Anticoagulant and Antithrombotic Activity of Maltodapoh, a Novel Sulfated Tetrasaccharide

DANIEL J. MARTIN, JOSEPH A. TOCE, PHILLIP J. ANEVSKI, DOUGLAS M. TOLLEFSEN and DANA R. ABENDSCHEIN

ABSTRACT

Orally bioavailable anticoagulants are needed that exhibit rapid and predictable onset and offset kinetics. This study was designed to determine whether maltodapoh, a novel sulfated bis-maltobionic acid amide, exhibits anticoagulant and antithrombotic activity in vivo after oral administration. Maltodapoh exhibited a dose-dependent increase in activated partial thromboplastin time (aPTT) in both rabbit and human plasma in vitro. Maltodapoh also induced a dose-dependent increase in aPTT when administered either i.v. or p.o. in rabbits. After a single oral bolus (3 mg/kg), aPTT increased 2- to 3-fold between 4 and 8 h and remained elevated for at least 24 h. This dose doubled the time to the onset of thrombotic occlusion after electrical injury to the carotid artery (from 52 ± 12 min in vehicle-treated, control rabbits, n = 7, to 98 ± 12 min in maltodapoh-treated animals, n = 7, P < .001) and reduced by 84% the weight of thrombus in the superior vena cava induced over 2 h after insertion of a thrombogenic copper wire and thread device (from 37 ± 10 mg in controls to 6 ± 3 mg in maltodapoh-treated animals, P < .001). Thus, based on the in vivo activity after oral administration, favorable kinetic profile and efficacy for inhibition of both arterial and venous thrombosis, further testing of this class of compounds appears warranted.

Anticoagulation in patients at high risk for thrombosis (e.g., those with unstable coronary disease or after hip and joint surgery) is a difficult problem affecting thousands of people annually. Heparin and its derivative, low-molecular weight heparin, although effective as an anticoagulant to prevent thrombosis, cannot be administered orally and is therefore difficult to maintain for extended therapy outside the hospital. Warfarin (Coumadin) is an orally bioavailable anticoagulant that inhibits production of vitamin K-dependent coagulation factors. However, this agent takes several days to reach therapeutic anticoagulant levels, requires frequent monitoring of anticoagulation and is subject to many drug-drug and dietary interactions. Furthermore, the effects on coagulation are not readily reversible once medication is discontinued. Thus, new orally active anticoagulants are needed that exhibit rapid and predictable onset and offset kinetics.

Sulfated bis-lactobionic acid amides have been shown to exhibit anticoagulant activity (Raake and Elling, 1989; Klauser et al., 1991). Based on this observation and interest in an orally active compound, Reliable Biopharmaceuticals (St. Louis, MO) generated a highly sulfated tetrasaccharide composed of two maltose sugars connected by a 1,3-diamino-2-propanol linker. This compound was named maltodapoh based on the combination of maltose (malto) and diaminopropanol (dapoh).

Our study was designed to determine whether maltodapoh exhibits anticoagulant activity in vitro and in vivo and whether it is active after oral administration. We first determined the in vitro concentration of maltodapoh required to increase the aPTT in plasma 2- to 3-fold above baseline levels; the target prolongation for currently used anticoagulants. This concentration was adjusted for an initial intravenously administered dosage in rabbits followed by orally administered dosages. The oral dosage of maltodapoh required to increase aPTT 2- to 3-fold was then tested for its efficacy to inhibit arterial and venous thrombosis.

Methods

Synthesis of Maltodapoh. Maltobionic acid was generated by electrochemical oxidation of maltose and was purified by ion exchange chromatography. The spray-dried disaccharide acid powder was then dehydrated by refluxing in dimethyl formamide/pentane. This conversion was monitored by HPLC until >90% of the material was converted to the lactone form. The disaccharide lactone was condensed with 1,3-diamino-2-propanol to form the bis amide of maltobionic acid, maltodapoh (Fig. 1). Finally, the amide was highly sulfated by reaction with sulfur trioxide pyridine complex in di-

ABBREVIATIONS: aPTT, activated partial thromboplastin time; PBS, phosphate-buffered saline; HPLC, high-pressure liquid chromatography; HCII, heparin cofactor II; ATIII, antithrombin III.
methyl formamide. The sulfated maltodapoh was purified with a combination of solvent precipitations and column chromatography. The identity and purity of the maltodapoh were assessed by elemental analysis, mass spectroscopy and size exclusion chromatography. Based on the carbon/sulfur ratio from elemental analysis, an average of 16.4 of the 17 available sites were sulfated resulting in a calculated molecular weight of 2504 Da. Mass spectroscopy of the maltodapoh showed a fragmentation pattern that was expected from a highly sulfated family of molecules with the assigned structure. Separation of maltodapoh on a size exclusion column with determination of concentrations in serial fractions by polarimetry revealed one major peak. Assay of the effect of each column fraction on aPTT in plasma in vitro showed a good correlation between maltodapoh concentration and the change in aPTT consistent with homogeneity of the purified material.

In Vitro Experiments. Maltodapoh was tested for anticoagulant activity in both human and rabbit plasma. Blood was withdrawn from the antecubital vein of consenting, healthy human volunteers and from the central ear artery of unanesthetized rabbits. The blood was placed in tubes containing 3.8% sodium citrate (one part citrate to nine parts blood) and centrifuged at 1000 × g for 10 min. Maltodapoh was prepared as a stock solution (1.5 mg/ml) and diluted serially with PBS. A 10-μl aliquot of diluted maltodapoh or 10 μl of PBS as a control was added to 100 μl of plasma for assay of aPTT as described below. aPTT for each dilution was measured in triplicate and the results averaged. Concentration versus aPTT experiments were repeated for both rabbit and human plasmas obtained from three different individuals.

To investigate the basis for the anticoagulant activity of maltodapoh, we tested its ability to stimulate inhibition of thrombin by HCII or ATIII. Human α-thrombin (10 nM, Haematologic Technologies, Essex Junction, VT), HCII (29 nM) or ATIII (51 nM) purified from human plasma as described previously (Tollefsen et al., 1983), and maltodapoh or heparin (Sigma Chemical Co., St. Louis, MO) at the final concentrations indicated were incubated in 0.1 ml of 0.02 M Tris-HCl containing 0.15 M NaCl and 1 mg/ml polyethylene glycol at pH 7.4 (TS buffer). Thrombin was added last to initiate the reaction. After 1 min, residual thrombin activity was determined by addition of 0.5 ml tosyl-Gly-Pro-Arg-p-nitroanilide (Boehringer Mannheim, Indianapolis, IN) followed by measurement of the rate of change in absorbance at 405 nm.

To determine whether maltodapoh stimulated inhibition of thrombin by HCII or ATIII in plasma ex vivo, aliquots (2.5 μl) of citrated rabbit plasma were incubated in 50 μl of TS buffer for 1 min with several concentrations of maltodapoh and a trace amount of 125I-labeled thrombin (0.3 pmol, ~13,000 cpm) prepared as described previously (Tollefsen and Blank, 1981). 125I-thrombin-HCII and 125I-thrombin-ATIII complexes were then separated by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions and detected by autoradiography as described (Tollefsen et al., 1983).

Animal Preparations. All procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996) and were approved by the Animal Studies Committee at Washington University. New Zealand White rabbits of either sex, weighing 3.0 to 3.8 kg were fasted for 12 to 24 hr and anesthetized with an intramuscular injection of 3.0 to 3.8 kg were fasted for 12 to 24 hr and anesthetized with an intramuscular injection of 50 mg/kg of ketamine and 18 mg/kg of xylazine, supplemented as needed. Body temperature was maintained by placing the animals on a heating pad. A 22-gauge catheter (Surflo, Terumo Medical Co. Elkton, MD) was inserted into a femoral vein or a pediatric feeding tube (Davol, Bard Co., Cranston, RI) was inserted through the mouth and into the stomach for delivery of maltodapoh or PBS vehicle. A catheter was inserted into a femoral artery or a jugular vein for the
In Vivo Experiments. To determine the effect of maltodapoh on coagulation after oral administration, the selected dosage dissolved in 5 ml of PBS was infused as a bolus into the femoral vein. Serial arterial blood samples were withdrawn before and 1, 5, 10, 30, 60 and 120 min after drug infusion for analysis of aPTT.

To determine the effect of maltodapoh on coagulation after oral administration, a baseline blood sample was collected from the jugular vein and the selected dosage dissolved in 5 ml of PBS was injected as a bolus into the stomach. The feeding tube was immediately withdrawn to avoid regurgitation. However, if regurgitation occurred, the experiment was stopped and the rabbit euthanized. Blood samples were collected before and 1, 2, 4, 8, 12 and 24 h after the administration of maltodapoh for analysis of aPTT. Rabbits regained consciousness after 1 to 2 h, and were returned to their cages after 4 h and permitted access to food and water.

To assess the effect of maltodapoh administered orally on in vivo thrombosis, preparations described previously for arterial and venous thrombosis were implemented simultaneously in the same animals (Fujii et al., 1991; Hollenbach et al., 1994). Briefly, after a 24-h fast and induction of anesthesia, an oral dosage of maltodapoh shown to increase aPTT 2-fold or PBS vehicle as a control was administered. For induction of arterial thrombosis, a common carotid artery was exposed and instrumented with a proximal Doppler flow probe and a distal transluminal needle electrode consisting of the tip of a 23-gauge needle crimped on the end of 30-gauge Teflon-insulated silver-coated copper wire (A-M Systems, Everett, WA). To avoid induction of cerebral infarction from embolism with thrombus, an arteriovenous shunt consisting of a 5-cm length of silanized polypropylene tubing (1.57 mm I.D., 2.41 mm O.D) was inserted between the carotid artery and the jugular vein. Mean flow velocity was decreased to 50% of the baseline level by tightening the ligature used to anchor the electrode on either side of the vessel. Five hours after oral administration of maltodapoh, identified as the interval needed to achieve a 2-fold increase in aPTT, electrical injury to the carotid artery was initiated by application of 250 μA of anodal current to the indwelling electrode for 2 h.

For induction of venous thrombosis, a 5-cm silanized polypropylene tube (1.57 mm I.D., 2.41 mm O.D) was advanced via a jugular vein into the superior vena cava. A preweighed 7 cm length of 34-gauge copper wire, looped at one end and with eight, 3-cm strands of cotton thread attached was then pushed through the catheter into the vena cava exposing the threads to the circulation. The catheter was flushed and clamped closed with a hemostat. Initiation of thrombosis was considered to begin at the time the wire with threads was advanced into the vessel, which coincided with the onset of electrical current in the carotid artery. Surgical bleeding time was measured and arterial blood samples were withdrawn before and 5, 6 and 7 h after oral administration of maltodapoh or PBS vehicle for assay of aPTT. After the 7-h sample, the resulting venous thrombi were removed, blotted dry and weighed. The effect of maltodapoh on arterial thrombosis was assessed by measuring the frequency of cyclic flow variations resulting from transient accumulation and dislodgment of platelets at the site of electrical injury. The incidence and time of onset of complete arterial occlusion, if it occurred, were also recorded.

aPTT and Surgical Bleeding Time. aPTT was measured with use of a Coag-A-Mate XM automated coagulation timer (Organon Teknika, Durham, NC). A sample of 100 μl of citrated plasma was warmed for 60 s to 37°C and then 100 μl of prewarmed activator reagent (Automated APTT, Organon Teknika) was added and allowed to incubate for 300 s. Prewarmed 20 mM calcium chloride (100 μl) was then added and aPTT was recorded as the time for clot formation. Surgical bleeding time was assessed by making an incision in the edge of the ear with a scalpel blade and immersing the ear in warmed saline. The time required for bleeding to stop (s) was taken as the bleeding time.

Statistical analysis. Results are expressed as the mean ± S.D. Interval changes in aPTT were compared between maltodapoh-treated and controls by multiple analysis of variance. An unpaired Student’s t test was used to compare the incidence and time of onset of arterial thrombosis and the weight of venous thrombus between the maltodapoh-treated and the control groups. A value of P < .05 was considered significant.

Results

Effects of Maltodapoh on Coagulation Assessed In Vitro. A concentration-dependent increase in aPTT was observed after the addition of maltodapoh to either rabbit or human plasma (Fig. 2). The amount of maltodapoh required to increase aPTT by 2-fold compared to baseline values was approximately four times higher in rabbit plasma (0.2 mg/ml) compared with human plasma (0.05 mg/ml), in part, because the baseline aPTT in rabbit plasma (59 ± 11 s) was nearly twice that of human plasma (37 ± 1 s).

In a buffer assay system, maltodapoh stimulated inhibition
of thrombin by HCII, but was approximately 20 times less active by weight than unfractionated heparin (Fig. 3A). Half-maximal inhibition was observed in the presence of 0.1 and 2 μg/ml of heparin and maltodapoh, respectively. In contrast, maltodapoh did not stimulate the inhibition of thrombin by ATIII over the concentration range of 0.2 to 2000 μg/ml (Fig. 3B). In rabbit plasma incubated with increasing concentrations of maltodapoh and trace amounts of 125I-thrombin, covalent 125I-thrombin-HCII complexes were detected at concentrations ≥10 μg/ml, but no 125I-thrombin-ATIII complexes were detected by SDS-PAGE and autoradiography (Fig. 3C).

**Effects of Maltodapoh on Coagulation Assessed In Vivo.** A 1 mg/kg intravenous bolus of maltodapoh in anesthetized rabbits appeared to represent a threshold dosage for increasing the aPTT 2-fold, with the increase showing some variation but persisting for at least 60 min (Fig. 4). Arterial blood pressure was unchanged throughout the 2-h observation interval in rabbits given the 1 mg/kg dosage (68 ± 12 mm Hg at baseline and 67 ± 4 mm Hg after 2 h, n = 4).

Oral administration of 1 mg/kg of maltodapoh in other rabbits allowed to regain consciousness after removal of the orogastric tube resulted in a nearly 2-fold increase in aPTT by 4 h, which was significant compared with baseline values by 8 h (Fig. 5). After 12 h, aPTT levels had returned toward baseline levels. Increasing the dosage to 3 mg/kg resulted in a nearly 3-fold increase in aPTT by 8 h with a 2-fold increase persisting for at least 24 h. However, increasing the dosage to 5 mg/kg appeared to attenuate the response with only a 2-fold increase in aPTT occurring by 8 h and returning towards baseline by 12 h similar to the response with 1 mg/kg.

**Effect of Maltodapoh on Thrombosis.** The incidence of complete arterial occlusion in the control group was 100% (7 of 7) compared to 71% (5 of 7) in rabbits that received 3 mg/kg of maltodapoh orally (P = NS). However, the average time to occlusion was nearly doubled in maltodapoh-treated animals compared with controls (Table 1). Maltodapoh also decreased the number of cyclic flow variations indicative of transient arterial occlusion in the first hour after induction of thrombosis compared with controls, but the difference was not significant. The number of cyclic flow variations in control animals during the second hour was diminished because most of the arteries were occluded. Venous thrombus weight was decreased significantly in maltodapoh-treated animals compared with controls (Table 1).

Surgical bleeding time was increased from 96 ± 25 s at baseline to 214 ± 74 s by 6 h (2.4-fold) in rabbits given maltodapoh (P = .01). No change in bleeding time was observed in control animals (110 ± 14 s at baseline and 106 ± 6 s at 6 h).

**Discussion**

Our data show that maltodapoh administered orally in rabbits exhibits good anticoagulant and antithrombotic activity. A clinically accepted level for anticoagulation based on a 2- to 3-fold increase in aPTT was achieved within 8 h from the time of oral administration of the compound. Furthermore, with a single oral dose of maltodapoh (3 mg/kg), anticoagulation persisted for at least 24 h (Fig. 5). Arterial and venous thrombosis induced beginning 5 h after administration of maltodapoh were both attenuated with a doubling of the time to occlusion of the carotid artery and a 6-fold reduction in the weight of thrombus in the superior vena cava after 2 h compared with untreated controls (Table 1). Based on the favorable kinetic profile, the efficacy of the agent to inhibit both arterial and venous thrombosis, and the lack of an effect on blood pressure, maltodapoh appears to meet most of the objectives for an orally active anticoagulant.

Anticoagulant effects have been demonstrated for other bis-aldonic acid amides including lactobionic acid (Klauser et al., 1991; Raake et al., 1989). However, until now the activity after oral administration of this class of compounds has not been examined. As with the lactobionic acid amides, maltodapoh is approximately 10-fold less potent than heparin as measured by aPTT in vitro (Toce JA and Anevski PJ, unpublished observations). Nevertheless, in the thrombosis preparations, the activity of maltodapoh appears comparable to that of heparin as well as warfarin, which does not reduce the incidence of thrombosis but retards the growth of thrombi (Foschi et al., 1993).

Similar increases in aPTT (1.5- to 2-fold) were observed when 1 mg/kg of maltodapoh was delivered either intravenously or orally. Interestingly, although the 3 mg/kg oral dosage of maltodapoh yielded an even greater increase in aPTT (2.5- to 3-fold) than the 1 mg/kg dosage, 5 mg/kg in-
duced less marked increases in aPTT levels and a more rapid return toward baseline levels (Fig. 5). This suggests that absorption of the compound was attenuated or that a competitor was formed with the higher dosage. Measurement of pH in the stomach (data not shown) indicated that there were no dose-related changes to account for the diminished anticoagulant effect.

It is not clear from our data whether native maltodapoh or a metabolite is the active moiety. However, based on the 2- to 4-h delay in the onset of action after oral administration, an active metabolite may be formed. Maltase is plentiful in the digestive tract of rabbits making degradation of maltodapoh a likely possibility (Marounek et al., 1995). One factor we noted that affected the level of the anticoagulant response was the duration of fasting. If the rabbits were not fasted 18 to 24 h before the administration of maltodapoh including preventing coprophagy, either no increase in aPTT was observed or the effect was delayed. Thus, conditions for the optimal absorption of maltodapoh as well as its biodistribution will need to be defined in additional studies.

The mechanisms responsible for the anticoagulant/anti-thrombotic activity of maltodapoh are unclear. When maltodapoh was added to rabbit plasma or purified human HCII and ATIII in vitro, it stimulated the inhibition of thrombin by HCII but not by ATIII (Fig. 3). The concentration of maltodapoh required to stimulate HCII activity was actually lower than that required to prolong the aPTT in vitro (cf., Fig. 2). Nevertheless, when $^{125}$I-thrombin was added ex vivo to plasma samples obtained from animals given 3 mg/kg of maltodapoh orally, there was no detectable increase in the

**Fig. 5.** Effect of maltodapoh on aPTT in vivo after oral administration of a bolus in rabbits allowed to regain consciousness. Bars represent the mean and S.D.

**TABLE 1**

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<tr>
<th>Rabbit No.</th>
<th>Time of complete occlusion (min)</th>
<th>Time of reopening$^a$ (min)</th>
<th>Time of reocclusion (min)</th>
<th>CFV (0–1 h)</th>
<th>CFV (1–2 h)</th>
<th>Wet weight thrombus (mg)</th>
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<td></td>
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<td>3 ± 2$^b$</td>
<td>3 ± 2</td>
<td>6.0 ± 2.7$^b$</td>
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CFV, Cyclic flow variations; n/a, not applicable.

$^a$After the first complete occlusion.

$^b$P < .001 compared with control.
rate of formation of $^{125}$I-thrombin-HCII complexes, even though the aPTT of the samples was prolonged (data not shown). This suggests that maltodapoh may prolong the aPTT in vivo by a mechanism that does not involve inhibition of thrombin by HCII. The sulfated bis-lactobionic acid amide, LW 10082, which was initially shown to stimulate HCII activity in vitro (Klauser, 1991), has more recently been found to delay the activation of factor X and prothrombin in plasma (Beguin et al., 1991; Ofosu et al., 1992) and to directly inhibit factor IXa/VIIIa complex (Xase) (Sugidachi et al., 1994). In addition, Klocking et al. (1991) have shown that bis-lactobionic acid amides administered i.v. increase the release of tissue-type plasminogen activator and thereby may exert some antithrombotic effect by enhancement of fibrinolysis. The extent to which these or other mechanisms account for the anticoagulant and antithrombotic effects of maltodapoh will require further study.

Our animal preparations of thrombosis have been described previously, but are particularly well suited for simulating human pathophysiology. The arterial preparation of electrical vascular injury has been shown to evolve platelet-rich thrombus similar to that observed in the coronary artery of patients with acute myocardial infarction (Fuji et al., 1991). The venous thrombosis preparation involving insertion of a thrombogenic copper wire and cotton thread apparatus into the superior vena cava has been shown to evolve a platelet-poor, fibrin and red cell-rich thrombus analogous to that observed in patients with deep venous thrombosis and pulmonary embolism (Hollenbach et al., 1994). Using this approach where blood flow was maintained appeared more physiological than previous preparations involving venous stasis.

In conclusion, we have shown that a novel, highly sulfated bis-maltobionic acid amide exhibits anticoagulant/antithrombotic activity after oral administration comparable to that achieved with available anticoagulants. Maltodapoh appears, however, to have the advantages of rapid onset and moderate duration that may facilitate its use in patients requiring acute anticoagulation. Further testing of this class of compounds appears warranted.

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References


Send reprint requests to: Dr. Dana R. Abendschein, Cardiovascular Division, Washington University School of Medicine, 660 South Euclid Avenue, Box 8086, St. Louis, MO 63110.