MDM2 inhibition sensitizes neuroblastoma to chemotherapy-induced apoptotic cell death

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Abstract
Novel therapeutic approaches are urgently needed for high-stage neuroblastoma, a major therapeutic challenge in pediatric oncology. The majority of neuroblastoma tumors are p53 wild type with intact downstream p53 signaling pathways. We hypothesize that stabilization of p53 would sensitize this aggressive tumor to genotoxic chemotherapy via inhibition of MDM2, the primary negative upstream regulator of p53. We used pharmacologic inhibition of the MDM2-p53 interaction with the small-molecule inhibitor Nutlin and studied the subsequent response to chemotherapy in neuroblastoma cell lines. We did 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and terminal deoxynucleotidyl transferase assays to measure proliferation and apoptosis in several cell lines (IMR32, MYCN3, and JF) treated with combinations of cisplatin, etoposide, and Nutlin. We found consistent and robust decreases in proliferation and increases in apoptosis with the addition of Nutlin 3a to etoposide or cisplatin in all cell lines tested and no response to the inactive Nutlin 3b enantiomer. We also show a rapid and robust accumulation of p53 protein by Western blot in these cells within 1 to 2 hours of treatment. We conclude that MDM2 inhibition dramatically enhances the activity of genotoxic drugs in neuroblastoma and should be considered as an adjuvant to chemotherapy for this aggressive pediatric cancer and for possibly other p53 wild-type solid tumors. [Mol Cancer Ther 2006;5(9):2358 – 65]

Introduction
Neuroblastoma is an aggressive pediatric malignancy accounting for >15% of all pediatric cancer deaths and represents a major therapeutic challenge in pediatric oncology (1, 2). Current therapy is inadequate for high-stage disease with <40% long-term survival and is associated with unacceptable dose-related toxicity (3). Aberrant expression of the MYCN oncogene plays a central role in neuroblastoma tumorigenesis and MYCN amplification is a major negative prognostic indicator (1, 4, 5). As part of our investigations into the molecular effectors of MYCN action, we found that MYCN regulates the MDM2 gene, which, in turn, inhibits the p53 tumor suppressor (6). Because the overwhelming majority of de novo neuroblastoma tumors are wild type at the p53 locus (7–9), we hypothesized that MDM2-mediated inhibition of p53 plays an important role in preventing apoptosis in neuroblastoma and contributes to disease progression. We also predicted that pharmacologic disruption of the interaction between MDM2 and p53 would inhibit p53 sufficiently to activate downstream apoptotic pathways in neuroblastoma.

The tumor suppressor p53 is a central regulatory molecule responsible for growth arrest upon cellular stress or DNA damage (10, 11). In the event of extensive DNA damage, p53 activates apoptotic pathways leading to cell death. The fact that ~50% of all human tumors harbor p53 mutations underscores the importance of disrupting p53 activity in the process of tumorigenesis and progression (5). In addition to the paucity of p53 mutations in neuroblastoma (8, 12), several studies have also shown that p53 downstream functions are intact in neuroblastoma and that, when activated, p53 is capable of inducing normal apoptotic responses to stress or DNA damage (7–9). Thus, it is likely that the signals activating or inhibiting p53 in neuroblastoma need to be altered to permit tumorigenesis (13).

The primary negative regulator of p53 is MDM2 (14). Through direct binding to p53, MDM2 initiates p53 ubiquitination, nuclear export, and inactivation of p53 transactivating functions (10, 15). Interestingly, p53 directly activates MDM2 transcription, leading to increased protein levels and decreased p53 function (10). This negative feedback loop is essential for controlled activation of p53 in cells that have limited damage and can self-repair. However, overexpression of MDM2 leads to insufficient p53 activity and contributes to tumorigenesis as shown in transgenic mouse models (16, 17) and in human tumors (5, 18). MDM2 is amplified in over one third of sarcomas that retain wild-type p53 (19). Several studies have also shown that Mdm2 haploinsufficiency prevents tumorigenesis in the Eμ-myc mouse model of lymphoma (20, 21). These studies, in particular, showed that B-cell apoptosis was enhanced by Mdm2 deficiency, resulting in reduced tumor formation and increased survival (22).
Pharmacologic inhibition of the MDM2-p53 interaction as a therapeutic approach is a subject of intense investigation (23–25). Recently, effective small-molecule inhibitors that competitively bind to MDM2 and prevent binding with p53 have been synthesized (23–25). In particular, the Nutlin compounds (e.g., Nutlin 3a and Nutlin 3b) were found to bind with high affinity to MDM2 and to inhibit the growth of p53 wild-type osteosarcoma xenografts (26). These compounds were identified in a high-throughput screen of MDM2 inhibitors, aided by high-resolution nuclear magnetic resonance structural analysis of the MDM2-p53 protein/protein interaction (ref. 24 and references therein).

Because MDM2-mediated ubiquitination of p53 is critical for initiation of p53 degradation, preventing the interaction of p53 and MDM2 leads to markedly diminished p53 degradation. Subsequent elevated levels of p53 can alter the transcriptional activity of p53 and induce cell cycle arrest (primarily through p21-mediated mechanisms) and apoptosis through transcriptionally dependent and independent mechanisms (27–29). These mechanisms have been shown to be primarily independent of p53 phosphorylation, because Nutlin treatment alone does not induce phosphorylation at p-Ser-15 (26) and earlier work showed that p53 phosphorylation is dispensable for p53-mediated transcriptional activation and apoptosis (30).

As a therapeutic modality, disrupting the interaction of MDM2 and p53 shows great promise. Nutlin 3a has been shown to be antiproliferative as a single agent in a variety of adult tumors with wild-type p53. These include chronic lymphocytic leukemia (31), acute myelogenous leukemia (32), lung cancer (33), as well as osteosarcoma and colon cancer (26, 27). As MDM2 inhibitors move to the clinic, reactivating p53 in tumors that have suppressed its antiproliferative activity may prove to be an important adjuvant to chemotherapy, sensitizing tumor cells to apoptosis in the presence of genotoxic damage (see refs. 25, 34 for recent reviews).

To test our hypothesis that neuroblastoma is sensitive to MDM2 inhibition, we studied the response of neuroblastoma cell lines to genotoxic chemotherapy with and without this treatment. Nutlin 3a, as a single agent, rapidly stabilized p53 and triggered cell death. Furthermore, we show that Nutlin-induced p53 stabilization dramatically sensitizes neuroblastoma cells to the effects of cisplatin and etoposide, resulting in apoptosis. Our data support the therapeutic use of MDM2 antagonists as agents used in combination with genotoxic drugs in the treatment of neuroblastoma.

Materials and Methods

Tissue Culture

The Tet21 and MYCN-3 were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% Tet-approved fetal bovine serum and 1% penicillin and streptomycin. The Tet21 cell line was obtained from Manfred Schwab (University of Heidelberg, Heidelberg, Germany). The MYCN-3 cell line was generated as follows: the MYCN cDNA was cloned into the pTRE2-Hygro vector (BD Biosciences, Bedford, MA) containing a tetracycline-responsive promoter. This construct was then transfected into a SHEP subclone stably expressing the TRE-response element and selected with hygromycin. The JF, IMR32, and HCT 116 lines were maintained in RPMI 1640 (Invitrogen), MEMs (Invitrogen), McCoy’s 5A medium, respectively, supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Cisplatin solution (50 mg/mL; American Pharmaceutical Partners, Inc., Los Angeles, CA) and etoposide (Sigma, St. Louis, MO), stored as a 50 mmol/L stock solution in DMSO at −20°C, were used. Nutlin 3a and 3b were provided by Dr. Vassilev (Roche, Nutley, NJ). All the drugs were diluted in serum-free medium before treatment of cells.

Western Blot

Antibodies used are as follows: α-p53 (Santa Cruz Biotechnology, Santa Cruz, CA), α-MDM2 (Calbiochem, San Diego, CA), α-actin (Sigma) monoclonal antibodies, and horseradish peroxidase–conjugated goat α-mouse (Sigma) antibody were used for Western blot analysis as described below.

Fifty-microgram aliquots of protein from cells treated with Nutlin and combinations of chemotherapy (see Results) were electrophoresed and transferred as described (6). Cells were directly lysed by boiling in MDM2 lysis buffer for 5 minutes and protein was quantified by Bradford assay before electrophoresis. Actin was used for loading control in all experiments.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were done as in ref. (35). Briefly, 0.5 × 10⁵ cells/mL were plated into 96-well microtiter plates, incubated at 37°C, and cultured overnight. Stock solutions of Nutlin 3a or 3b in DMSO were diluted in PBS and added to the appropriate concentration by serial dilutions. PBS alone, medium alone, and medium plus DMSO were control wells for each dilution. Plates were incubated for 72 hours and then centrifuged for 5 minutes at 1,000 rpm. Half the medium (135 μL) was removed and replaced with fresh medium. Then, 15 μL MTT were added to each well. Plates were shaken in the dark for 5 minutes, incubated for an additional 4 hours, and read at 550 nm in the plate reader.

Terminal Deoxynucleotidyl Transferase Apoptosis Assay

IMR32, JF, and MYCN3 cells were plated in 10-cm dishes at 2 million cells per dish and grown overnight. The cells were exposed to drug combinations for 24 hours. Briefly, cells were harvested in 1× PBS and incubated in 3 mL of 1% formaldehyde-PBS for 15 minutes on ice. The cells were fixed in 70% ethanol-PBS overnight at −20°C. Next, 50 μL of the terminal deoxynucleotidyl transferase (TdT) reaction mix were added to each sample consisting of 10 μL of 5× reaction buffer, 1.5 μL TdT, 5 μL CoCl₂, 0.5 μL biotin-16-dUTP, and 33 μL dH₂O. The samples were incubated for 1 hour at
37°C and then washed with PBS. For fluorescence-activated cell sorting analysis, 100X avidin FITC buffer (4X SSC, 2.5 μg/mL avidin DCS FITC (Vector Laboratories, Burlingame, CA), 0.1% Triton X-100, and 5% nonfat dry milk) was then added to each sample. The samples were incubated in the dark at room temperature for 30 minutes and washed in 1 mL 1X PBS + 0.1% Triton X-100. The cells were resuspended in PBS and transferred through a 70 μm cell filter into a fluorescence-activated cell sorting tube for analysis. Fluorescence-activated cell sorting analysis was done using CellQuestPro and standard software.

**Results**

**Nutlin 3a Shows a Specific Antitumor Activity through p53 Stabilization in Neuroblastoma Cells**

We first tested the ability of the active Nutlin enantiomer, Nutlin 3a, to activate the p53 pathway in neuroblastoma cells. Western blot analysis showed that Nutlin 3a is able to induce a strong, specific, and rapid stabilization of p53 in neuroblastoma cell lines (Fig. 1A and B). We used the inactive enantiomer, Nutlin 3b, which has ~200-fold lower MDM2 binding affinity, as a control for off-target drug effects (26).

Because neuroblastoma is treated with multidrug chemotherapy, we also wanted to test the effect of Nutlin combined with agents commonly used for neuroblastoma treatment. Incubation of exponentially growing IMR32 cells with a low (2 μmol/L) or high dose (10 μmol/L) of Nutlin 3a for 1 hour led to a significant increase in p53 protein level. In contrast, cisplatin or cisplatin plus Nutlin 3b had little effect on the p53 level. However, the low dose of Nutlin 3a significantly increased stabilization of cisplatin-induced p53. Nutlin 3b had no effect on p53 levels, as previously shown (Fig. 1A and B).

Because p53 stabilization plays a critical role in regulating both cell proliferation and apoptosis, we next examined the effect of the MDM2 inhibitor on these cell activities. IMR32 cells were treated with increasing doses of Nutlin 3a or Nutlin 3b, and cell viability and apoptosis were assessed by MTT and TdT assays, respectively. The analysis of cell growth and viability showed a potent and dose-dependent antiproliferative activity of the active enantiomer A compared with the enantiomer B (IC50 3.25 versus 52 μmol/L), confirming the selective modulation of the p53 pathway by Nutlin 3a (Fig. 1C). Similarly, Nutlin 3a induces a significant and dose-dependent apoptotic effect as a single agent. No apoptosis was observed with Nutlin 3b at these doses (Fig. 1D).

**Nutlin 3a Potentiates the Antitumor Effects of Conventional Chemotherapeutic Agents in Neuroblastoma Cells**

Next, we tested the effects of the combined treatment of Nutlin and cisplatin on cell growth viability and apoptosis to determine if inhibition of the p53-MDM2 interaction might potentiate the antitumor effect of conventional...
chemotherapy in neuroblastoma. Both N-type (neuronal) IMR32 and JF, and S-type (stromal) MYCN3, were simultaneously treated with increasing low dose of cisplatin and fixed low dose (2 \( \mu \text{mol/L} \)) of active or inactive enantiomer of Nutlin 3a for 72 hours. MTT analysis showed that treatment with Nutlin 3a plus cisplatin significantly reduced cell viability in all cell lines compared with treatment with cisplatin alone (Fig. 2). The Nutlin 3b control showed no effect. IC\(_{50}\) values in different neuroblastoma cell lines after single-agent or combined treatment are summarized in Table 1. The matched p53 wild-type HCT116+ and p53 null HCT116+ cell lines were also tested to confirm the expected p53-dependent activity of Nutlin. Because MYCN expression can increase MDM2 expression, two MYCN-inducible cell lines, Tet21 and MYCN3, were tested to determine if changes in MYCN level altered the response to Nutlin. The IC\(_{50}\) did not change significantly upon MYCN induction, suggesting that the effect of Nutlin was sufficiently potent at these doses to overcome the 2- to 3-fold increase in MDM2 seen upon MYCN induction in these cell lines (Table 1; ref. 6).

To assess the contribution of low dose of Nutlin 3a to apoptosis induced by chemotherapy, we did TdT assays after treatment with Nutlin 3a or Nutlin 3b plus chemotherapy. IMR32 cells were incubated with increasing doses of cisplatin or etoposide for 24 hours. TdT analysis shows that the addition of low-dose Nutlin 3a consistently increases apoptosis in combination with etoposide (Fig. 3A) or cisplatin (Fig. 3B). We showed a similar effect in the JF and MYCN3 cell lines as shown in Fig. 4A. Thus, our data show that the addition of Nutlin 3a to chemotherapy both decreases cell viability as measured by MTT assay and increases sensitivity to apoptosis due to genotoxic chemotherapy.

Because combined cisplatin and etoposide is commonly used for induction therapy in neuroblastoma (1), we further tested this combination plus Nutlin 3a (Fig. 4B). Although the addition of etoposide to cisplatin increased apoptosis at 24 hours, a dramatic increase was noted upon addition of Nutlin 3a. It should be emphasized that the doses of cisplatin and etoposide used in these studies induced minimal apoptosis as single agents. Figures 3 and 4 illustrate that further stabilization of p53 with inhibition of MDM2 significantly sensitizes neuroblastoma to these chemotherapeutic agents.

Nutm 3a-Mediated Apoptosis Occurs through Rapid p53 Stabilization in Neuroblastoma Cells

To further define the mechanism of action of Nutlin 3a in neuroblastoma, we analyzed p53 protein levels in IMR32 cells at different time points after treatment. IMR32 cells were incubated with low-dose cisplatin; Nutlin 3a or 3b alone; or in combination for 1, 4, and 8 hours. p53 expression was then assayed by immunoblot. Treatment with higher doses of Nutlin 3a (10 \( \mu \text{mol/L} \)) was included as a positive control for p53 stabilization. We show that increases in p53 protein levels could be detected as early as 1 hour after exposure to Nutlin 3a (Fig. 5A). We also show increased MDM2 levels at 4 hours resulting from p53-driven expression of MDM2 (Fig. 5B). These findings

Table 1. IC\(_{50}\) values (\( \mu \text{mol/L} \)) for single-agent or combined treatment in neuroblastoma cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cisplatin</th>
<th>Nutlin 3a</th>
<th>Nutlin 3b</th>
<th>Cis + Nut3a</th>
<th>Cis + 3b</th>
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<tbody>
<tr>
<td>HCT116 wt</td>
<td>7</td>
<td>3.5</td>
<td>0.39</td>
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<td></td>
</tr>
<tr>
<td>HCT116 null</td>
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<td>40</td>
<td>4</td>
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<tr>
<td>IMR-32</td>
<td>0.5</td>
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<td>51.6</td>
<td>0.01</td>
<td>0.5</td>
</tr>
<tr>
<td>JF</td>
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<td>2.5</td>
<td>0.09</td>
<td>0.7</td>
<td></td>
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<tr>
<td>Tet21 Ind</td>
<td>21.5</td>
<td>5</td>
<td>&gt;100*</td>
<td>6.25</td>
<td>20.3</td>
</tr>
<tr>
<td>Tet21-NI</td>
<td>23.8</td>
<td>20</td>
<td>&gt;100*</td>
<td>20.3</td>
<td>20.3</td>
</tr>
<tr>
<td>MYCN3-Ind</td>
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<td>3</td>
<td>31.5</td>
<td>0.09</td>
<td>1.13</td>
</tr>
<tr>
<td>MYCN3-NI</td>
<td>4</td>
<td>3</td>
<td>31.5</td>
<td>0.17</td>
<td>1.5</td>
</tr>
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</table>

NOTE: Data were derived from MTT assays done as described in Materials and Methods. Tet 21 and MYCN 3 are tet-inducible cell lines. IMR-32 and JF (SK-N-JF) are MYCN-amplified cell lines. Nutlin 3a consistently reduced the IC\(_{50}\) of cisplatin between 10- and 50-fold depending on cell lines tested. Although less sensitive to cisplatin than the other cell lines, there is a trend toward increased sensitivity in the MYCN-induced version of the Tet21 cell line.

Abbreviations: Ind, induced; NI, noninduced; wt, wild type.

*The highest dose tested was 100 \( \mu \text{mol/L} \).
illustrate that the expected dynamic autoregulation of MDM2 by p53, and p53 by MDM2 (36), is active in neuroblastoma. Nutlin 3a and cisplatin treatment yielded higher p53 levels than Nutlin alone consistent with the apoptosis data (Figs. 3 and 4), and Nutlin 3b did not affect p53 levels as shown previously (Fig. 5C).

It is likely that the early rapid increase in p53 seen with Nutlin 3a leads to irreversible activation of the apoptotic pathway before the later increase in MDM2 levels can down-regulate this response in neuroblastoma cells.

Discussion

Our results show that targeting of the p53-MDM2 interaction with MDM2 inhibitors limits proliferation and sensitizes neuroblastoma cells to drug-induced apoptosis. The presence of wild-type p53 in the majority of neuroblastomas at diagnosis (13) represents a potent molecular target for therapy. p53 controls the activity of multiple genes involved in cell cycle, apoptosis, and cell senescence regulation (for review, see ref. 10). One major function of p53 is activation of the G1-S and G2-M checkpoints of the cell cycle through transcriptional stimulation of p21 (for review, see ref. 37). A second important function is that p53 transcriptionally activates multiple proapoptotic pathways (such as those downstream of BAX), which forms mitochondrial pores leading to release of cytochrome c, activating downstream caspases leading to apoptosis (38). Not only is p53 not mutated in neuroblastoma, but also...
these downstream pathways are functional (8, 12, 39–41). These findings suggest that upstream inhibition of p53 activation in the face of ongoing metabolic stress is necessary for circumvention of p53-mediated cell cycle arrest and apoptosis in rapidly proliferating neuroblastoma cells.

As a key oncogene driving the development of neuroblastoma, MYCN transcriptionally activates MDM2 expression (6), suggesting that elevated MDM2 partially contributes to controlling p53 activity in neuroblastoma, both during tumorigenesis and progression. The importance of MDM2 in neuroblastoma is an area of active research. For example, Keshelava et al. (42) has shown elevated MDM2 expression is associated with multidrug resistance in some neuroblastoma lines. Other studies have shown that MDM2 degrades wild-type p53 in neuroblastoma cells (9, 43). Isaacs et al. (44) has shown the importance of MDM2 activity in neuroblastoma by showing that its ubiquitin ligase activity is rate limiting in the degradation of p53 in neuroblastoma. In addition, relapsed neuroblastoma has an increased frequency of MDM2 amplification (45). Taken together with the data presented above, these findings show that the p53-MDM2 pathway is intact in neuroblastoma and that disruption of the p53-MDM2 interaction in the presence of functional downstream pathways causes cell cycle arrest and apoptosis in neuroblastoma cells.

To date, the Nutlin compounds have been used to show effective cell cycle arrest and apoptosis upon disruption of MDM2-p53 in other p53 wild-type adult tumors [e.g., chronic lymphocytic leukemia (31), multiple myeloma (46), and lung cancer (33)], and p53 wild-type osteosarcoma (26). In these other tumors, maximum levels of p53 and rates of apoptosis are found after 24 to 48 hours of treatment (27). We find much more rapid and robust levels of p53 stabilization in neuroblastoma that suggests to us that neuroblastoma is particularly sensitive to this approach. Furthermore, because >98% of neuroblastoma has functional p53, MDM2 inhibition should be applicable to all neuroblastoma tumors during initial therapy.

MYCN is necessary and sufficient for tumorigenesis in a tissue-specific mouse model of neuroblastoma (47). In concordance with the human data, the majority of tumors from these mice are p53 wild type.3 The mechanism by which MYCN contributes to evasion of p53-directed apoptosis in neuroblastoma is still undefined. We have presented data supporting the hypothesis that MYCN-driven expression of MDM2 contributes to p53 inhibition in neuroblastoma (13). There are several studies in the literature suggesting that MYCN both sensitizes cells to apoptosis and permits proliferation and expansion of neuroblastoma cells (reviewed in refs. 8, 48). MYCN can sensitize cells to cytotoxic drug–induced apoptosis (49). Additionally, MYCN induces apoptosis in B lymphocytes when expressed from the immunoglobulin enhancer Eμ in transgenic mice (50).

In light of the pleotropic effects of MYCN influence on both apoptosis and proliferation, we expected to see a change in apoptosis response to Nutlin upon MYCN induction in our conditional cell lines. However, no significant change was seen in the two MYCN-inducible cell lines used in this study. We speculate that MYCN changes in apoptosis sensitivity are overridden by removal of MDM2-mediated suppression of p53. Alternatively, these SHEP-derived lines do not accurately reflect the

3 Dr. Michael Hogarty, Children’s Hospital of Philadelphia, personal communication.
sensitivity of de novo MYCN-amplified tumors. However, it is clear that all neuroblastoma cell lines tested, independent of their MYCN status, were extremely sensitive to combined therapy.

In this study, we show that Nutlin 3a causes growth inhibition of neuroblastoma cells. In the presence of genotoxic stress with cisplatin, Nutlin 3a induces between an 8- and 50-fold change in IC50 compared with cisplatin alone. We further show that Nutlin 3a induces apoptosis in all the neuroblastoma cell lines tested. Moreover, addition of Nutlin 3a results in a marked decrease in the dose of the two genotoxic drugs, cisplatin and etoposide, required to induce apoptosis in these cells, broadening the therapeutic index of these drugs. Our data are particularly significant in light of the deleterious side effects of current therapy for neuroblastoma. These morbidity includes cardiac dysfunction, sensorineural deafness, second malignancies, and growth disturbances (1, 3, 51, 52). The possibility of improved neuroblastoma cell kill in conjunction with attenuated morbidity in children with neuroblastoma warrants further investigation of these compounds in vivo models and possibly clinical pilot studies.

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References


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