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Structure-based design of a thiazolidinedione which targets the mitochondrial protein mitoNEET

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ABSTRACT

Several PPAR-gamma agonists containing a thiazolidinedione moiety (referred to as glitazones) have been proposed to be neuroprotective and appear to alter mitochondrial function. Recently, a search for mitochondrial proteins that bind pioglitazone identified a novel protein, mitoNEET, which was later shown to regulate the oxidative capacity of the mitochondria. This identified an alternative target for the glitazones suggesting a possible new drug target for the treatment of neurodegenerative diseases. Molecular docking studies employing the reported crystal structure revealed five possible binding pockets on mitoNEET. We focused on two sites based on their physical characteristics. Using binding information gained from the analysis of two glitazones docked in these pockets, we designed and synthesized a ligand (NL-1) that would preferentially bind to site 1. Based on [^{3}H]-binding data of the glitazones and comparisons to computer generated K_is, we were able to predict that site 1 was likely the target of the glitazones. NL-1 uncoupled isolated mitochondrial complex I respiration with an IC_{50} of 2.4 μM and inhibited state III respiration up to 45%. To investigate the ability of NL-1 to block rotenone initiated free radicals from complex I, we found it was able to protect the human neuronal cell line SH-SY5Y against rotenone induced cell death. These data demonstrate that mitoNEET is a viable target for the design and synthesis of novel therapeutic agents aimed at altering mitochondrial function.

Mitochondria play a large role in many neurodegenerative diseases. Since neuronal cells are very sensitive to changes in intracellular energy status, many of the neurodegenerative diseases such as Parkinson's and Alzheimer's diseases have aberrant mitochondrial activity associated with them.[1–3] What still remains unclear however, is exactly how and when mitochondria contribute to the death of neuronal cells.[4] In spite of this, mitochondria are an attractive drug target to develop neuroprotective compounds. Recently, a new mitochondrial protein, mitoNEET, was identified as a target of the glitazones.[5–10] The binding of ligands to this protein was found to alter the oxidative capacity of mitochondria and reduced utilization of complex I substrates. Considering complex I is a major source of reactive oxygen species (ROS) in mitochondria, reducing the production of ROS through this mechanism may be useful in treating a variety of diseases such as stroke, Alzheimer's and Parkinson's disease. For example, studies have shown that the glitazones are able to prevent oxidative damage after cerebral reperfusion injury.[9] However, the exact role of mitoNEET in this protection still needs to be clarified.

To date, the physiological function of mitoNEET is unknown. Clinical studies show that the agonist activity at PPAR-γ receptor by pioglitazone (Fig. 1) does not account for all of its beneficial effects.[5] It has been suggested that the PPAR-γ receptor-independent activity of these glitazones is due to their interactions with mitochondria. In recent studies, it was found that troglitazone causes mitochondrial membrane depolarization[11] and inhibits complex 1 respiration.[12] Glitazones also regulate microglial activation[13] suppress BACE-1 expression[14] decrease tau phosphorylation,[15] increase mitochondrial efficiency and number, and protect against MPP+ toxicity in SH-SY5Y cells.[16] These findings support the use of glitazones for both Alzheimer's disease[17] and Parkinson's disease.[18,19] If mitoNEET is the mitochondrial target of these drugs, it would suggest that compounds aimed specifically at it may have great value in treating these diseases. This study demonstrates the first mitoNEET directed identification of a compound aimed at altering mitochondrial activity.

The crystal structure of mitoNEET was recently elucidated by two groups independently.[7,9] MitoNEET was found to form a homodimer and contained two Fe molecules, each coordinated by three cysteines and one histidine. The location of mitoNEET on the outer membrane combined with its redox potential suggests it may play an important role in the regulation of mitochondrial oxidation.

To identify possible binding pockets on mitoNEET, we used the SiteFinder module in MOE 2008.10 (Chemical Computing Group)

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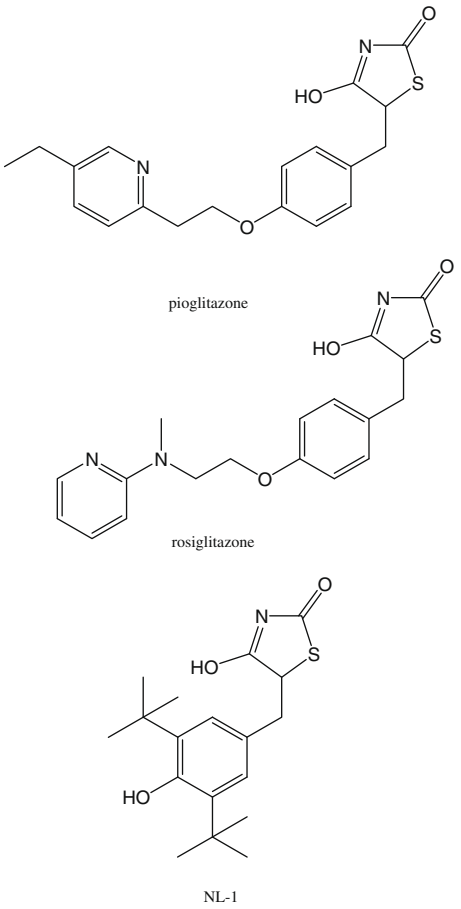

Figure 1. Structure of the gliatazones pioglitazone and rosiglitazone, and the TZD NL-1.

which uses alpha spheres which are either hydrophobic or hydrophilic and probes the surface of protein for possible binding pockets. We identified five possible sites illustrated in Figure 2A and the amino acids associated with each pocket are shown in Table 1. Taking into consideration the different sizes of the pockets, we focused our attention on sites 1 and 2. Site 2 correlates with the pocket already proposed by others as a possible binding site[7] near the 2Fe–2S site. After docking, the predicted pK_is of the glitazones showed site 1 having a higher affinity than site 2. However the differences were small and further experimentation would be required to help identify the actual binding site.

Based on our modeling data, we investigated a novel ligand (NL-1) that was selective for site 1 over site 2. The TZD NL-1 (Fig. 1) was synthesized as described previously.[20,21] NL-1 contained bulky *t*-butyl groups on the phenolic ring which should reduce its affinity to site 2 versus site 1. This is due to the orientation that NL-1 needs to obtain in order to fit into the binding pockets. This orientation in site 1 hinders critical H-bonds between the *para*-hydroxyl group and LYS-78 and reduces overall H-bonding to the TZD ring. From docking studies at site 1, the major interaction for NL-1 was found to be hydrogen bonding with residues LYS-B78 (with the hydroxyl group on the aromatic ring), LYS-A42 (with the TZD nitrogen and ketone) and ARG-B76 (with the hydroxyl of the TZD) (Fig. 3). In site 2, only TRP-A75 was seen to interact with NL-1.

Rosiglitazone and pioglitazone shared similar binding interactions with mitoNEET. However, unlike NL-1, these two compounds showed additional aromatic interactions with the binding pockets. In site 1, LYS-B89 and LYS-A42 seemed to provide the major interactions with the phenolic aromatic ring on pioglitazone, while ARG-B76 and MET-B44 provided hydrogen bonding interactions. In pocket 2, ARG-A76 provided both hydrogen bonding and aro-

Table 1
Residues and size of possible binding pockets on the mitoNEET protein

Site	Size	Residues
1	24	Chain 2:MET44; LEU47; ARG76; HIS90; GLU93; THR94 Chain 1:LYS42;ALA43
2	20	Chain 1:HIS48; ILE49; TRP75; ARG76; SER77; LYS78
3	14	Chain 2: PRO54 Chain 1: ASN91; GLY95; ASP96; ASN97
4	11	Chain 2: GLY95; ASP96; ASN97 Chain 1: PRO54
5	17	Chain 2: MET62; GLU63 Chain 1: MET62; GLU63; LYS105

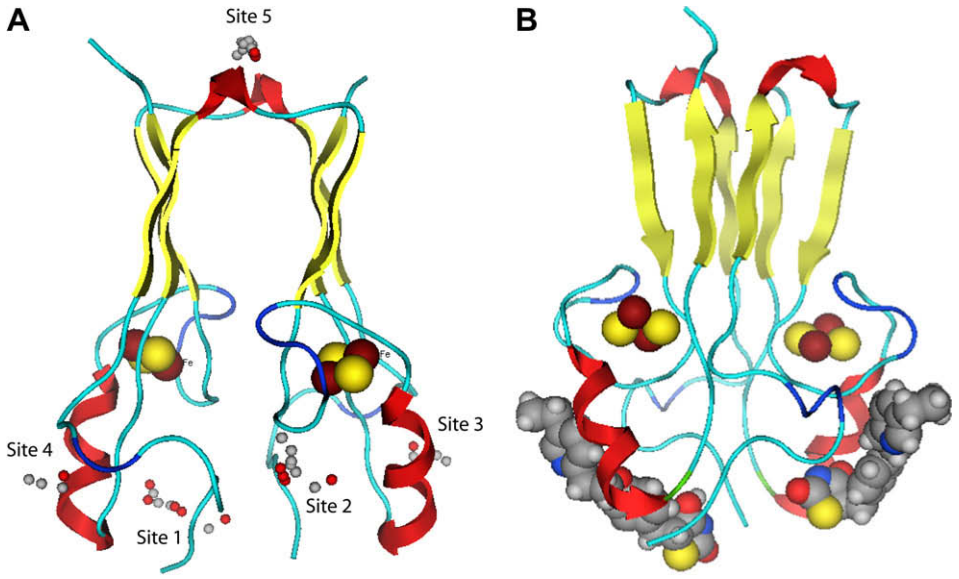

Figure 2. Possible binding sites found in mitoNEET using SiteFinder (MOE, Chemical Computing Group). (A) Identification of the five possible sites. (B) Pioglitazone docked into sites 1 and 2.

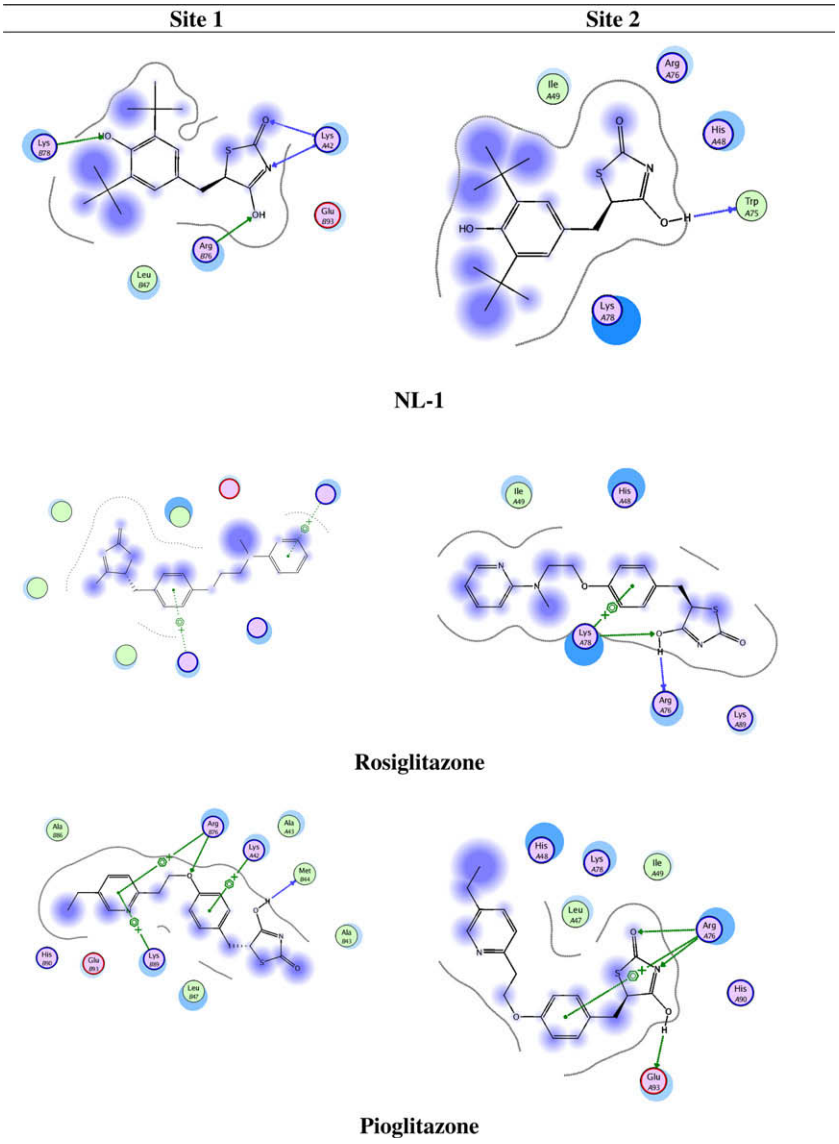

Figure 3. Docked poses of NL-1, rosiglitazone, and pioglitazone at sites 1 and 2 in mitoNEET, as predicted by SiteFinder (MOE).

matic interactions and GLU-A93 formed hydrogen bonds with pioglitazone. The predicted binding affinities for these compounds are given in Table 2 along with the binding affinities experimentally obtained using [^{3}H]-rosiglitazone.

To complement the docking studies, we conducted binding studies using liver mitochondrial suspensions and [^{3}H]-rosiglitazone. The IC_{50} value for rosiglitazone was 1.1 μM and 0.9 μM for NL-1 (Table 2). Interestingly, the binding of rosiglitazone was similar to that observed for pioglitazone (1.0 μM).[5] The predicted pK_i change for NL-1 from site 1 to site 2 was 4.78–2.77, respectively (Table 2). This two log-fold change in binding constant allowed for the differentiation of affinities to these sites. This was accomplished by plotting the experimentally determined IC_{50} to the calculated pK_is of NL-1, pioglitazone, and rosiglitazone. As shown in Figure 4, a correlation between the predicted p*K*i and IC_{50} was $r^2 = 0.98$ and 0.01 for sites 1 and 2, respectively. These data strongly suggest that site 1 is the higher affinity binding site for the glitazones.

Table 2
Predicted binding affinities (pK_i) and IC_{50} values for [^{3}H]-rosiglitazone binding for selected TZDs at docking sites 1 and 2 of mitoNEET

	Predicted pK_i		IC_{50} of [^{3}H]-rosiglitazone displacement (μM)
	Site 1	Site 2	
Rosiglitazone	2.41	3.19	1.1
Pioglitazone	3.65	4.43	1.0
NL-1	4.78	2.77	0.9

The modeling data argues that the thiazolidinedione group is important for the binding affinity of both the glitazones and NL-1 to mitoNEET. Hydrogen bonds are formed between pocket amino acids and the TZD oxygens and nitrogen. In addition, the phenolic ring forms a pi–pi interaction that gives additional stability for binding. This area of the TZDs provides ideal opportunities for modification that can enhance binding and selectivity to this protein. NL-1 lacks the aromatic moieties that are attached to the phenolic ring that are found in pioglitazone and rosiglitazone. The lack of additional affinity to mitoNEET by the glitazones supports the hypothesis that the major interactions between all these compounds and mitoNEET stem from the hydrogen bonding and accepting groups located on the TZD moiety. The benefit of removing these aromatic chains is that one may reduce the affin-

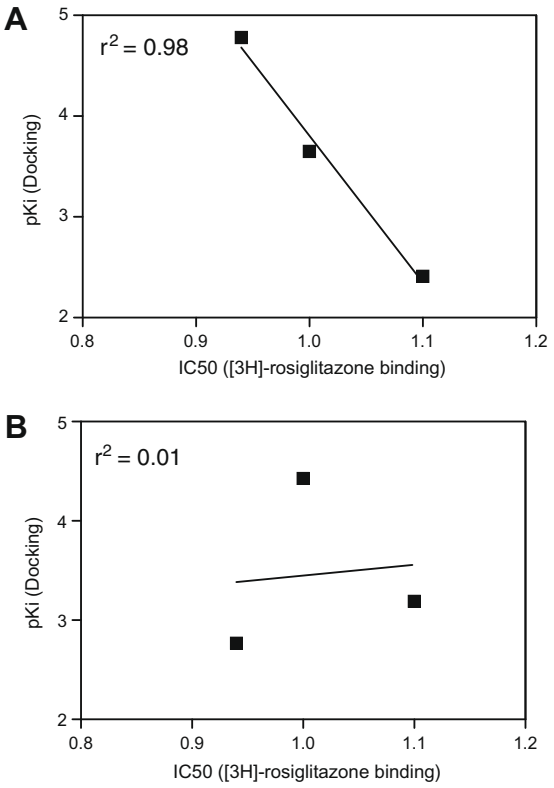

Figure 4. Correlations between the predicted binding (pK_i) and experimentally determined IC_{50}s of [^{3}H]-rosiglitazone binding. (A) The correlation plot of pioglitazone, rosiglitazone, and NL-1 for site 1. (B) The correlation plot of pioglitazone, rosiglitazone, and NL-1 for site 2.

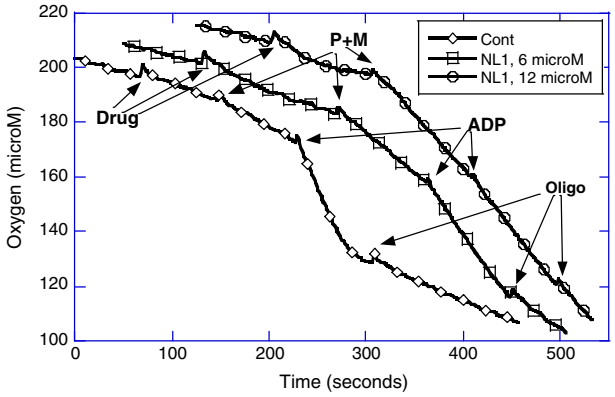

Figure 5. NL-1 alters mitochondrial state II, III and IV respiration. Traces represent vehicle (control, open diamonds), NL-1 at 6 μM, (open squares), and NL-1 at 12 μM (open circles) treated mitochondria. Pyruvate (P) and malate (M) were added as substrates for Complex I to initiate state II respiration. State III respiration was initiated by the addition of ADP and state IV respiration was initiated with the addition of oligomycin as described in the text. Each experiment was repeated at least three times from different mitochondrial preparations.

ity of compounds away from PPAR-γ's Y-shaped binding pocket. Since mitoNEET is a dimer, it was not surprising that we identified two possible binding sites (sites 1 and 2) on opposite sides of the protein. We observed that these two binding sites share some common amino acids, but were distinctly different in their distance and orientation in relation to the iron as well as their binding specificities. When we measured the total energy of the protein before docking pioglitazone into site 1, the energy was 3123 kcal/mol. When we docked pioglitazone in site 1 the energy become much lower, to 1063 kcal/mol, and after docking another molecule of pioglitazone in site 2, the energy went down further, to 838 kcal/mol. This observation suggests that even though the binding affinity of pioglitazone is not very strong (1 μM), the significant stabilizing effects seen on mitoNEET may be due to the binding of two molecules of pioglitazone to one mitoNEET (Fig. 2B).

To validate pharmacological function of NL-1, we determined its ability to alter mitochondrial respiration. Respiration was carried out as described previously[22] and NL-1 was evaluated in this experiment for its pharmacological effect (Fig. 5). Figure 6A shows the relationship between respiratory control ratio (RCR, the ratio between state III and state IV respiration) and NL-1 concentration. The RCR decreased from approximately 7 to 1 with increasing NL-1 demonstrating an uncoupling of mitochondrial respiration. An IC_{50} of 2.4 μM was obtained for NL-1's ability to uncouple the mitochondrial respiration (Fig. 6A). NL-1 also reduced the maximal respiration rate as measured by state III respiration by 45%, as shown in Figure 6B. Altering mitochondrial function in this way may underlie the neuroprotective effects observed with the glitazones. When taking into consideration that complex I is a major site for the production of reactive oxygen species in the mitochondria, reducing its activity could reduce the amount of free radicals generated from this site. This reduction may also contribute to the neuroprotective properties observed with the glitazones in preventing cell death after ischemic-reperfusion injury.[23,24]

To further evaluate the pharmacological effect of NL-1, we investigated its neuroprotective potential against rotenone-induced cell death in the human neuronal cell line SH-SY5Y. Rotenone interacts with complex I of the mitochondria resulting in the release of ROS that lead to the death of cells. In our experiments, cell death was evaluated by the release of lactate-dehydrogenase into the conditioned media (LDH Kit, Caymen USA). Assays were done in a 96-well plate with 5×10^4 cells/well. The cells were pre-incubated with NL-1 for 1 h before the addition of 250 nM rotenone. LDH release was measure 24 h later. As shown in Figure 7, NL-1 was able to dose-dependently reduce rotenone-induced LDH release from these neuronal cell cultures showing a 40% reduction in cell death. These data support our hypothesis that NL-1 is reducing the formation of ROS by altering complex I activity as indicated by its effects on mitochondrial respiration. It is also possible that the phenol moiety of NL-1 may be acting as a lipid peroxyl radical scavenger which may contribute to the protection of these cells. However, initial SAR studies suggest that the phenol moiety does not play a significant role in protecting cells from rotenone induced toxicity (data not shown). Taken together, the respiration, binding studies and cytoprotection data indicates NL-1 is mediating its effects primarily through mitoNEET.

In summary, we have identified a potential binding site for the glitazones on mitoNEET. This allowed for the first rational drug design aimed at producing ligands to this protein. Docking studies indicated that the anchor of the glitazones to mitoNEET appears to be the thiazolidinedione group and pi–pi interactions to the first aromatic ring. Our analysis suggests that alterations around the phenolic aromatic ring may provide additional selectivity to this protein. In addition, the mitoNEET ligand (NL-1) was able to alter complex I mediated mitochondrial respiration and act as a protective agent against a mitochondrial toxin. With mitoNEET representing a novel drug target for the treatment of metabolic and neurodegenerative diseases, these data provide the initial characterization of the proposed binding site to help guide future drug design.

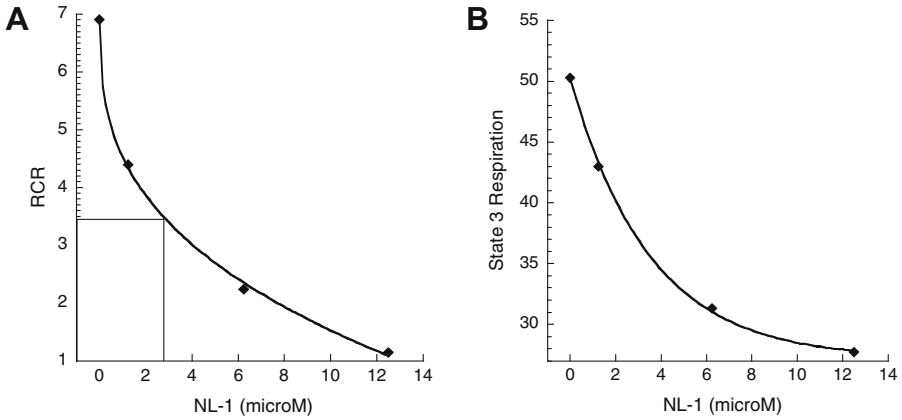

Figure 6. (A) NL-1 reduced respiratory control rate (RCR) demonstrating uncoupled mitochondrial respiration with an IC_{50} of 2.4 μM. (B) The inhibited state III respiration by NL-1 by approximately 45%. Figure is a representative graph of data extracted from a single dose–response study using NL-1.

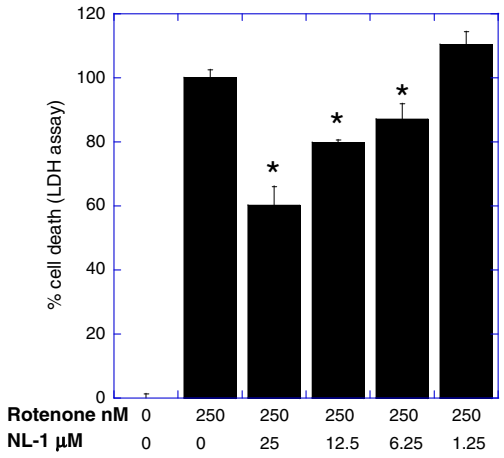

Figure 7. NL-1 inhibits rotenone-induced toxicity in SH-SY-5Y cells as determined by a reduction in LDH release into the conditioned media. $^{*}P$ <0.05 statistical significance as determined by a one-way ANOVA with Student–Newman–Keuls post-test. Each bar represents mean ± S.E.M., where $n = 3$. This experiment was repeated in triplicate.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.12.088.

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