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Caenorhabditis elegans Model to Test the Effect of Pharmacological Drugs on IGF-1/insulin Signalling Pathway

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Abstract

Many pharmacological drugs have been reported to alter insulin signalling in the body resulting in altered blood glucose levels. Drug induced hypoglycaemic or hyperglycaemic effect may lead to adverse effects especially in diabetic patients. Treating ailments of diabetic patients has always remained challenging for the clinicians due to unexplored effect of many drugs on insulin signalling. Insulin/insulin like growth factor-1 signalling (IIS) pathway is highly conserved between *Caenorhabditis elegans and humans. In both C. elegans and humans IIS pathway is involved in regulating fat storage. C. elegans* dauer formation is regulated primarily via IIS pathway and is triggered by adverse environmental conditions. In this paper we proposed the use of *C. elegans* dauer formation as a vital strategy to check the drug interaction with IIS. Activity of DAF-2 and DAF-16 are the key regulators of IIS in *C. elegans.* Aspirin, silymarin and pravastatin drugs have been reported to alter blood glucose levels using animal models and clinical reports. To test the efficacy of our model we tested the effect of these drugs on IIS by using dauer formation as a read-out. Our results report that aspirin and silymarin decreased dauer formation whereas pravastatin enhanced it; the effect was mediated through *daf-16* signalling. Our results thus report that *C. elegans* dauer formation can be used as an effective readout for drug and IIS pathway interaction.

Keywords: Diabetes; Drug; Dauer; *daf-2*; *daf-16*; IGF/insulin signalling; *Caenorhabditis elegans*

Abbreviations: IIS: Insulin/insulin-like Growth Factor-1 Signalling; NGM: Nematode Growth Medium; FOXO: Forkhead Box O Transcription Factors; HNF: Hepatocyte Nuclear Factors; SD: Standard Deviation

Introduction

Diabetes mellitus (DM) commonly referred as diabetes is characterized by high blood glucose level. Insulin insufficiency or ineffective insulin termed as insulin resistance results in diabetes [1,2]. Diabetes or altered insulin signalling increases vulnerability to other ailments like hypertension, cardiovascular diseases, hepatic diseases, neuropathy, nephropathy, foot diseases, stroke, eye complications etc [1-7]. Many of the routinely prescribed pharmacological drugs have been reported to alter blood glucose levels [8-16]. Drug-induced hyperglycaemia poses a serious threat for diabetic patients even resulting in life threating complications at times [8,9]. Due to unexplored effect of many drugs on insulin signalling treating ailments of diabetic patients has always been challenging for the clinicians.

Some of the routinely prescribed anti-pyretic, analgesic and anti-inflammatory drugs such as aspirin (salicylates), ibuprofen and meclofenamic acid have been reported to show hypoglycaemic effect [11,13,15]. Diabetic patients are prone to cardiovascular diseases like hypercholesterolaemia and atherosclerosis [3]. Statins drugs prescribed for lowering the blood cholesterol levels work by inhibiting HMG-CoA reductase (or 3-hydroxy-3-methyl-glutaryl-CoA reductase) which acts as a rate limiting step in the mevalonate pathway for cholesterol synthesis [14]. Statins like simvastatin, lovastatin, pravastatin have been reported to modulate insulin signalling [10,14]. Hepatic pathologies are also common in patients with diabetes [5]. Silymarin used as an herbal medication for hepatic pathologies also shows hypoglycemic effect [12,16]. ACE inhibitor (Angiotensin-converting-enzyme inhibitor) drugs used for treating hypertension has been reported to result in severe hypoglycemia in diabetic patients [17]. Some other drugs like β-blockers, pentamidine, fluoroquinolones are also reported to show hypoglycaemic effect [18-20]. These drugs alter the blood glucose levels by mechanisms such as altering insulin secretion, insulin sensitivity, altering glucose metabolism or enhancing hypoglycaemic effect of anti-diabetic drugs [8,9,20,21]

Insulin/insulin-like growth factor-1 signalling (IIS) pathway is highly conserved between Caenorhabditis elegans and humans [22]. Insulin like peptides has been reported to be encoded by thirty seven C. elegans genes [23]. C. elegans INS-1 gene out of the 37 reported genes has most closest resemblance to human insulin [23]. Similarly, C. elegans DAF-2 shows 36% similarity to human insulin receptor and is C. elegans orthologue of human insulin/IGF receptor [24]. daf-2 signalling is involved in reproductive growth, dauer formation and life span extension [24-27]. daf-2 activity is dependent on daf-16 which is an ortholog of human hepatocyte nuclear factor 3 (HNF-3)/

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forkhead family of transcription factors (FOXO) [28]. DAF-16 acts as a negative regulator of IIS pathway, DAF-2 activation results in DAF-16 phosphorylation and blocking its nuclear translocation and inactivation [24,25,28]. Over-expression of *ins-1* and human insulin results in enhanced dauer formation and increased life-span in *C. elegans daf-2(e1365)* mutant [23]. As in humans where reduced insulin signalling results in diabetes and increased fat deposition similarly, in *C. elegans* insulin signalling has been reported to modulate fat deposition and longevity [29].

C. elegans undergo normal reproductive development during favorable conditions whereas during adverse environmental conditions like temperature stress, overcrowding and food deprivation development is diverted towards dauer formation [30-32]. Apart from IIS pathway TGF-β pathway mediated through IIS also influence dauer formation [32]. Testing the effect of pharmacological drug on IIS pathway and glucose metabolism is difficult and time consuming using rodents and other higher animal models. In this paper we proposed the use of *C. elegans* dauer formation as an index to find the drug interaction with IIS pathway. We analyzed the effect of three drugs aspirin, silymarin and pravastatin on dauer formation. Use of *C. elegans* dauer formation index can prove out as an effective strategy for preliminary check for the drug interaction with IIS pathway.

Experimental Procedure

Strains

C. elegans strains were maintained at 20°C on nematode growth media (NGM) agar plates seeded with *E. coli* strain OP50 [33]. The following animals were used in the study*: N2, daf-16(mu86), daf-2(e1370), daf-2(e1368), muEx108 (daf-16a*::GFP/*daf-16b*KO, pRF4(rol-6(su1006)). All the strains were obtained from Caenorhabditis Genetics Center (CGC) which is funded by the NIH National Center for Research Resources (NCRR).

Drug treatment and dauer assay

Drug treatment plates were prepared by pouring 200 µl of drug solution (100 µM final concentration prepared in water) on the OP50 NGM agar plates. Plates were allowed to dry and were used for dauer assay.

Eggs were transferred to OP50 seeded NGM agar plate supplemented with drug (100 µM) or without drug (control). The plates were incubated at suitable conditions as required for dauer formation. After 4-5 days, number of dauers formed was counted manually using a dissecting microscope and further confirmed by 1% SDS treatment. Results of five independent experimental trials were used to report the mean percentage of dauer formed.

DAF-16::GFP localization assay

Synchronized population of L1 animals of transgenic DAF-16: GFP line was cultured under control and in presence of drug (100 µM) at 20°C. After 3 days DAF-16::GFP animals were sensitized by shifting them to 34°C for 5 min, and monitored for nuclear localization of DAF-16. Short time heat exposure sensitize the localization of DAF-16::GFP facilitating the distinction between cytoplasmic verses nuclear localization of DAF-16.The number of animals that show DAF-16 nuclear localization were counted using Olympus BX51 fluorescent microscope at 20X magnification. Result was expressed as mean percentage ± S.D (standard deviation) of animals that show nuclear localization of DAF-16.

Results

Drug treatment altered percentage of dauer formation in wild type animals

In the wild type animals, dauer formation is strongly induced at a temperature of 27°C [30]. We examined effect of silymarin, aspirin and pravastatin drug on dauer formation. Silymarin is a hepatoprotectant prescribed for treating liver cirrhosis, acute liver intoxication, and chronic hepatitis [12,16]. Aspirin is a salicylate drug used as an analgesic, antipyretic, and anti-inflammatory medication [15]. Pravastatin is used for lowering cholesterol and preventing cardiovascular disease [10,14].

To test the effect of drugs on dauer formation (Figure 1A and 1B), synchronized eggs of wild type animals were seeded on plates with drugs silymarin (100 µM), aspirin (100 µM), pravastatin (100 µM) and control plates (without drug treatment) at 27°C. After 3 days of incubation the number of dauer present on the respective plates was counted manually and further confirmation of dauer was done by SDS treatment.

Results of dauer assay were represented as mean ± S.D by averaging the results of five independent trials. The mean percentage ± S.D of

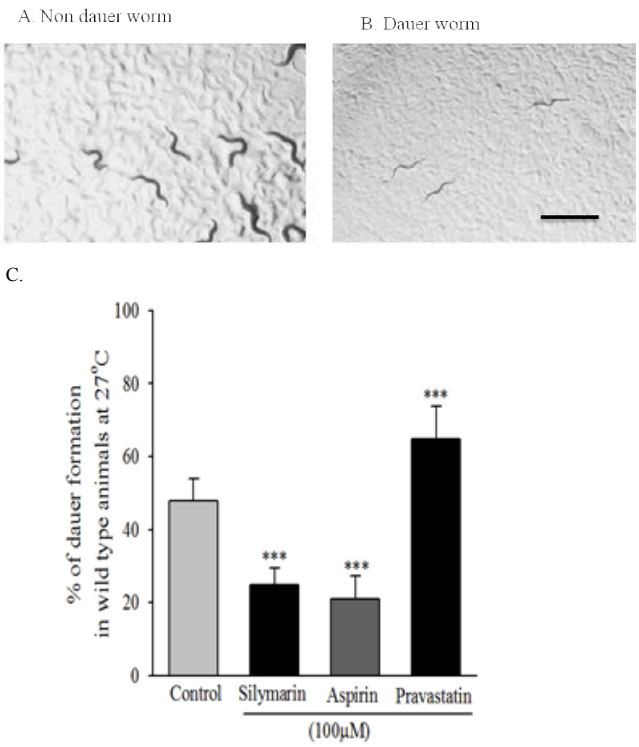

Figure 1: Drug treatment modulates dauer formation at 27°C in wild type animals. (A-B) Morphology of dauer animals. Representative image of non dauer (A) and dauer animal (B). Scale bar, 1 mm. (C) Percenatge of dauer formation at 27°C in wild type animals with effect to drug treatment. Synchronised eggs of wild type animals were grown on 100 µM drug treated (aspirin, silymarin, pravastatin) and untreated control plates. The proportion of dauer larvae formed was scored after 4-5 days. Dauers are identified based on morphology and resistance to 1% SDS. Data is represented as mean ± S.D. (***p<0.0001, t-test values; N>150). N represents the number of animals. Results of five independent experimental trials were used for analysis.

dauer formation on control plates was 47.8 ± 6.1 whereas the drug treated plates showed respective percentage of 24.8 ± 4.7 ($p<0.0001$, t-test) for silymarin, 21.1 ± 6.2 ($p<0.0001$, t-test) for aspirin, and 64.8 ± 8.9 ($p<0.0001$, t-test) for pravastatin (Figure 1C). Silymarin and aspirin drugs showed a statistically significant effect on suppressing dauer formation whereas, pravastatin treatment significantly enhanced dauer formation.

Drug modulates dauer formation by effecting DAF-16/FOXO

C. elegans arrest in dauer stage is regulated by the IIS pathway. DAF-2 a transmembrane protein which functions as insulin receptor and its effector DAF-16 has been reported to regulate dauer formation [26-28]. To determine the effect of silymarin, aspirin and pravastatin drugs on IIS pathways we performed dauer assay in insulin like receptor mutant *daf-2(e1370), daf-2(e1368)* and insulin like pathways effector mutant *daf-16(mu86). daf-2(e1370)* and *daf-2(e1368)* strain is a constitutive dauer mutant at permissive temperature of 25°C [24,25]. At non permissive temperature of 15-20°C it survives as a normal non dauer animal [25]. *daf-16(mu86)* has large deletion mutation due to which life span extension and dauer formation is hampered [28].

The dauer formation of *daf-2(e1370)* and *daf-2(e1368)* animals was performed at 22°C. Dauer formation in *daf-2(e1370)* and *daf-2(e1368)* control animals was comparable to wild type control (at 27°C) (Figure1C, 2A, 2B). Results of dauer assay were represented as mean ± S.D. by averaging the results of five independent trials. The mean percentage of dauer formation of *daf-2(e1370)* on control plates was 41.8 ± 6.1 whereas the drug treated plates showed respective percentage of 1.8 ± 0.7 ($p<0.0001$, t-test) for silymarin, 4.9 ± 1.2 ($p<0.0001$, t-test) for aspirin, and 78.8 ± 11.9 ($p<0.0001$, t-test) for pravastatin (Figure 2A). Whereas, mean percentage of dauer formation of *daf-2(e1368)* on control plates was 37.68 ± 5.2 whereas the drug treated plates showed respective percentage of 1.6 ± 0.6 ($p<0.0001$, t-test) for silymarin, 3.6 ± 1.1 ($p<0.0001$, t-test) for aspirin, and 68.8 ± 10.6 ($p<0.0001$, t-test) for pravastatin (Figure 2B). We also checked the dauer formation of *daf-2(e1370)* and *daf-2(e1368)* at 24°C, similar to 22°C aspirin and silymarin drug showed a statistically significant effect on suppressing dauer formation whereas, pravastatin treatment significantly enhanced dauer formation at 24°C (data not shown).

The dauer formation of *daf-16(mu86)* animals was performed at 27°C. The mean percentage ± S.D of dauer formation on control plates was 28.8 ± 9.1 whereas the drug treated plates showed respective percentage of 25.8 ± 8.7 ($p=0.32$, t-test) for silymarin, 29 ± 8.2 ($p=0.45$, t-test) for aspirin, 27.2 ± 8.9 ($p=0.55$, t-test) for pravastatin (Figure 2C). All the drugs tested did not show any significant change in dauer formation in *daf-16* null mutant.

As per the above results it is likely that drug is modulating *daf-16* signalling directly or indirectly. To confirm further the role of DAF-2 or DAF-16 in modulating dauer formation we checked the effect of drugs on dauer formation using *daf-2(e1368); daf-16(mu86)* double mutant. Similar to *daf-16(mu86)* testing drug using double mutant of *daf-2(e1368); daf-16(mu86)* did not effect dauer formation with the respective values similar to control (Figure 2D). Results thus suggest that drug is modulating *daf-16* signalling and wild type copy of DAF-16 is vital for the drugs to show its effect.

Drugs induce nuclear translocation of DAF-16

All the drugs tested showed dauer formation is regulated by effecting DAF-16/FOXO signalling pathway. Nuclear translocation of DAF-16 results in enhanced life span and dauer formation. To check the effects of drugs tested on DAF-16 nuclear localization we examined the effect of the drugs on DAF-16::GFP localization. Results of DAF-16::GFP localization assay were represented as mean ± S.D by averaging the results of five independent trials carried out with about 200 animals per trial. The mean percentage ± S.D of nuclear localized DAF-16::GFP animals on control plates was 44.8 ± 7.1 whereas the drug treated plates showed respective percentage of 21.8 ± 6.7 ($p<0.0001$, t-test) for silymarin, 26.8 ± 5.2 ($p<0.0001$, t-test) for aspirin and 68.8 ± 11.99 ($p<0.0001$, t-test) for pravastatin (Figure 3A and B). Results of DAF-16: GFP nuclear translocation is consistent with the trends observed for percentage of dauer formed in effect of the drugs treatment (Figure 1C).

Discussion

IIS pathway is conserved across species, and play similar roles in *C. elegans* and humans. In both *C. elegans* and humans this signalling pathway is involved in nutrient utilization and storage [22]. During favourable environmental conditions IIS pathway drives *C. elegans* for normal reproductive life cycle whereas during food deficit it leads to dauer formation [24,30]. Steps in insulin signalling mediated through DAF-2 in *C. elegans* and insulin receptor in humans have certain steps in common which are mediated through PI3 and AKT signaling. Insulin signalling in *C. elegans* prevents nuclear translocation of DAF-16 keeping it in inactivated state; similarly in humans binding of FOXO/HNF-3 to insulin response sequence is inhibited by insulin signalling

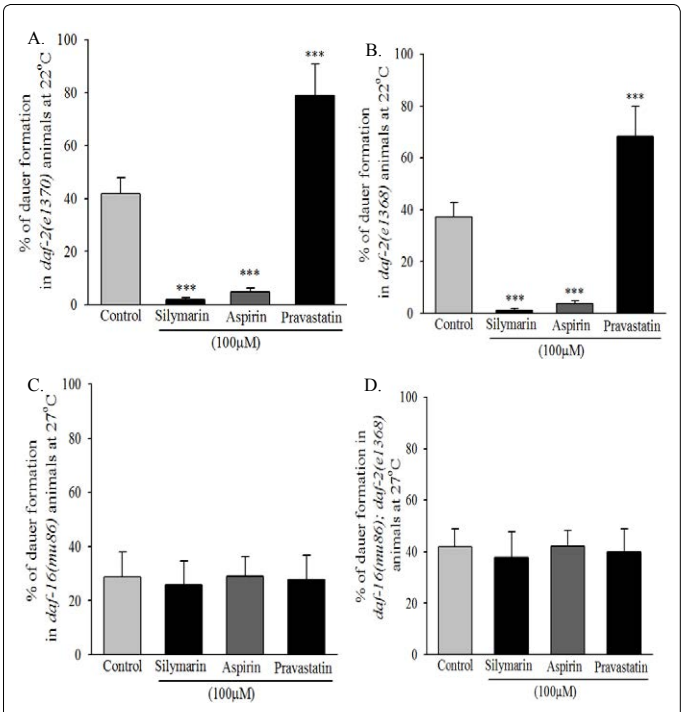

Figure 2: Drug modulates dauer formation by effecting insulin/IGF signalling pathway (A) Effect of drug treatment on dauer formation in *daf-2(e1370)* mutant at 22°C. (B) Effect of drug treatment on dauer formation in *daf-2(e1368* mutant at 22°C. (C) Effect of drug treatment on dauer formation in *daf-16(mu86*) mutants at 27°C. (D) Effect of drug treatment on dauer formation in *daf-2(e1368);daf-16(mu86*) double mutants at 24°C. 100 µM of aspirin, silymarin and pravastatin drug concentration was used. The proportion of dauer larvae formed was scored after 4-5 days. Dauers are identified based on morphology and resistance to 1% SDS. Data is represented as mean ± S.D. (***$p<0.0001$, t-test values; N>150). N represents the number of animals. Results of five independent experimental trials were used for analysis.

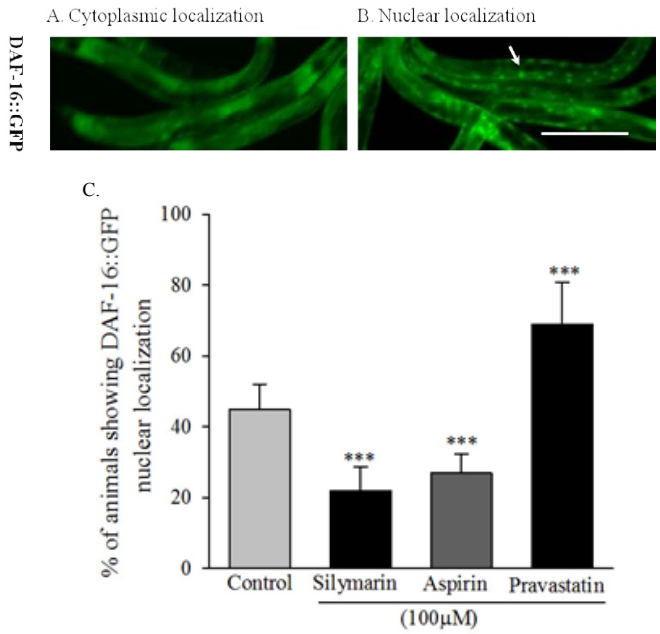

Figure 3: Drug treatment effects nuclear localization of DAF-16::GFP. (A-B) Representative picture of DAF-16::GFP animals showing DAF-16 localization in cytoplasm (A) and nucleus (B). Scale bar: 200 µm, arrow mark denotes nuclear region with DAF-16::GFP. (C) Effect of drug treatment on nuclear localization of DAF-16::GFP. L1 animals of DAF-16::GFP transgenic line was cultured under control and in the presence of drug (100 µM) at 20°C. Young adult animals were sensitized by shifting to 34°C for 5 min and monitored for nuclear localization of DAF-16 using fluorescent microscope at 20X. Nuclear localization of DAF-16 is expressed as mean percentage ± S.D.(***p<0.0001, t-test values).

[22]. Nuclear translocation of DAF-16 in *C. elegans* promotes longevity, stress tolerance and dauer formation whereas nuclear translocation of FOXO in humans is associated with gluconeogenesis, cell cycle arrest, apoptosis, DNA repair etc [27,28,34].

Over-expression of INS-1 and expression of human insulin enhances dauer formation in both wild type and *daf-2* mutant animals [23]. Hexosamine signalling pathway has been reported to be involved in type 2 diabetes in humans, it also influence IIS in *C. elegans* [35,36]. Two of the enzymes involved in regulating this pathway O-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA) are highly conserved and are present in *C. elegans* (OGT-1 and OGA-1) as well [35,36]. Impairment of O-GlcNAc cycling as well as mutation in gentic loci encoding OGA has been reported to result in insulin resistance and type 2 diabetes [35,36]. OGA-1 and OGT-1 mutation in *C. elegans* has been reported to effect macronutrient storage as well as dauer formation [35,36]. All these lines of evidences demonstrate effectiveness of *C. elegans* as a model to study insulin signalling pathways and diabetes.

In this paper we used *C. elegans* dauer formation phenotype as the readout to study the effect pharmaceutical drugs on insulin signaling. We tested the interaction of three drugs aspirin, silymarin and pravastatin with *C. elegans* IIS signaling. All the three drugs effected dauer formation mediated through *daf-16* pathway. Silymarin and aspirin drugs showed decrease in the dauer formation by suppressing the DAF-16 nuclear translocation and promoting insulin signaling. Hypoglycemic effect of both silymarin and salicylates like aspirin has already been reported, thus both the drugs are reported to reduce insulin resistance [12,13,15,16]. Whereas, pravastatin resulted in increased DAF-16 nuclear translocation and enhanced dauer formation. Effect of pravastatin along with other statins drugs has been reported to improve insulin sensitivity [10,14]. All the three drugs tested modulated dauer formation and have been reported to be hypoglycemic [10-16]. Difference in percentage of dauer formation seen between aspirin/silymarin versus pravastatin may be due to the reported role of aspirin and silymarin in conferring stress tolerance which may be the reason to suppress dauer formation.

Aspirin has been reported to extend *C. elegans* life span by effecting AMPK andDAF-16/FOXO pathways [37,38]. *C. elegans* life span extension by aspirin has been reported to be mediated through DAF-16 as there was no increase in life span observed in *daf-16(mu86)* null mutant [38]. We also observed that in null mutant of *daf-16* dauer formation was not altered. Moreover change in nuclear localization of DAF-16 during aspirin treatment for longevity could not be reported [38]. We observed a significant decline in DAF-16 nuclear localization when animals were treated with aspirin (Figure 3B). Not very distinctive (3.2%) change has been reported in life span with effect to aspirin treatment in *daf-2(e1370)* animals [38] whereas; we observe a significant decrease in dauer formation in *daf-2(e1370)* mutants. All together these results suggest that aspirin has different effect on modulation of IIS with age. At early stage of development it enhances insulin signalling and suppresses *daf-16*, while during adult stage it inhibits IIS or activates DAF-16 activity. In both the cases (dauer formation and life span) the effect is modulated through the DAF-16/FOXO, downstream effector molecule of IIS.

Use of rodents or other higher animal models for testing the effect of drug on glucose metabolism requires considerable times and involves various invasive methods. Use of dauer formation strategy as a read out to check the effect of drugs on insulin signalling can act as an easy and quick method for preliminary screening of drugs. The current assay is able to predict drugs interaction with IIS pathway but cannot directly predict its hypoglycaemic or hyperglycaemic effect on the human subjects. Other signalling pathways which influence *C. elegans* IIS pathways and interaction of other drugs with glucose metabolism should be further investigated to develop a comprehensive *C. elegans* model for drug testing and diabetes.

Authors' Contributions

JK, AA, KCP conceived and designed the experiments. JK, KCP performed the experiments. JK, AA, KCP analyzed the data. JK and AA discussed the data. AA and JK wrote the manuscript. BP and VKS gave valuable suggestions and feedback.

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