Single-Dose Toxicity Study of Hepatic Intra-arterial Infusion of Doxorubicin Coupled to a Novel Magnetically Targeted Drug Carrier

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The toxicity of a single hepatic intra-arterial administration of doxorubicin (DOX) coupled to a magnetically targeted drug carrier (MTC) was evaluated in a swine model. MTC is a microparticle composite of elemental iron and activated carbon. MTC-DOX is a new formulation of doxorubicin absorbed to the MTC and is designed for site-specific delivery to a solid tumor in the presence of an externally applied magnetic field. The magnetic field induces extravasation of MTCs through the vascular wall, leading to localization and retention in the tissue at the targeted site. Eighteen swine were assigned to 6 treatment groups, including 3 control groups (vehicle control, doxorubicin, MTC), and 3 experimental groups that received the MTC-DOX preparation. Animals were given a single administration of test article, evaluated over 28 days, and then sacrificed. Signs of toxicity were monitored via clinical status, total body weight, gross and microscopic pathology, and serum chemistries. Angiography was used to determine the extent of any embolization present. There were no adverse effects observed in the DOX-alone group. Biologically significant, treatment-related gross and microscopic lesions were limited to the targeted area of the liver only in groups receiving ≥75 mg of MTC (with or without doxorubicin). The severity of liver necrosis correlated to the severity of embolization following treatment. Doxorubicin was not freely circulating in any of the MTC-DOX groups, suggesting successful localization to the targeted site. The no-adverse-effect level (NOAEL) was determined to be the MTC-DOX low-dose group.

Key Words: targeted drug delivery; magnetic targeting; microparticle; chemotherapeutic drug; intra-arterial delivery; liver toxicology; hepatic chemotherapy; doxorubicin.

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver (Munoz and Lincell, 1982) and the most common cancer in some geographic areas such as Asia and Africa (Simonetti et al., 1991). In the absence of demonstrable extrahepatic metastasis, surgical resection is usually only considered for those patients with good hepatic reserve, who present with a small single tumor located in an easily accessible region within the liver. Only a small percentage of those patients presenting with HCC are eligible for resection or transplantation (Colleoni et al., 1998). It has been widely reported that no single agent or combination of agents, given systemically, leads to greater than 25% response rates or has any significant impact on survival (Allgaier et al., 1998; Okada, 1998). Systemic chemotherapy has been limited by the overall toxicity, and by multiple other mechanisms (e.g., multiple drug resistance of tumor cells, tumor architecture limiting access of drug to tumor cells, large volume of distribution of the drug), resulting in sub-optimal dosing (Bradley et al., 1988; Goldstein et al., 1989). In contrast to these sub-optimal results for systemic chemotherapy, there has been a number of encouraging reports on regional chemotherapy. Recent studies have shown increased hepatic uptake of chemotherapeutic agents such as doxorubicin (Kohz et al., 1995), that have led to substantially enhanced responses when the agent was administered regionally (Lee, 1977).

Doxorubicin has been the most widely used anti-neoplastic agent as a single-agent systemic therapy regimen for HCC, with reported response rates of up to 15% (Chlebowski, 1984; Ihde, 1977). Doxorubicin has also been one of the most widely reported agents used in localized therapy approaches. In combination with ethiodized oil (Ethiodol, Savage Laboratories, Melville, NY) and gelatin sponge (Gel Foam, Pharmacia UpJohn, Kalamazoo, MI), doxorubicin has shown response rates of up to 50%, but still has not shown any survival advantage (Carr et al., 1997). Chemoembolization, using absorbable gelatin sponge, has sometimes demonstrated hepatic toxicity associated with its use, which has recently been ameliorated with the use of degradable starch microparticles (Fossheim et al., 1997). It has been widely reported that if surgical resection or transplantation is not an option for the patient, local or regional approaches using transcatheter arterial chemoembolization (TACE) or percutaneous ethanol injections (PEI) are the preferred treatments of choice (Colleoni et al., 1998; Nakamura et al., 1997).

Regional therapy achieved through targeted drug delivery could potentially improve efficacy by increasing the drug concentration in the tumor while limiting systemic drug concentrations that produce systemic toxicities. The use of magneti-
tially targeted carriers (MTC, from FeRx, Inc., Arvada, CO) for drug delivery aims to target drugs to specific sites with selective catheterization and direct application of a magnetic field to the desired site, in order to achieve prolonged release of high, localized concentrations of drug by retention of MTCs in the region of interest.

MTCs combine elemental iron with activated carbon in microparticles in a size range of 0.5–5 μ. The activated carbon is capable of adsorbing and desorbing pharmaceutical agents such as doxorubicin. The elemental iron component of the microparticles allows for targeting and local retention after hepatic arterial administration, by placement of an external magnet placed on the body surface. Doxorubicin (DOX) absorbed to MTCs (MTC-DOX) can thus be administered by selective catheterization of one of the hepatic artery branches feeding an HCC lesion. Placement of an external magnet over the region of the tumor allows for efficient targeting of the MTC-DOX (Goodwin et al., 1999).

The purpose of this study was to evaluate the toxicity profile of MTC-DOX in the swine model, which was chosen because of the similarity of the human and swine vascular anatomy. Signs of toxicity were monitored via clinical status, total body weight, gross and microscopic pathology, and serum chemistries. Angiography was used to determine the extent of any embolization-related vascular occlusion in the treated arterial bed.

MATERIALS AND METHODS

Animals. Female Yorkshire, non-tumor bearing, domestic swine were obtained from S & S Farms (San Diego, CA). The animals were laboratory-bred and were experimentally naive at the outset of the study. Animals selected for use in this study were as uniform in age and weight as possible. They were 3 to 4 months of age, and their body weights ranged from 23 to 29 kg (mean = 26.8 kg). All animals were acclimated to laboratory conditions for a minimum of 7 days prior to study initiation. All animals were handled according to “Principles of Laboratory Animal Care” and the “Guide for the Care and Use of Laboratory Animals.” NIH Publication No. 80–23, revised 1985.

Materials. A vial containing 100 mg of sterile MTCs was incubated at room temperature (18 to 25°C) with 8 mg (4 ml) of doxorubicin (2 mg/ml) for 30 min to allow adsorption. The MTC-doxorubicin suspension was then diluted with 16 ml of vehicle (manufactured by Pharmaceutical Development Center, Charleston, SC and comprised of 10% mannitol, USP, and 0.5% sodium carboxymethylcellulose) and sonicated for 30 s using a Cole-Palmer Ultrasonic Cleaner (Vernon Hills, IL) prior to administration. The resulting solution had a suspension concentration of 0.4 mg/ml of doxorubicin and 5.0 mg/ml of MTC. The suspension was used within 6 h of preparation. Doxorubicin-HCl Injection, USP was purchased from Fujisawa USA (Deerfield, IL). A rare-earth NdFeB permanent 5-kgauss magnet (Magnet Sales, Inc., Culver City, CA) was housed in a flexible magnet holder (FMH, FeRx Incorporated, Arvada, CA), which allowed steady positioning of the magnet at the desired location.

Experimental protocol. A total of 18 animals were randomly assigned to 6 treatment groups of 3 animals each. Each animal received a single dose of either control or test material by hepatic intra-arterial infusion. The animals were evaluated for changes in clinical signs (appetite, stooling, activity level), body weight (measured prior to dosing and on Days 7, 14, 21, and 29 [pre-terminally]), and serum laboratory indices. All animals were euthanized on Day 29, except for two that required early sacrifice. A full necropsy was conducted on all animals that survived to the end of the study, and a partial necropsy was conducted on those animals that were sacrificed early. Tissues from the liver, stomach, lung, heart, and spleen were collected for histopathological evaluation.

Group assignments and dose levels. Animals were dosed using a fixed concentration of the test article. Low-, medium-, and high-MTC-DOX doses varied as a function of the infusion volume. Each group consisted of 3 animals. The vehicle control group received a dose volume of 45 ml. The high-DOX control group received 18 mg of doxorubicin in a dose volume of 45 ml (0.73 ± 0.04 mg DOX/kg body weight). The dose solution had a concentration of 0.4 mg/ml of doxorubicin. The high-MTC control group received 225 mg of MTC in a dose volume of 45 ml (8.85 ± 0.83 mg/kg). The dose solution had a concentration of 5.0 mg/ml of MTC drug carrier. The MTC low-DOX group received 2 mg of DOX and 25 mg of MTC in a dose volume of 5 ml (0.08 ± 0.00 mg of DOX/kg, 1.01 ± 0.03 mg of MTC/kg). The medium-MTC-DOX group received 6 mg of DOX and 75 mg of MTC in a dose volume of 15 ml (0.22 ± 0.01 mg of DOX/kg, 2.79 ± 0.16 mg of MTC/kg). The high-MTC-DOX group received 18 mg of DOX and 225 mg of MTC in a dose volume of 45 ml (0.72 ± 0.06 mg of DOX/kg, 8.94 ± 0.71 mg of MTC/kg). The low-, medium-, and high-MTC-DOX groups all received a concentration of 0.4 mg/ml of doxorubicin and 5.0 mg/ml of MTC drug carrier.

Catheterization procedure. The animals were fasted overnight (approximately 12–15 h) prior to the procedure. In preparation for the procedure, each animal was weighed and pre-anesthetized with 150 mg ketamine and 150 mg xylazine. Under general anesthesia, the right femoral artery was cannulated using standard percutaneous techniques. Animals were administered 5000 IU of heparin systemically, prior to delivery as prophylaxis against catheter or test article induced thrombosis. Under fluoroscopy, a 5 French angled Glidecath (Boston Scientific Corp., BSC, Natick, MA) and a 0.035-inch angled Glidewire (BSC) were inserted into the celiac artery. The common or proper hepatic artery was catheterized, and angiography was performed to select a segmental branch of the hepatic artery that provided adequate accessibility to the targeted lobe of the liver. The right, middle, or left hepatic artery, or segmental branch thereof, was then catheterized with a microcatheter (Fas Tracker 325 catheter, BSC) and microwire (Taper 22 wire, BSC). Angiography was then performed to verify catheter placement.

Magnet placement and depth measurements. Using angiography, location of the external magnet was determined by placing a 2-inch diameter metal disk on the ventral surface of the pig, positioned centrally over the targeted segment, and approximately 1–2 cm distal to the catheter tip. The disk was outlined on the skin surface to guide placement of the magnet. Once the magnet position was determined, a depth from the catheter tip to the center point of the magnet was determined by angiography. Following the angiography procedures, the north pole of the magnet was centered in the marked position on the surface of the animal and was kept in position during the entire infusion procedure (groups 3, 4, 5, 6) and for an additional 15 min following the final infusion.

Test material infusion. The test-article dose volume was infused as repeated cycles of 7.5-ml infusions at an infusion rate of 2 ml/min. The cycles were repeated every 15 min until all of the dose volume was administered.

Post-infusion angiography. At the end of the infusion, an angiogram was performed to verify the patency of the arteries in the selected lobe of the liver. Angiography was performed through the microcatheter. The microcatheter was then removed and repeat angiography of the common or proper hepatic artery was performed through the 5-French catheter to determine the patency of the hepatic artery.

Toxicokinetic analysis and serum chemistries. Aliquots of approximately 2.0 ml of whole blood were collected in EDTA-containing tubes from all animals in Groups 2, 4, 5, and 6 on Day 0 prior to dosing, and at 15, 30, 45, 60, 90, 120, and 180 min post-dose. The samples were mixed immediately by inverting at least 6 times, and then centrifuged. Analysis of plasma doxorubicin levels were quantified by high-pressure liquid chromatography (HPLC)
For each surviving animal, multiple serum chemistry, hematology, and coagulation parameters were measured on Days 1, 3, 7, 14, 21, and 28. Dunnett’s t-test was used to analyze for inter-group differences. Student’s t-test was used to test pairwise combinations of inter-group and time point differences.

RESULTS

The first part of the study examined tolerance to the MTC-DOX. Individual body weight and body weight changes for all animals surviving to the scheduled sacrifice on Day 29 were recorded. Weight changes were similar in all 3 controls (vehicle 2.8 ± 0.8 kg, doxorubicin 3.0 ± 1.2 kg and MTC 2.8 ± 0.9 kg), and the MTC-DOX low-dose groups (2.8 ± 0.6 kg). There was less body-weight gain in the MTC-DOX medium (1.3 ± 1.0 kg)- and high-dose groups (1.0 kg). In the MTC-DOX high-dose group, only one animal survived to day 29. Of the 2 other animals, one was sacrificed at Day 14 and weighed 1.5 kg less at that time. The third animal was sacrificed too early to obtain weights.

Clinical Signs and Mortality

The vehicle and doxorubicin controls showed no clinical symptoms and survived to Day 29 for sacrifice. In both the MTC control group and MTC-DOX low-dose group, 2 animals had reduced appetite and no stool for Day 1 only. No other changes were noted in these animals and all survived to Day 29. The MTC-DOX medium-dose group had no abnormal behavior and all survived to Day 29.

All 3 animals in the MTC-DOX high-dose group had serious clinical signs. Two of the 3 animals (nos. 1 and 10) had reduced appetite, no stool, loose stool, and lethargy during Days 1–4. On Day 5, one animal (no. 1) developed a temperature of 106°F and jaundice. This animal was sacrificed and partially necropsied on Day 5. Another animal (No. 16) had reduced appetite, diarrhea, and soft stool starting on day 11 and developed focal skin lesions located primarily in the head region by day 14. The animal was euthanized on that day.

Serum Chemistries

Intergroup analysis of the clinical pathology parameters, as compared to the vehicle control group, showed statistically significant changes in serum chemistry (LDH, CPK, bilirubin, AST [SGOT], and serum iron) and hematology parameters (monocytes, neutrophils, lymphocytes) for the MTC-DOX high-dose group only (Table 1).

Compared to pre-dose and post-dose levels, elevations in mean AST were present in all groups. However, none of these elevations reached statistical significance in the doxorubicin and MTC control groups or MTC-DOX low- and medium-dose groups. Only the MTC-DOX high-dose group, in which AST levels were nearly 20 times higher than pre- and post-dose levels, reached statistical significance on days 1 and 3 (p < 0.05). The changes in levels of AST over time, in all groups, correlates with the time course of hepatic necrosis. AST levels rose rapidly and peaked, correlating with acute hepatocellular damage after dosing, before gradually declining to normal levels by Day 28.

LDH activity was elevated in all groups in a manner similar to that observed with AST and only the MTC-DOX high-dose group had statistically significant elevations (Days 1 and 3). CPK was also elevated in all groups, similar to AST and LDH. Elevations in CPK values were statistically significant from the vehicle control only in the MTC-DOX high-dose group (Day 1). Serum bilirubin elevations were also only significant in the MTC-DOX high-dose group, most predominant on Day 3, and were considered related to bile stasis in the affected livers. Transient increases in serum iron, statistically significant only in the MTC-DOX high-dose group (Days 1 and 3), were considered to be related to the iron component of the MTC carrier.

There were transient changes in certain hematology parameters, including neutrophils, lymphocytes, and monocytes, that were statistically significant from the vehicle control, primarily in the MTC-DOX high-dose group. These changes were considered dose-related. There were no measurable differences

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Mean Clinical Pathology Results for MTC-DOX High-Dose Group</th>
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<tbody>
<tr>
<td>Clinical chemistry</td>
<td>Pre-dose</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>27</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>500</td>
</tr>
<tr>
<td>CPK (U/L)</td>
<td>1667</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>0.0</td>
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<tr>
<td>Serum iron (mg/dl)</td>
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</tr>
<tr>
<td>Hematology</td>
<td></td>
</tr>
<tr>
<td>Neutrophils (/μl)</td>
<td>12982</td>
</tr>
<tr>
<td>Lymphocytes (/μl)</td>
<td>7205</td>
</tr>
<tr>
<td>Monocytes (/μl)</td>
<td>297</td>
</tr>
</tbody>
</table>

(OREAD BioSafety, Farmington, CT). For each surviving animal, multiple serum chemistry, hematology, and coagulation parameters were measured on Days 1, 3, 7, 14, 21, and 28. Dunnett’s t-test was used to analyze for inter-group differences. Student’s t-test was used to test pairwise combinations of inter-group and time point differences.
between groups for partial thromboplastin and prothrombin times.

**Toxicokinetic Analysis**

Groups receiving MTC-DOX (low, medium, or high dose) showed little or no circulating levels of doxorubicin over the 3-h sampling period, whereas the doxorubicin control group had high levels of doxorubicin throughout the same period (Fig. 1). These results suggest that the drug remained localized primarily to the targeted site in the MTC-DOX treatment groups.

**Pathology**

**Targeted liver.** Microscopic changes were primarily limited to the targeted region of the liver in those groups receiving MTC particles. The severity of these changes was in proportion to the dose of MTC particles, with the most severe liver changes in groups receiving the highest dose of MTC particles (MTC control and MTC-DOX high-dose group). Portal bridging fibrosis (bands of fibrous connective tissue connecting adjacent portal areas) was a prominent change in the MTC control group but absent in the DOX control group. Bile pigment, peribiliary fibrosis, neutrophilic inflammation of bile ducts, and bile duct rupture (Fig. 2) were present only in the groups receiving 75 mg of MTC particles or greater (MTC control, MTC-DOX medium- and high-dose groups). Chronic/
active inflammation was only seen in those animals receiving the high dose of MTC particles (MTC control and MTC-DOX high-dose groups). Extravasation of MTC particles into the portal area tissue (including the walls of the hepatic artery branches) was noted in all animals receiving MTC particles. Existence of MTC particles in the Kupffer cells of the hepatic lobule was noted in all groups receiving high-dose MTC-DOX and in one animal in the low MTC-DOX group (group 4). In most animals, multinucleated giant cells were associated with the presence of MTC particles in the portal area tissue (Fig. 3). In the targeted liver, severe and moderate necrosis of entire hepatic lobes was noted in the MTC-DOX high-dose group (Fig. 4) and MTC control group respectively. Other groups had no observable necrosis except one animal in the medium-dose group that had mild necrosis.

**Non-targeted liver.** In groups receiving high doses of MTC particles (MTC control and MTC-DOX high-dose groups), MTC particles were seen in the hepatic artery, portal areas, and hepatic lobules (Kupffer cells) in the non-targeted regions of the liver. The presence of these particles, however, was not associated with any microscopic damage to the liver. Bile-stasis in the non-targeted region of the liver was present in only one animal receiving the MTC-DOX high dose and was considered to be secondary to the severe changes occurring in the targeted region of the liver in that animal. No other groups had particles outside of the targeted region.

**Other tissue.** MTC particles were present within submucosal arteries in the stomach of a single animal in the MTC-DOX high-dose group and was associated with minimal accumulation of multinucleated giant cells. In this same group, 2 animals had bronchopneumonia with severe lung inflammation and bacteria in the bronchi. In one animal, pleural fibrosis and pleura inflammation were associated with the pneumonia. Neutrophilic inflammation of the pericardium in these animals was also most likely due to bacterial infection. Granulomatous inflammation or neutrophilic inflammation in the spleens of both animals was likely an extension of inflammation in other tissues of the body.

A second part of this research focused on the mechanical embolization by the MTC-DOX injection. Representative pre- and post-infusion angiograms from the DOX and MTC control groups, and the MTC-DOX low- and high-dose groups are presented in Figures 5a through 5f, respectively. The relative extent of post-treatment embolization in each group was determined by angiography and was graded as none, minor, moderate, or significant. The vehicle control group had no observable embolization (in one animal, angiography was not performed, due to dislodgement of the catheter as the animal woke up following the procedure). All animals in the DOX control group had no measurable embolization, except one with minor embolization. In the MTC control group, the first animal had minor, the second had moderate, and the third had significant embolization. None of the 3 animals in the MTC-DOX low-dose group had observable embolization. Two animals in the MTC-DOX medium-dose group had minor embolization. One animal had no observable embolization. All animals in the MTC-DOX high-dose group had significant embolization of the hepatic arterioles that resulted in severe hepatic necrosis.

**DISCUSSION**

Toxicokinetic results indicate that doxorubicin is circulating at minimal levels in all the groups receiving MTC-DOX. This suggests that the MTC particles effectively localized doxorubicin to the targeted site where the magnet was placed.

The animals tolerated the MTC-DOX in a dose-related fashion. They gained less and less weight as MTC-DOX dosage was increased from low to high. Weight gain in the vehicle, doxorubicin, and MTC controls were comparable to the MTC-DOX low-dose group. MTC-DOX medium- and high-dose groups had significantly lower weight gains.

The MTC-DOX high-dose group was the only group that had statistically significant elevations in AST indicating more severe hepatocellular damage compared with all other groups. Unlike the MTC-DOX high-dose group, neither the doxorubicin nor the MTC control group had significant AST elevations, suggesting a possible synergistic effect between MTCs and doxorubicin that increases the severity of hepatocellular damage. While this synergy resulted in increased toxicity in normal liver tissue, it is possible that this effect will also be applicable to rapidly dividing neoplastic cells, thereby increasing efficacy of treatment. Further study is needed to verify this synergy in a tumor model. Similarly, elevations in LDH, CPK, bilirubin, neutrophils, lymphocytes, and monocytes were only significant in the MTC-DOX high-dose group.

Portal bridging fibrosis, peribiliary fibrosis, neutrophilic inflammation of bile ducts, and bile duct rupture were present only in the groups receiving 75 mg of MTC particles or greater (MTC control and MTC-DOX medium- and high-dose groups) and not in the MTC-DOX low-dose group. Such changes may result from extravasation of MTC out of the hepatic artery causing local inflammation.

Hepatic lobule necrosis is a concern. The liver has a dual blood supply from the hepatic artery and portal vein and is therefore not easily infarcted. The necrosis observed may be due to incomplete extravasation of MTC particles out of the hepatic artery and subsequent blockage of the hepatic artery branches, concurrent with occlusion of the portal vein due to portal fibrosis and inflammation. Necrosis was observed only in groups receiving 75 mg of MTC particles or greater. It is interesting to note that given the more porous nature of vasculature in tumors such as hepatocellular carcinoma, it is possible that extravasation of MTC particles would be more complete and necrosis may not occur until higher amounts of MTCs are used.

The toxicity profile of a single administration of MTC-DOX
FIG. 5. Comparison of pre- and post-treatment hepatic arteriograms following administration of test article to determine patency of hepatic arterioles. (a) Shows hepatic angiogram prior to infusion of doxorubicin (DOX) in a DOX control animal. (b) Pre-infusion main hepatic angiography of MTC control animal. (c) Post-infusion angiography following 6 infusion cycles of 37.5 mg MTC/cycle, for a total of 225 mg of MTC particles. Angiography depicts significant embolization in the arterial branches feeding the targeted segments. (d) Pre-infusion selective angiography of MTC-DOX low-dose animal. (e) Pre-infusion main hepatic angiogram of MTC-DOX high-dose animal. (f) Post-infusion angiogram following administration of 18 mg doxorubicin/225 mg MTC, delivered in 6 infusion cycles of 3 mg DOX/37.5 mg MTC. Significant embolization can be observed in the arterial branches feeding the targeted segments.
targeted to the liver in normal swine has been demonstrated. Based upon the gross and the microscopic pathology, the NOAEL was determined to be 25 mg MTC/2 mg doxorubicin, the level of the MTC-DOX low-dose group.

Since a relevant in vivo liver tumor model was not readily available, this study was conducted in a non-tumor model. Consideration should be made for the potential differences in MTC-DOX toxicity between normal and neoplastic cells. It is hypothesized that the MTCs should more readily extravasate from the neovasculature of a tumor, where the lesion is often hyper-vascular and the endothelial lining is relatively more porous.

The minimal serum concentration of doxorubicin, as compared with controls when doxorubicin was coupled to MTCs, suggests that the use of magnetic targeted carriers successfully localized the drug to a targeted site. The toxicity profile of MTC-DOX in a swine model has been demonstrated and a NOAEL was determined to be 25 mg MTC/2 mg doxorubicin. Levels higher than this result in hepatic necrosis and embolization of arteries. Future studies of the efficacy and safety profile of magnetic targeted carriers in tumor models are necessary to further our understanding of a promising targeted drug-delivery technique.

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REFERENCES


