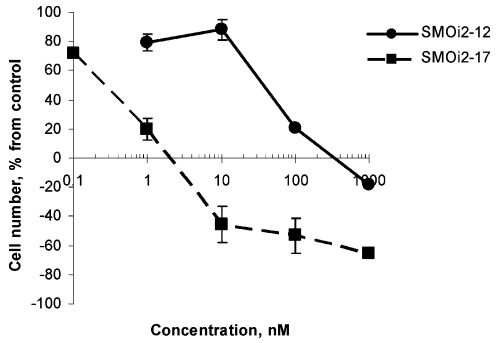

Figure 4. Toxicity of the second intracellular loop derivatives determined by MTT assay in SK-Mel2 melanoma cells after 48 h of exposure to compounds.

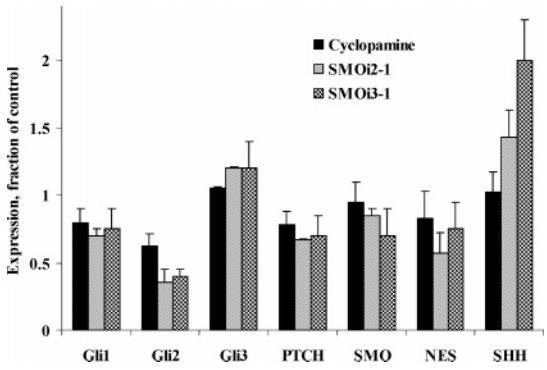

Figure 5. Gene expression changes caused by 48 h of exposure to 5 μM cyclopamine, SMOi3-1, and SMOi2-1 determined in prostate cancer cell line DU145 by quantitative PCR.

group of C-terminal homocysteine (SMOi2-21) totally abolished the activity, thus rendering it unsuitable for receptor identification.

All palmitoylated peptides had growth inhibition curves that became flat at higher concentrations or went up indicating lowering of activity with increased concentrations (Figure 3). The presence of a lipid moiety suggests that the peptides may be prompt to micelle formation. Fluorescence studies with the WMC 77 compound whose fluorescence increases more than 40-fold upon transition from the aqueous to the hydrophobic environment[31] have shown that the critical micelle concentration is 1 μM for all peptides studied (Supporting Information). Micellization may be responsible for lowering the effective concentration of free peptides in solution and subsequent apparent reduction in potency. The majority of peptides also precipitated in the medium at concentrations higher than 10 μM.

In an attempt to obtain metabolically stable analogues of the SMO antagonist, we synthesized retroinverso derivatives of the most potent all-L antagonists. SMOi2-16, SMOi2-17, and SMOi2-20 were constructed of all-D-amino acids and had reversed sequences compared to SMOi2-8 and SMOi2-12 (Table 1). To keep the fatty acid on the same termini of the peptide, ϵ-palmitoyl-D-lysine was added to the C-termini of the peptides. Both SMOi2-16 and its truncated versions SMOi2-17 and SMOi2-20 were even more potent in inhibiting and killing melanoma cells than their all-L parent peptides (Table 1, Figure 4).

To prove that growth inhibitory effects of the peptides are due to down-regulation of the Hedgehog pathway, we conducted an analysis of the expression of genes that are known markers of the pathway, Gli-1, Gli-2, Gli3, Ptch, Shh, SMO, and NES. The changes in gene expression were similar to that of a known and well characterized SMO antagonist cyclopamine (Figure 5). To avoid significant cell death, nonoptimized, less active peptides were used in the assay that utilized DU145 prostate cancer cells.

Discussion

Analogues of the intracellular loops of GPCRs are known to inhibit or activate the signaling of the target proteins by binding to G-proteins that are downstream effectors for this class of receptors and/or by interacting with the C-terminal tails, which are essential for receptor signaling.[25−29,32,33] We assumed that similar mechanisms may allow analogues of SMO intracellular loops to influence Hedgehog pathway. The compounds caused very significant growth inhibition in cancer cells dependent on this pathway. Mechanistically, the effects can be due to interaction with the rest of SMO molecule or with unknown downstream effectors. We are currently trying to identify the molecular targets of loop derivatives utilizing immobilized peptides.

The higher activity of retroinverso analogues could be attributed to several factors. It has been shown that retroinverso peptides adopt somewhat different conformations in solutions and are frequently more rigid than their all-L counterparts.[34,35] Circular dichroism spectra (data not shown) are consistent with the higher degree of folding of retroinverso peptides in aqueous solutions and in membrane-mimicking micelles. Thus, the higher activity of the retroinverso version of i2 derivatives can be due to changes in the structure that favor more efficient binding. The higher metabolic stability of all-D peptides is another obvious feature that can contribute to higher apparent biological activity, and pharmacology studies that are in progress will allow testing of this possibility.

Cyclopamine was proven to be an effective antitumor agent in a number of tumor models.[19,36−38] It was found to effectively eliminate skin lesions caused by basal cell carcinomas when applied topically.[39] Topically applied cyclopamine is also considered to be one of the most promising agents for the treatment of psoriasis,[40,41] which is known to be accompanied by activation of Hedgehog pathway.[42] The higher than cyclopamine potency of SMO derivatives makes them promising drug candidates. Their mechanism of action is significantly different from the mechanisms of currently known SMO antagonists, and they are likely to work even further downstream the Hedgehog signaling pathway. Lower plasma stability of peptides may be advantageous when local topical treatment is needed. Quick degradation in circulation may significantly reduce potential side effects caused by circulating agents.

In conclusion, synthetic analogues of the second and third intracellular loops of the SMO protein potently inhibit growth of breast and prostate cancer cells, melanoma, and possibly other types of cancer cells. The inhibitors down-regulate the expression of the genes of the Hedgehog pathway. The most potent of the compounds is the derivative of the N-terminal half of the second intracellular loop. The peptide is 10 amino acid residues long and has an IC_{50} in the nanomolar range. Its metabolically stable analogues, retroinverso peptides, are even more potent. Novel SMO inhibitors are promising drug candidates for use in cancer therapy. By analogy with other palmitoyl peptides that are skin-permeable and widely used in creams and ointments, they have the potential to find application as topical agents in treating and preventing skin malignancies and in the treatment of psoriasis. The approach described may have wide applications for the rational design of inhibitors for other

membrane proteins with conserved and unique primary structures in juxtamembrane regions.

Experimental Section

Peptide Synthesis. The peptides were synthesized by solid-phase peptide synthesis on a 433A peptide synthesizer (Applied Biosystems, Foster City, CA) equipped with a conductivity monitoring unit utilizing Fmoc amino acid derivatives (AnaSpec, San Jose, CA). The synthesis was performed with conditional blocking of unreacted amino groups with acetic anhydride for easier purification of the resulting peptides. Peptides were cleaved from the resin with 87.5% trifluoroacetic acid containing 5% water, 5% thioanisol, and 2.5% triisopropylsilane, precipitated with cold diethyl ether, washed five times with ether, and dried in vacuum overnight. Peptides dissolved in dimethylformamide were purified by HPLC on a preparative (25 mm × 250 mm) Atlantis C18 reverse-phase column (Agilent Technologies, Palo Alto, CA) in a gradient of 0.05% trifluoroacetic acid in water and acetonitrile containing 0.05% trifluoroacetic acid. The fractions were analyzed by electrospray LC/MS on an Agilent 1100 series instrument (Agilent Technologies, Palo Alto, CA) with the use of a Zorbax 300SB-C18 Poroshell column and a gradient of 5% acetic acid in water and acetonitrile. Only fractions containing more than 95% pure product were combined and freeze-dried. Peptides were dried from 5% acetic acid to ensure conversion into acetate salts. The purity and structure were further confirmed by LC/MS with separation on a Zorbax 300SB-C18 analytical column.

Addition of Fatty Acids to the C-Termini of Peptides. For L-peptides containing ϵ-palmitoyl-Lys on the C-terminus, commercially available Fmoc- ϵ-palmitoyl-L-Lys (AnaSpec, San Jose, CA) was utilized. Fmoc-ϵ-palmitoyl-D-Lys is not commercially available. It was synthesized on the resin utilizing orthogonally protected Fmoc-D-Lys(ivDde) (*N*-α-Fmoc-*N*-ϵ-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl-D-lysine) (Novabiochem). After attachment of the amino acid to Rink-amide resin, the ivDDE protection group was removed by treatment with a hydrazine/imidazole mixture in NMP as previously described.[43] The resin was washed with NMP and reacted with 10-fold excess of palmitic acid/HBTU/HOBt in NMP for 2 h. After the resin was washed with NMP, the synthesis was continued utilizing standard protocols on the peptide synthesizer.

Drug Toxicity Assay. DU145, PC3, MCF7, or Mel-SK-2 cells (American Type Culture Collection, Manassas, VA) were inoculated in 96-well plates at 200−400 cells/well in DMEM medium containing 10% fetal bovine serum and allowed to attach for 24 h. An amount of 100 μL of cell suspension was used for each well, and 100 μL of inhibitor in medium at 2× concentration was added the next day and kept in the CO_2 incubator for 48 h. While the drugs were added, assays were performed on extra reference plates to determine the cell population density at time 0 (T_0). The cells were stained with Promega Non-Radioactive Cell Proliferation Assay Kit (MTT) according to the manufacture's protocol. The absorbance of the wells was determined at 544 nm by a FLUOstar/POLARstar Galaxy (BMG Lab Technologies GmbH) microplate reader. The assays were performed on control (*C*) and test (*T*) cells. Cellular responses were calculated from the data using the following formula: $100 \times [(T - T_0)/(C - T_0)]$ for $T > T_0$ and $100 \times [(T - T_0)/T_0]$ for $T < T_0$. T_0 corresponds to cell density at the time of drug addition.

Acknowledgment. This research was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research.

Supporting Information Available: Mass spectrometry results and HPLC data for all compounds described, protocol and data for critical micelle concentration determination, and protocol for gene expression analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM0705657