Bisphosphonates used for the treatment of bone disorders inhibit squalene synthase and cholesterol biosynthesis

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Abstract Some bisphosphonates used for the treatment of bone disorders are also potent inhibitors of squalene synthase, a critical enzyme for sterol biosynthesis. Among seven drugs tested, YM 175 (cycloheptylaminomethylene-1,1-bisphosphonic acid) was the most potent inhibitor of rat liver microsomal squalene synthase (K_i = 57 nM) and sterol biosynthesis from [^{14}C]mevalonate in rat liver homogenate (IC_{50} = 17 nM). EB 1053 (3-(1-pyrolidino)-1-hydroxypropylidene-1,1-bisphosphonic acid) and PHPBP (3-(1-piperidino)-1-hydroxypropylidene-1,1-bisphosphonic acid) were less potent inhibitors in both these assays. Pamidronate and alendronate were poor inhibitors of squalene synthase (IC_{50} >10 μM) but were potent inhibitors of sterol biosynthesis from mevalonate (IC_{50} = 420 and 168 nM, respectively), suggesting that the latter two agents may have inhibited other enzymes involved in the synthesis of farnesyl pyrophosphate from mevalonate. Etidronate and clodronate were inactive in both these assays. YM 175 also inhibited sterol biosynthesis in mouse macrophage J774 cells (IC_{50} = 64 μM) and in rats, when administered acutely, it inhibited cholesterol biosynthesis in the liver (ED_{50} = 30 mg/kg, s.c.). Structural modifications on YM 175 to enhance cell permeability may result in a new class of cholesterol-lowering agents.—**Amin, D., S. A. Cornell, S. K. Gustafson, S. J. Needle, J. W. Ullrich, G. E. Bilder, and M. H. Perrone.** Bisphosphonates used for the treatment of bone disorders inhibit squalene synthase and cholesterol biosynthesis. *J. Lipid Res.* 1992. **33:** 1657–1663.

Supplementary key words YM 175 • pamidronate • alendronate • etidronate • clodronate • J774 cells

Bisphosphonates are compounds containing a P-C-P bond and are thus related to pyrophosphate (P-O-P), but are resistant to metabolic degradation. The bisphosphonates are well known inhibitors of bone resorption and as such are used for the treatment of several bone disorders (e.g., osteoporosis, Paget's disease, tumor osteolysis) (1–4). The mechanism of action of bisphosphonates is not clear. More than one mode of action may be involved, e.g., inhibition of osteoclast differentiation, osteoclast cytotoxicity, inhibition of mineral matrix dissolution. Results of studies on cultured cells have shown that bisphosphonates are taken up by bone cells, and a variety of intracellular effects have been reported including effects on cell number, production of lactic acid, alkaline phosphatase activity, fatty acid production, collagen and proteoglycan synthesis, PGE_2 production, and resorption of bone particles by macrophages (see reviews 1, 5, 6). We report here that some bisphosphonates used for the treatment of bone disorders are also potent inhibitors of squalene synthase, a critical enzyme for cholesterol biosynthesis.

EXPERIMENTAL PROCEDURES

Materials

The following compounds were synthesized by medicinal chemists at Rhône-Poulenc Rorer: YM 175 (cycloheptylaminomethylene-1, 1-bisphosphonic acid; other name used: CAMBP); EB 1053 (3-(1-pyrolidino)-1-hydroxypropylidene-1, 1-bisphosphonic acid); PHPBP (3-(1-piperidino)-1-hydroxypropylidene-1, 1-bisphosphonic acid, patent No:4871720, Assignee: Ciba-Geigy Corporation, 1989); pamidronate (3-amino-1-hydroxypropylidene-1, 1-bisphosphonic acid, APD, AHPrBP); alendronate (4-amino-1-hydroxybutylidene-1, 1-bisphosphonic acid, ABP,

Abbreviations: YM 175, cycloheptylaminomethylene-1, 1-bisphosphonic acid; EB 1053, 3-(1-pyrolidino)-1-hydroxypropylidene-1, 1-bisphosphonic acid; PHPBP, 3-(1-piperidino)-1-hydroxypropylidene-1, 1-bisphosphonic acid; pamidronate, 3-amino-1-hydroxypropylidene-1, 1-bisphosphonic acid; alendronate, 4-amino-1-hydroxybutylidene-1, 1-bisphosphonic acid; etidronate, 1-hydroxyethylidene-1, 1-bisphosphonic acid; clodronate, disodium dichloromethylidene-1, 1-bisphosphonic acid; FFA, free fatty acids; FPP, farnesyl pyrophosphate.

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AHBuBP); etidronate (1-hydroxyethylidene-1, 1-bisphosphonic acid, HEBP, EHDP); clodronate (disodium dichloromethylidene-1, 1-bisphosphonic acid, Cl_2MBP); FPP.

Sodium [^{14}C]octanoate (NEC-092H, 55 Ci/mol), sodium [^{14}C]acetate (NEC 084H, 59 Ci/mol), [^{14}C]mevalonolactone (NEC-679, 50 Ci/mol), [^{3}H]squalene (NET-645, 29 Ci/mmol), [^{3}H]FPP (NET-1042, 20 Ci/mmol), [^{3}H]cholesterol (NET-139, 50 Ci/mmol) and Aquasol-2 were purchased from DuPont/NEN, Boston, MA. Cholesterol, lanosterol, squalene, octanoate, acetate, DL-mevalonic acid lactone, ATP, and NADPH were purchased from Sigma Chemical Co., MO. Petroleum ether, chloroform, and ethanol were purchased from Fisher Scientific Co., Fair Lawn, NJ.

Methods

Microsomal enzyme preparation. Fresh livers from male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 150–200 g were collected after exsanguination. All subsequent procedures were performed at 4°C. The liver was homogenized in phosphate buffer (50 mM, pH 7.4). Cellular fractions (10,000 *g* supernatant and microsomes) were separated as described by Popjak (7). The 10,000 *g* supernatant was aliquoted and stored at −80°C. Microsomes were prepared by two consecutive centrifugations (100,000 *g*) and then resuspended in the phosphate buffer. Microsomes were rehomogenized with a motor-driven Teflon pestle to yield a uniform suspension (15–30 mg protein/ml), aliquoted, and stored at −80°C until use. No loss in the enzyme activity was observed over a 2-month period.

Squalene synthase assay. The procedure was a modification of that described earlier (7, 8). The assay was performed in 1 ml of 50 mM phosphate buffer, pH 7.4, containing 10 mM $MgCl_2$, 0.5 mM NADPH, microsomes (8 μg protein), a bisphosphonate dissolved in distilled water, and substrate [^{3}H]FPP (0.5 μM, 0.27 Ci/mmol) in a 16 × 125 mm glass screw-cap tube. All components except [^{3}H]FPP were preincubated for 10 min at 37°C. The reaction was initiated by the addition of [^{3}H]FPP. After 10 min at 37°C, the reaction was terminated by the addition of 1 ml 15% KOH in ethanol. The tubes were incubated at 65°C for 30 min to solubilize proteins. The mixture was extracted with 5 ml petroleum ether for 10 min. After freezing the lower aqueous phase, the organic phase was transferred to glass tubes containing 2 ml distilled water. After washing, the lower aqueous phase was frozen and the petroleum ether phase was removed and counted with 10 ml Aquasol using a Beckman LS-9000 scintillation counter. DPM values were adjusted against a blank (no enzyme). The squalene synthase reaction was linear under the above assay conditions.

For kinetic studies, microsomes were incubated for 5 min with [^{3}H]FPP (0.7 Ci/mmol). After terminating the reaction, squalene (10 μg) was added. Other procedures were similar as described above. Extraction efficiency with an internal standard [^{3}H]squalene was 95%.

In some experiments internal standards cholesterol, lanosterol, and squalene (10 μg each) were added after terminating the reaction. Products were extracted in petroleum ether and then evaporated to dryness under N_2. The residue was resuspended in 20 μl chloroform and spotted on plastic silica gel thin-layer plates (Kodak 13181). The plates were eluted with chloroform. Product(s) were determined by scanning with a Bioscan System 200 Imaging Scanner. After visualization with iodine, the lipid spots were cut out and counted with Aquasol. Over 95% of the radioactivity was present in a peak corresponding to squalene (R_f 0.81).

Inhibition of sterol biosynthesis in a cell-free system. The assay procedure was similar to that described by Popjak (7). The assay was performed in 1 ml of oxygenated phosphate buffer (50 mM, pH 7.4) containing 4 mM ATP, 10 mM $MgCl_2$, 0.5 mM NADPH, a bisphosphonate dissolved in distilled water, 150 μg rat liver microsomal protein, 2 mg supernatant fraction from the 10,000 *g* centrifugation of liver homogenate, and [^{14}C]mevalonic acid (500 μM, 0.4 Ci/mol) in a 16 × 125 mm glass screw-cap tube. The tube containing all components except [^{14}C]mevalonate was incubated for 10 min at 37°C. The reaction was initiated by the addition of [^{14}C]mevalonate. The tubes were flushed with 95% O_2/ 5% CO_2, capped, and incubated at 37°C in a metabolic shaker at 120 oscillations/min for 90 min. The reaction was stopped by addition of 1.0 ml 15% KOH in ethanol and internal standards cholesterol, lanosterol, and squalene (10 μg each) were added. The tubes were saponified at 75°C for 2 h and extracted with 5 ml of petroleum ether for 10 min. The lower aqueous phase was frozen in a dry ice/alcohol mixture, and the petroleum ether phase was removed, washed with 2 ml of distilled water, and then evaporated to dryness. The ^{14}C-labeled sterols synthesized were separated by thin-layer chromatography on plastic silica gel plates (Kodak 13181) and processed as described above.

Inhibition of cholesterol biosynthesis in J774 cells. J774 cells, a monocyte-macrophage cell line derived from a mouse tumor cell, were obtained from ATCC (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics (100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B). Cells were seeded at a concentration of 2 × 10^6 cells/35 mm. After 3 days, the media was replaced with 1 ml serum-free media. Eighteen hours later, YM 175 dissolved in saline was added, followed 2 h later with [^{14}C]octanoate (300 μM, 4 μCi/well). The amount of sterols synthesized in the next 2 h was determined as described earlier. Two sterol products were observed (61% in cholesterol and 39% in lanosterol). Results are presented as inhibition in total

sterol synthesis compared to the vehicle-treated control value.

Ex vivo inhibition of cholesterol biosynthesis in rat livers. The assay was similar to that described earlier by Endo et al. (9). YM 175 was dissolved in saline and administered subcutaneously to male Sprague-Dawley rats (200–250 gm). After 1 h, rats were killed, livers were removed and transferred to oxygenated Krebs-Ringer-bicarbonate buffer (pH 7.4). Livers were chopped into 0.8-mm^2 slices using a McIlwain tissue slicer (Brinkmann Instruments, Westbury, NY), and suspended in the same buffer. Aliquots of the suspension containing about 100 mg tissue were pipetted, in triplicate, into culture tubes that contained [^{14}C]sodium octanoate (300 μM, 6.67 Ci/mol) or [^{14}C]sodium acetate (1 mM, 2 Ci/mol). The assay volume was 1 ml. The tubes were gassed with 95% O_2/5% CO_2 for 10 sec, stoppered with a serum cap, and incubated at 37°C in a metabolic shaker at 150 oscillations/min for 90 min. The reaction was stopped by addition of 1.0 ml 15% KOH in ethanol and an internal standard [^{3}H]cholesterol (30,000 dpm) was added to determine recovery, which ranged from 70 to 80%. Radioactivities in various lipid spots were quantitated as before. Over 90% of the total radioactivity was present in the cholesterol spot. Results are presented as percent inhibition of cholesterol biosynthesis compared to vehicle-treated control value. In one experiment, the residue remaining after the ether extractions was acidified, and ^{14}C-labeled fatty acid synthesized was extracted with petroleum ether and counted as described earlier (10).

Statistical analyses. The IC_{50} and the K_i values were calculated using a computer program by Tallarida and Murray (11). In the rat ex vivo assay, drug-treated values were compared with the vehicle-treated values as group data (11).

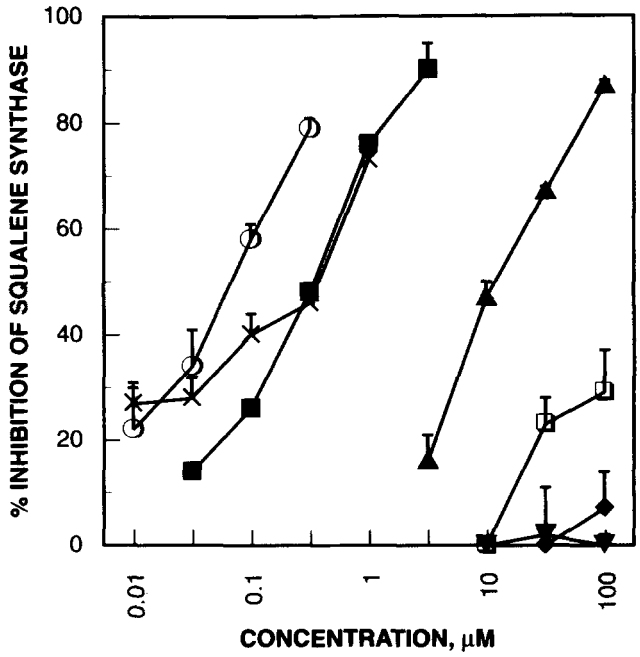

Fig. 2. Inhibition of rat liver microsomal squalene synthase by various bisphosphonates. Rat liver microsomal enzyme (8 μg protein) and FPP (0.5 μM) were incubated in phosphate buffer (50 mM, pH 7.4) for 10 min at 37°C. Results are presented as mean percent inhibition (± SEM, n = 3) of squalene synthesis by various bisphosphonates: YM 175 (○), EB 1053 (X), PHPBP (■), pamidronate (▲), alendronate (□), etidronate (▼), and clodronate (◆). The calculated IC_{50} values are shown in Table 1.

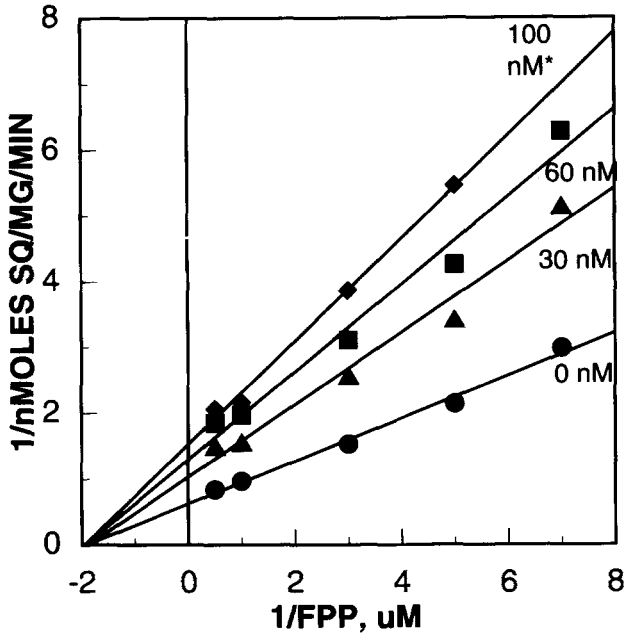

Fig. 1. Lineweaver-Burk analysis of the inhibition of rat microsomal squalene synthase by YM 175. Each point represents mean of duplicate determination. Various concentrations of YM 175 were used (*). Computer-generated regression lines for the data points are shown. The calculated K_m for FPP was 0.5 μM and the K_i for YM 175 was 57 nM.

RESULTS

Inhibition of rat microsomal squalene synthase

The K_m value for squalene synthase as determined by a double reciprocal plot was 0.5 μM FPP (**Fig. 1**). Inhibition of squalene synthase by various bisphosphonates is shown in **Fig. 2,** and calculated IC_{50} values are summarized in **Table 1.** The most potent bisphosphonate was YM 175 (IC_{50} = 64 nM). YM 175 was a noncompetitive inhibitor of squalene synthase (Fig. 1) with a calculated K_i value of 57 nM. EB 1053 and PHPBP were less potent inhibitors (IC_{50} = 208 and 311 nM, respectively). Pamidronate and alendronate were very poor inhibitors while etidronate and clodronate were inactive.

TABLE 1. Inhibition of squalene synthase and sterol biosynthesis by various bisphosphonates

$$H_2O_3P\text{-}\overset{R'}{\underset{R}{C}}\text{-}PO_3H_2$$

Drug	Structure R	Structure R'	Squalene Synthase	Sterol Biosynthesis
YM 175	H	NH⟨⬡⟩	64 nM	17 nM
EB 1053	HO	CH_2CH_2N⟨⬠⟩	208 nM	113 nM
PHPBP	HO	CH_2CH_2N⟨⬡⟩	311 nM	74 nM
Pamidronate	HO	$CH_2CH_2NH_2$	12,300 nM	420 nM
Alendronate	HO	$(CH_2)_3NH_2$	29%/100 μM	168 nM
Etidronate	HO	CH_3	0%/100 μM	0%/10 μM
Clodronate	Cl	Cl	7%/100 μM	0%/10 μM

Inhibition of rat microsomal squalene synthase (Fig. 2) and inhibition of sterol biosynthesis from [^{14}C]mevalonate in a cell-free system (Fig. 3) are shown as IC_{50} (nM) or % inhibition.

Inhibition of cholesterol biosynthesis in a cell-free system

Inhibitors of squalene synthase were also potent inhibitors of cholesterol and other sterol biosynthesis from radiolabeled mevalonate in a cell-free system. The IC_{50} values for YM 175, EB 1053, and PHPBP for the inhibition of total sterols were 17, 113, and 74 nM, respectively (**Fig. 3,** Table 1). Alendronate and pamidronate were relatively poor inhibitors of squalene synthase, but were relatively more potent inhibitors of sterol biosynthesis (IC_{50} = 168 and 420 nM, respectively). Etidronate and clodronate were inactive (0% inhibition at 10 μM).

Four products were observed in the petroleum ether extract: cholesterol (R_f 0.27, 5-10% of total sterols), lanosterol (R_f 0.42, 7-18%), a peak near squalene (probably squalene epoxide, R_f 0.73, 15-30%) and squalene (R_f 0.81, 50-70%). Similar extent of inhibition in these products was observed with each bisphosphonate (results not shown). This indicates that the inhibition occurred prior to the synthesis of squalene.

Inhibition of cholesterol biosynthesis in J774 cells

YM 175 completely inhibited sterol biosynthesis at 300 μM concentration (**Fig. 4**) with a calculated IC_{50} value of 64 μM. Similar IC_{50} values were also observed in two other experiments with slightly different assay conditions (results not shown). The inhibition in sterol biosynthesis was not associated with cytotoxicity as determined by protein determination and by trypan blue stain.

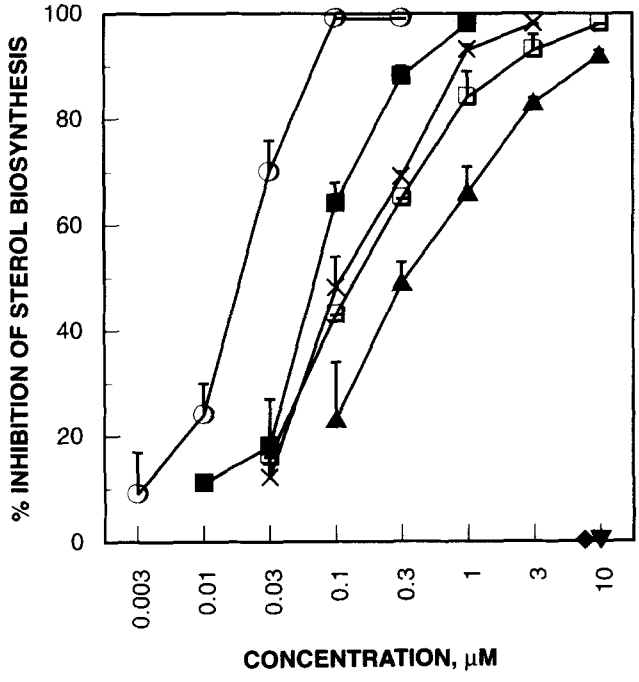

Fig. 3. Inhibition of sterol biosynthesis by bisphosphonates in a cell-free system. Drugs were incubated with rat liver microsomal and cytosolic fractions, ATP, $MgCl_2$, NADPH, and [^{14}C]mevalonate. The amount of four sterols synthesized in 90 min was determined (see Methods). Results are presented as mean percent inhibition of total sterols synthesized ± SEM (n = 3) in the presence of various bisphosphonates: YM 175 (○), EB 1053 (X), PHPBP (■), pamidronate (▲), alendronate (□), etidronate (▼), and clodronate (♦). Calculated IC_{50} values are presented in Table 1.

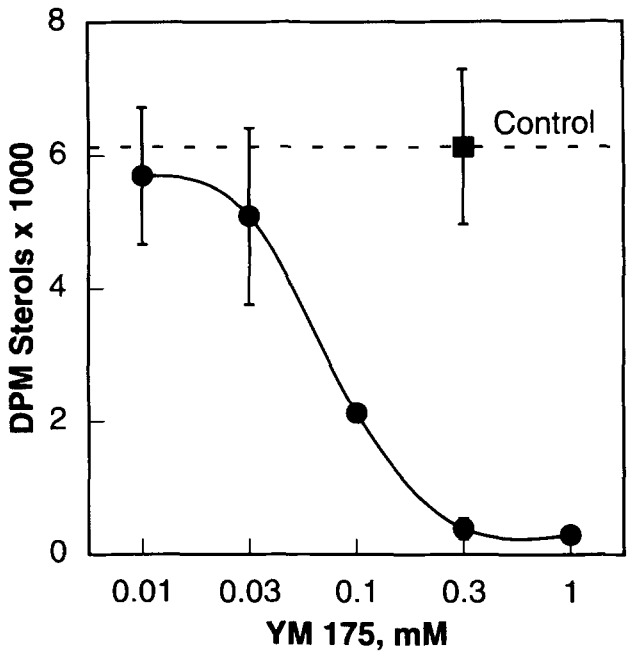

Fig. 4. Inhibition of cholesterol biosynthesis in J774 cells. Mouse macrophage J774 cells were incubated with YM 175 at the indicated concentrations. Two hours later, [^{14}C]octanoate was added. The amount of cholesterol and lanosterol synthesized in next 2 h was determined. Total sterol synthesis in the saline-treated wells (■) and in the YM 175-treated wells (●) are shown as mean ± SEM, n = 4.

Inhibition of cholesterol biosyntheses in rats

YM 175 administration resulted in a significant inhibition of cholesterol biosynthesis in the liver with an estimated ED_{50} value of 30 mg/kg, s.c. (**Table 2**). After 100 mg/kg, s.c., sufficient quantity of the drug was present in the liver to cause a complete inhibition (98%) of cholesterol biosynthesis. As we previously observed (10) for a selective HMG-CoA reductase inhibitor, lovastatin, YM 175 selectively inhibited the cholesterol biosynthesis and the free fatty acid (FFA) synthesis was not affected (Table 2).

Octanoate and acetate are precursors for cholesterol biosynthesis. Externally added acetate does not reach the mitochondrial pool. However, octanoate is rapidly oxidized intramitochondrially to acetyl-CoA, which feeds both the intra- and extramitochondrial metabolic pathways (12). Similar inhibition in the cholesterol biosynthesis was observed with YM 175 when octanoate or acetate was used as a substrate (Table 2). This indicates that YM 175 did not inhibit the oxidation of octanoate to acetyl-CoA in the cell.

DISCUSSION

This study shows that some bisphosphonates are potent inhibitors of squalene synthase. YM 175 is the most potent inhibitor, which is 50-fold more potent than other known squalene synthase inhibitors with IC_{50} values of 3–12 μM (8, 13, 14).

YM 175 appears to inhibit squalene synthase by a different mechanism compared to FPP-mimic inhibitors as described by Biller et al. (13, 14). Bisphosphonates are potent ion chelators and thus may chelate Mg^{2+}, a cofactor for the squalene synthase reaction. One may speculate that YM 175 may interfere with the action of Mg^{2+}. This seems unlikely as the concentration of Mg^{2+} in our assay was 2×10^5 times higher than the IC_{50} value for YM 175. A second possibility is that bisphosphonates may mimic the pyrophosphate of FPP to inhibit squalene synthase. One would expect that all bisphosphonates should have a similar ion-chelating or pyrophosphate-mimicking property. However, we observed that only some bisphosphonates were potent inhibitors. FPP-mimic inhibitors are competitive inhibitors of squalene synthase (13, 14), while YM 175 was a noncompetitive inhibitor of this enzyme. Thus, YM 175 binds to a site on the enzyme other than the site for the attachment of FPP. Squalene synthase is a two-step reaction. It is possible that YM 175 may mimic

TABLE 2. Ex vivo inhibition of cholesterol biosynthesis in rats

YM 175 (mg/kg)	Assay I (n = 9)	Assay II (n = 8)	Assay III	
			Cholesterol (n = 9)	FFA (n = 9)
0	20912 ± 4938	19987 ± 2618	7053 ± 462	35360 ± 3533
30		9155 ± 2171[a] (54% I)	3015 ± 323[a] (57% I)	32794 ± 1618 (7% I)
100	325 ± 87[a] (98% I)			

YM 175 was injected subcutaneously in rats. One hour later, the liver was removed. Cholesterol and FFA biosynthesis from radiolabeled octanoate (Assay I and Assay II, 300 μm, 6.67 Ci/mol) or acetate (Assay III, 1 mM, 2 Ci/mol) in the liver slices was determined as described in Methods. Results are presented as mean lipid synthesis (dpm/mg protein per h ± SEM) and as percent inhibition (% I) compared to respective mean control value; n = number of rats used for each treatment.

[a] $P < 0.05$ compared to the respective control values.

the carbocationic intermediates (8) in the second step of the squalene synthase reaction. Poulter et al. (8) have shown inhibitory activity of three ammonium analogs designed to mimic the electrostatic and topological properties of the proposed intermediates. Our data suggest that the aliphatic side chain containing a positively charged amine plays a role in enzyme inhibition. Among the bisphosphonates tested (Table 1), drugs without an amine chain, e.g., clodronate and etidronate, were not inhibitors of squalene synthase. The primary amines pamidronate and alendronate were poor inhibitors. In contrast, the bisphosphonates with an aliphatic substitution containing secondary and tertiary amines were potent inhibitors of squalene synthase. Thus, it seems reasonable to conclude that the amine side chain on the bisphosphonate plays a very important role in the inhibition of squalene synthase. Studies are in progress to determine the site of inhibition by YM 175.

Some bisphosphonates (e.g., pamidronate, alendronate) were more potent inhibitors of sterol biosynthesis in comparison to their relative squalene synthase inhibitory activity (Table 1). The reason for this difference is not known. One possible explanation is that some bisphosphonates may have inhibited other enzyme(s) involved in the synthesis of FPP from mevalonate.

The mechanism(s) by which bisphosphonates inhibit bone resorption is unknown but may include the inhibition of osteoclast differentiation or recruitment or toxic elimination of osteoclasts (15–17). Bisphosphonates, when added in the presence of bone mineral, are cytotoxic to macrophages (18). We demonstrated that some bisphosphonates are potent inhibitors of sterol biosynthesis in a cell-free system and YM 175 inhibited cholesterol biosynthesis in mouse macrophage J774 cells. In vivo, YM 175 is expected to accumulate in bone phagocytic cells. As complete inhibition of sterol biosynthesis results in cell death (19, 20), phagocytic accumulation of bisphosphonates in osteoclasts may lead to complete inhibition of sterol biosynthesis and may contribute to cytotoxicity. There appears to be a relationship between the sterol biosynthesis inhibitory activity (Table 1) and the effectiveness for inhibition of bone resorption. For example, YM 175 was 10 times as potent as pamidronate and 100 times as potent as etidronate (21). EB 1053 was 50 times more potent as an inhibitor of bone resorption compared to pamidronate (22). The relative potencies for inhibition of bone resorption in rat were alendronate > pamidronate > clodronate (17). Pamidronate was more cytotoxic to osteoclasts compared to etidronate and clodronate (15). In general, potent inhibitors of sterol biosynthesis were also potent inhibitors of bone resorption. One should be cautious, however, in over-simplification of these data as more than one mode of action is believed to be involved in inhibition of bone resorption (6, 23) and clodronate, which is not a sterol biosynthesis inhibitor, is an effective cytotoxic agent (24). Future research might be best directed towards elucidating the relationship between inhibition of sterol biosynthesis in bone cells and the possible cytotoxicity observed in osteoclasts by various agents.

YM 175 inhibited cholesterol biosynthesis in the liver after subcutaneous administration to rats. To our knowledge, this is the first demonstration that a squalene synthase inhibitor reduced cholesterol biosynthesis in vivo. In general, bisphosphonates are highly charged molecules and are poorly absorbed when given orally (25). Thus, the therapeutic utility of the bisphosphonate class of squalene synthase inhibitors as cholesterol-lowering agents is questionable. Structural modification of YM 175 to make phosphonates more lipophilic and liver-selective may result in a new class of cholesterol-lowering agents.

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