A Phase I-II Preoperative Biomarker Trial of Fenretinide in Ascitic Ovarian Cancer

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Abstract

Purpose: To evaluate study feasibility, toxicity, drug concentrations, and activity of escalating doses of the synthetic retinoid fenretinide (N-(4-hydroxyphenyl)retinamide (4-HPR)) in ovarian cancer by measuring serum CA125 and cytormorphic biomarkers in cancer cells collected from ascitic fluid before and after treatment.

Methods: Twenty-two naïve patients with ascitic ovarian cancer were treated with escalating doses of 4-HPR at 0, 400, 600, and 800 mg/d for 1 to 4 weeks before surgery. Changes in the proportion of proliferating cells expressed by Ki67 and computer-assisted cytormorphic variables (nuclear area, DNA index, and chromatin texture) were determined in ascitic cells. Drug levels were measured by high-performance liquid chromatography.

Results: Doses up to 800 mg/d were well tolerated, and no adverse reactions occurred. There was no effect of 4-HPR on changes in serum CA125, Ki67 expression, which were assessed in 75% of subjects, and cytormorphic variables, which were assessed in 80% of subjects. Plasma retinol levels were significantly lower in affected women than healthy donors. 4-HPR plasma concentrations increased significantly with increasing doses and attained a 1.4 μmol/L concentration with 800 mg/d. Drug levels in malignant ascitic cells and tumor tissue were higher than in plasma but were 50 and 5 times lower, respectively, than in carcinoma cells treated in vitro with 1 μmol/L 4-HPR.

Conclusions: Cell biomarkers can be measured in ascitic cells to assess drug activity. Under our experimental conditions, 4-HPR did not show activity in advanced ovarian cancer cells. However, clinical evidence supports further investigation of fenretinide for ovarian cancer prevention. (Cancer Epidemiol Biomarkers Prev 2006;15(10):1914–9)

Introduction

Ovarian cancer is the sixth most common cancer in the world with 165,000 new cases being estimated every year (1, 2). Because two thirds of patients present with advanced disease at diagnosis and the majority of these patients will develop recurrence (1, 2), intense effort has been focused on prevention and early detection to reduce ovarian cancer mortality.

Over the past years, retinoids have been studied as cancer therapeutic and preventive agents (3-6), and N-(4-hydroxyphenyl)retinamide (4-HPR) has emerged as one of the most promising compounds (4). In vitro, 4-HPR inhibits the growth of several human cancer cell lines, including ovarian cancer cells (4, 7). This agent has also shown antitumor activity in ovarian cancer animal models (8, 9). Furthermore, retinoid receptors have been associated recently with ovarian cancer prognosis, providing further evidence for their use in the clinic (10).

4-HPR acts through both retinoid receptor-dependent and retinoid receptor-independent mechanisms (11-13). Whereas the classic retinoids often induce differentiation, 4-HPR induces distinct biological effects, including generation of reactive oxygen species and promotion of apoptosis, which is mediated, at least in part, by an increase of the intracellular levels of ceramide (14-16). 4-HPR also lowers circulating insulin-like growth factor-I levels, which are associated with increased risk of premenopausal breast cancer, prostate, colorectal, and ovarian cancer (17).

A key issue is the optimal biological dose of 4-HPR, which has not been established. In vivo studies indicate that 4-HPR-induced apoptosis follows a dose-response relationship and is triggered by retinoid concentrations higher than 1 μmol/L, the level achieved in the blood with 200 mg/d (21). It is therefore crucial to further explore the clinical toxicity and the activity of escalating doses of 4-HPR to establish the most suitable dose for future clinical trials.

Here, we present the results of a phase I-II study of 4-HPR administered for 1 to 4 weeks before surgery in newly diagnosed ascitic ovarian carcinoma. Aims of the study were as follows: (a) to assess the toxicity of 4-HPR at different dose levels; (b) to measure drug and metabolite concentrations in plasma and tumor tissue and to compare plasma retinol levels
in affected women and healthy control subjects; (c) to measure the variations in serum CA125 levels; and (d) to determine the feasibility of assessing selected biomarkers in cancer cells collected from ascites before and after 4-HPR treatment.

Patients and Methods

Participants. Patients with newly diagnosed ascitic ovarian cancer were recruited. Eligibility criteria for entry were as follows: (a) clinical, instrumental, and cytologic evidence of newly diagnosed ascitic advanced ovarian cancer; (b) written informed consent before any study-specific screening procedure; and (c) performance status ≤2 on the WHO scale. Exclusion criteria were as follows: (a) any prior treatment for ovarian cancer; (b) ophthalmologic and retinal disorders with particular attention to night vision and dark adaptation impairment; (c) grade 3-4 dermatologic disorders; and (d) grade 3-4 hematologic, metabolic, hepatic, and renal dysfunctions.

Treatment. The study was approved by the Institutional Review Boards of the recruiting centers (European Institute of Oncology and San Gerardo Hospital). Patients were treated with escalating doses of 4-HPR at 0, 400, 600, and 800 mg/d for 1 to 4 weeks before surgery. This period was considered appropriate by the local Institutional Review Boards as the average waiting list for ovarian cancer surgery on both centers was ~4 weeks. In the absence of significant National Cancer Institute Common Toxicity Criteria grade 4 toxicity, a total of six patients was planned for each dose level. Treatment with 4-HPR, 100 mg capsules, was taken during each meal according to the following schedule: (a) 400 mg/d, 2 capsules bid; (b) 600 mg/d, 2 capsules tid; and (c) 800 mg/d, 2+2+4 capsules.

Toxicity Assessment. Toxicity was assessed on day 0 and then weekly until surgery using the National Cancer Institute Common Toxicity Criteria version 2.0 (National Cancer Institute Cancer Therapy Evaluation Program: Common Toxicity Criteria, which is available online).9

Measurements of Retinol, 4-HPR, and Metabolite Concentrations. Peripheral blood and ascites were collected before treatment and at surgery; ovarian vein blood and ovarian tumor were collected at surgery ~24 hours after the last 4-HPR dose. Blood samples from 44 blood female donors (age range, 39-67 years), with no evidence of ovarian tumor, were collected and analyzed for comparison of plasma retinol levels. A2780 human ovarian carcinoma cells, grown in the presence of 1 μmol/L 4-HPR and analyzed for intracellular drug concentrations as described previously (22, 23), were tested for comparison of drug levels in ascitic cells and tumor tissue. The concentrations of retinol, 4-HPR, its main metabolite \textit{N}(4-methoxyphenyl)retinamide (4-MPR) and the recently identified metabolite 4-oxo-4-HPR (23) were determined by high-performance liquid chromatography as described previously (21-23). The protein content was evaluated using the bicinechonic acid protein assay reagent (Pierce, Rockford, IL) in aliquots of 0.2 mL plasma or ascitic fluid, and tumor tissue samples and ascitic cells were homogenized. The reference standard of 4-HPR and 4-MPR were supplied by the R.W. Johnson Pharmaceutical Research Institute (Spring House, PA); the reference standard of 4-oxo-4-HPR was kindly supplied by Dr. R.W. Curley (The Ohio State University, Columbus, Ohio); retinol, used as reference standard, was obtained from Sigma (St. Louis, MO).

Measurements of Biomarkers in Ascitic Cells. Enriched preparations of ovarian tumor cells were obtained from ascites on discontinuous Ficoll-Hypaque gradient as described previously (24). Each preparation was morphologically assessed for purity. The changes in the proportion of apoptotic cells in the ascites collected at time 0 and on completion of 4-HPR treatment were measured by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL), in cells visualized under light microscopy and by DNA flow cytometry (25). Ki67 immunoreactivity was assessed using the MBI monoclonal antibody and a DAKO Autostainer Universal Staining System (DakoCytomation, Glostrup, Denmark). Immunoreactivity was evaluated as the percentage in all adequate specimens by counting all positive epithelial cell nuclei over the total number of epithelial cells.

Cytomorphometry. Cytomorphometric studies were done on Papanicolaou-stained cytologic slides containing the ascitic cells using previously described methodology (26, 27). Each slide was bleached and stained applying the Feulgen method (Becton Dickinson, San Jose, CA). The morphometric features, DNA content, and chromatin texture analyses of all cytologic slides were done using a CAS 200 image analyzer (Becton Dickinson). The CAS 200 digitalized the bright-field absorption into a series of pixels that were quantified from absorbance readings. Absorbances of rat hepatocyte nuclear DNA were used as a standard external control of known diploid DNA content. Specifically, the configurable run length is a count of the pixels within the cells whose gray level values differ from those of their left and right neighbors. Using routinely accepted standards for nuclear atypia, such as size, shape, and hyperchromasia, all the well-preserved epithelial nuclei were captured for quantitation. The mesothelial and inflammatory cells were not considered for analysis. Manually segmented nuclear images were obtained when nuclear images “touched” each other and interpreted as a single nuclear image by the instrument’s software.

The software “Cell Sheet” (Bacus Laboratories, Lombard, IL) was used to measure morphometric features, DNA content, and chromatin texture in each nucleus. In particular, the following features were evaluated: morphometric variables (nuclear area, perimeter, minimum and maximum diameter, and shape); cyometric variables related to DNA content (DNA index and picograms of DNA); Markovian chromatin texture features (run length, configurable run length, SD, valley, slope, and peak).

Statistical Analysis. The Kruskall-Wallis test was used to assess the effect of 4-HPR on the change in CA125 and Ki67 expression before and after treatment. To avoid multiple comparisons, nuclear area, DNA index, and the configurable run length were selected \textit{a priori} as the most representative end point biomarkers, one in each category of cytomorphometric analysis. For each cytomorphometric end point, the median value per each subject before and after 4-HPR treatment was calculated and the nonparametric Wilcoxon rank-sum test was applied to the percentage change [A\% = (after – before) / before]. The median percentage change of the DNA index and the configurable run length and the median difference between the logarithms of the nuclear area calculated for each subject were fitted with a linear regression model. To include all measures on multiple cells per each subject, the configurable run length and the log area of the nucleus of the single cells were fitted with a multilevel linear model, with the intercept fitted as random variable at subject level. The model was not applicable to DNA index because its distribution was not normal.

Results

Patient Characteristics. A total of 22 patients were recruited into the study (7 patients at 0 (control) level, 6 patients at 400 mg/d, 4 patients at 600 mg/d, and 5 patients at 800 mg/d

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9 http://cebp.cancer.gov/reporting/ctc.html
because of limited drug availability]. Median time of treatment with 4-HPR was 13.3 days. The main patient characteristics are listed in Table 1. The median age was 62 years (range, 45–77 years). Only two patients were diagnosed at an early stage (International Federation of Gynecology and Obstetrics IC and IIIC, respectively). All the other cases presented with advanced disease (stage III-IV). Distribution of histotypes was typical for epithelial cancer of the ovary, with the majority of papillary serous tumors. Patients 15 and 21 had a diagnosis of carcinoid tumor and fibrosarcoma, respectively, on the definitive pathology report and were therefore excluded from all analyses, except for toxicity. All patients had normal laboratory values at baseline, including hemogram, liver and renal function, and lipid profile. All had elevated levels of CA125 at baseline.

**Toxicity.** None of the 15 patients treated at 400, 600, and 800 mg/d dose level of 4-HPR experienced any dermatologic or ophthalmologic adverse reaction. No serious adverse events were observed.

**Retinol, 4-HPR, and Metabolite Concentrations.** Measurements of the concentrations of retinol, 4-HPR, and its two metabolites 4-MPR and 4-oxo-4-HPR were done before treatment and at surgery in plasma, ascites, and tumor tissue of 17 of 22 patients (5 patients at 0 mg/d, 6 patients at 400 mg/d, 2 patients at 600 mg/d, and 4 patients at 800 mg/d). Patient 16 was not included because she had not taken the drug the last day before surgery. The mean (±SD) pretreatment retinol levels of 17 patients with advanced ovarian cancer were 383 ± 197 ng/mL versus 596 ± 118 ng/mL in 44 healthy control subjects (P < 0.001, Student’s t test).

As reported previously (21), 4-HPR treatment caused a 80% to 90% reduction of retinol levels in plasma and a similar reduction was observed in ascitic fluid (Fig. 1). The concentrations of 4-HPR, 4-MPR, and 4-oxo-4-HPR in peripheral vein, ovarian vein, ascitic fluid, ascitic cells, and tumor tissue are reported in Fig. 2. In peripheral blood, 4-HPR concentrations slightly increased with increasing doses and were on average 0.87 (equivalent to 341 ng/mL), 1.11 (435 ng/mL), and 1.38 (540 ng/mL) μmol/L at 400, 600, and 800 mg/d, respectively. For each dose, the concentrations of 4-MPR and 4-oxo-4-HPR were slightly lower than the parent drug. In blood from the ovarian vein, the concentrations of 4-HPR, 4-MPR, and 4-oxo-4-HPR were lower than in peripheral blood, indicating drug retention in the ovary and the tumor. In ascitic fluid, the concentrations of 4-HPR and 4-oxo-4-HPR were lower than in plasma, whereas the concentrations of 4-MPR were higher. Ascitic cells had a very low drug content (lower than 0.1 μmol/mg protein), mostly at the limit of detectability, which was similar at all three doses and without evidence of 4-oxo-4-HPR presence. In solid tumor specimens, drug concentrations increased with increasing doses, although a high interpatient variability was observed with the highest dose. The concentrations of the drug and the two metabolites were, on average, higher in tumor than in plasma after 600 and 800 mg/d (data not shown). The concentrations of 4-HPR found in tumors of patients treated with 800 mg/d, which resulted in 1.38 μmol/L 4-HPR plasma concentrations, were compared with those found in A2780 cells, a human ovarian carcinoma cell line (7). The mean (±SD) concentrations of 4-HPR in *in vitro* grown carcinoma cells were 2.6 ± 0.6 μmol/L (i.e., 50 and 5 times lower than ascitic cells and patient tumors, respectively; data not shown).

**Modifications of CA125 and Laboratory Values during Treatment.** As shown in Table 2, treatment with 4-HPR did not affect the levels of serum CA125 compared with no treatment nor was there evidence of a linear dose-response effect. No effect of 4-HPR at any of the investigated dose levels was observed on laboratory data, including blood cell count, lipid profile (cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides), and liver function tests (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and γGT).

![Figure 1. Columns, mean retinol concentrations before (white columns) and after (black columns) 4-HPR treatment in peripheral vein, ovarian vein, and ascitic fluid; bars, SD.](image-url)
Tumor Cell Proliferation. The percentage of cell proliferation was assessed in 15 of 20 (75%) patients because of the poor number of neoplastic cells in some of the ascitic samples. Ki67 values before and after 4-HPR treatment at each dose level are detailed in Table 2. There was no indication of an antiproliferative effect of 4-HPR on tumor cells obtained from ascitic fluid. Although the percentage of apoptotic cells before and after 4-HPR using the TUNEL assay had been selected as a study endpoint, this evaluation was not feasible due to the extreme variability and inconsistency of the data.

Cytomorphometry. A summary of the median values of nuclear area, configurable run length, and DNA index at each dose level before and after treatment, together with their percentage change, is presented in Table 3. Figure 3A-C describes the variation of the median value of nuclear area, configurable run length, and DNA index in each of the 16 (80%) assessable subjects. Compared with baseline, the median configurable run length had a 23%, statistically significant reduction over time in the no treatment arm ($P = 0.01$), whereas no significant variation was observed in the 4-HPR arm ($P = 0.03$ for the time by treatment effect). The median nuclear area followed a similar trend, although the difference between treatment arms was not significant. Using a multilevel linear model, the estimates of the percent change of the three variables were in agreement with those obtained with the linear regression analysis, except that the different effect of no treatment versus 4-HPR became significant also for the percent change of nuclear area (data not shown).

Discussion

Advanced ovarian carcinoma remains a lethal disease and represents a major therapeutic challenge. Effective chemoprevention strategies are therefore demanded. Recent observations suggest that retinoids may be effective for cancer treatment and prevention (10), and preclinical (7-9) and clinical (18-20) data support the use of 4-HPR for ovarian cancer prevention.

Our study shows that cytormorphometric biomarkers can be measured in ascitic fluid in ~80% of subjects to assess drug activity in short-term preoperative trials. This is important given the known difficulty to conduct efficient phase I-II trials in ovarian cancer. We also show that the administration of 4-HPR up to 800 mg/d in patients with advanced epithelial ovarian cancer is well tolerated. No dermatologic or ophthalmologic effects occurred at a daily dose higher than the 200 mg dose used in chemoprevention trials (18, 20, 28). These findings are consistent with those reported in other series in adults, where 4-HPR proved to be well tolerated at doses of 400 mg/d (29), and with recent results in children with advanced neuroblastoma, where no major side effects were observed up to the 4000 mg/m² daily dose (30).

Under the conditions of our study, 4-HPR did not show any noticeable biological activity against ovarian cancer. Specifically, no effect was observed on the change in CA125 levels nor on the percentage of proliferating cells as measured by Ki67 staining. The expression of Ki67 has prognostic significance in stage III ovarian cancer (31) and can discriminate between borderline and advanced serous ovarian neoplasms (32). Whereas the extent of reduction in serum concentrations of CA125 during neoadjuvant chemotherapy has been associated with better survival in advanced ovarian cancer (33), there are no data using Ki67 changes, so the actual clinical implications of our findings are unclear. We attempted also to assess the induction of apoptosis in carcinoma cells in the ascitic fluid using TUNEL, but this proved to be technically unreliable for a high degree of background necrosis and was not pursued further in this exploratory study. In addition, the cytomorphometric analysis did not provide evidence for a biological activity of 4-HPR as nuclear area, DNA index, and chromatin texture were not modulated by the retinoid. In fact, a 23% significant reduction over time of configurable run length was observed in the control arm, whereas no significant variation was noted in the 4-HPR arms. We have no clear explanation

Table 2. CA125 serum levels and Ki67 expression in ascitic cells before and after 4-HPR treatment

<table>
<thead>
<tr>
<th>4-HPR (mg/d)</th>
<th>CA125</th>
<th>Ki67</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (range)</td>
<td>n</td>
</tr>
<tr>
<td>0</td>
<td>1,830 (159-5,047)</td>
<td>7</td>
</tr>
<tr>
<td>400</td>
<td>2,551 (164-9,220)</td>
<td>6</td>
</tr>
<tr>
<td>600</td>
<td>1,430 (243-2,694)</td>
<td>3</td>
</tr>
<tr>
<td>800</td>
<td>5,080 (1,540-11,056)</td>
<td>4</td>
</tr>
</tbody>
</table>
for this observation, except for the occurrence of reactive changes following paracentesis, which might have affected DNA features. In addition, part of the variations could be due to the separation procedure, which was not successful in all subjects. Our findings underline the importance of an untreated control group in these biomarker trials to control for different sources of variability.

One explanation for the lack of activity is that therapeutically active concentrations of 4-HPR were not attained at the doses used in this study. Our results of drug measurements showed that the plasma concentrations of 4-HPR slightly increased with increasing doses and were ~1 μmol/L. The concentrations of the recently identified polar metabolite 4-oxo-4-HPR (23) were similar to the parent drug, whereas concentrations of the recently identified polar metabolite 4-oxo-4-HPR (23) were similar to the parent drug, whereas concentrations of 4-MPR were lower in plasma but higher in the ascites. The high lipophilicity of 4-MPR might account for its accumulation in the ascitic fluid. From the ratios of the drug concentrations found in tumors and plasma, it can be concluded that the drug accumulated in tumor tissue at 600 and 800 mg/d. In spite of this, the drug concentration found in ascitic cells and tumor tissue of patients treated with 800 mg/d were 50 and 5 times lower, respectively, than those found in ovarian tumor cells grown in vitro in the presence of 4-HPR concentrations similar to those found in plasma (22). Therefore, higher intracellular drug concentrations may be needed to achieve the tumor growth-inhibitory effects observed in vitro. Importantly, the ovarian tumor cell line A2780 herein investigated is extremely sensitive to 4-HPR, inasmuch as the IC50 is achieved with 1 μmol/L, whereas ~10 μmol/L was the IC50 in different ovarian cell lines (7). This observation is in line with the finding that 4-HPR had antitumor activity in mice with ovarian tumors when given i.p. (i.e., locally) and not orally (8). Altogether, these results suggest that higher 4-HPR plasma concentrations should be achieved in humans. This seems to be feasible because 10 μmol/L 4-HPR plasma levels have been attained in children without major side effects (30).

Another explanation for the absence of 4-HPR activity could be the short treatment duration (median, 13.3 days), which prevented the attainment of a full clinical activity. In particular, 1 week of treatment may probably be a too short period to see any biomarker modulation. Moreover, the study was underpowered to search for a time-response relationship. In addition, the biomarkers measured in this study might not be the most predictive of the biological and anticancer effects of the drug. In view of the in vitro evidence that induction of apoptosis may be an important mechanism for the anticancer activity of 4-HPR, it would clearly be useful to accurately assess cancer cell apoptosis during treatment. We are currently testing alternative methods to determine apoptosis in cells harvested from ascitic fluid to address this issue.

Finally, a likely explanation for the absence of 4-HPR activity is that advanced ovarian carcinoma cells have acquired in vivo genetic and/or epigenetic changes in critical genes, which result in 4-HPR resistance. For example, retinoic acid receptor β is subject to transcriptional silencing (34) and cellular retinol-binding protein 1 is lost (35) in a subset of ovarian cancers. Interestingly, 4-HPR has been shown to activate both retinoic acid receptor β (36) and cellular retinol-binding protein 1 (23) in ovarian cancer cells.

Interestingly, our findings confirm previous studies showing that patients with established ovarian tumors have low retinol plasma levels compared with healthy controls (37, 38). Although contradictory results have been found in prospective studies (39, 40), our data support the notion that vitamin A and its derivatives play a key role in ovarian carcinogenesis (10) and provide further rationale for assessing their effect in prospective studies.

Although the present study was not able to show a noticeable activity of 4-HPR in ovarian cancer, we have shown

Table 3. Effect of fenretinide on nuclear area, configurable run length, and DNA index in ascitic cancer cells (median and range)

<table>
<thead>
<tr>
<th></th>
<th>No treatment (n = 6)</th>
<th>4-HPR, 400 mg (n = 4)</th>
<th>4-HPR, 600 mg (n = 3)</th>
<th>4-HPR, 800 mg (n = 3)</th>
<th>4-HPR, all doses (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNAindex</td>
<td>Baseline 2.56 (2.13-2.78)</td>
<td>2.69 (2.47-3.06)</td>
<td>2.54 (2.40-2.88)</td>
<td>2.84 (2.69-2.85)</td>
<td>2.70 (2.40-3.06)</td>
</tr>
<tr>
<td></td>
<td>Post-treatment 2.21 (0.53-2.61)</td>
<td>2.70 (2.53-2.82)</td>
<td>2.71 (2.59-2.86)</td>
<td>2.76 (2.62-2.79)</td>
<td>2.74 (2.53-2.86)</td>
</tr>
<tr>
<td>Configurablerunlength*</td>
<td>Baseline 1.06 (0.78-1.58)</td>
<td>0.94 (0.91-1.06)</td>
<td>1.02 (0.99-1.4)</td>
<td>1.00 (0.99-1.05)</td>
<td>1.00 (0.91-1.40)</td>
</tr>
<tr>
<td></td>
<td>Post-treatment 1.09 (0.76-2.08)</td>
<td>1.04 (0.96-1.09)</td>
<td>1.08 (0.98-1.83)</td>
<td>1.06 (0.90-1.67)</td>
<td>1.07 (0.90-1.83)</td>
</tr>
</tbody>
</table>

*Count of the pixels within the cells whose gray level values differ from those of their left and right neighbors.
†Ratio of DNA peak over rat epatocyte DNA peak.
the feasibility of this approach. Although our results are preliminary in nature and provide the rationale for a refined follow-on study, further investigation of this compound in ovarian cancer is warranted. Accumulated data from in vitro and animal systems suggest a strong synergy between 4-HPR and cytotoxic agents, such as cisplatin (8, 9). The limited toxicity of 4-HPR should encourage trials of combination therapy. Should positive data emerge from studies in advanced ovarian cancer, these could form the platform for subsequent trials of adjuvant treatment after complete remis-

sion following surgery and chemotherapy and particularly for primary prevention in high-risk subjects.

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References

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