Brief Articles

Osteoadsorptive Bisphosphonate Derivatives of Fluoroquinolone Antibacterials

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Bisphosphonates conjugated to fluoroquinolone antibacterials through an intermediate carbon had better activity than conjugates lacking the carbon. Virtually all molar-based activity of these esterified bisphosphonate derivatives was identical to that of its parent. De-esterified free-acid forms retained good activity against most Gram-negative bacteria, but not against Gram-positives. A free-acid derivative remained bound to washed bone and completely inhibited *Staphylococcus aureus* growth. The more potent parent, ciprofloxacin, failed to bind significantly, and bacterial growth occurred.
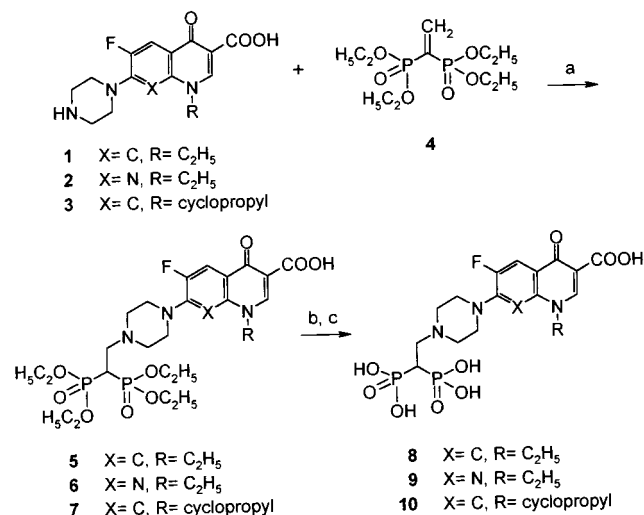
Introduction

Bisphosphonates, carbon analogues of pyrophosphate, strongly chelate metal ions and adsorb to bone.[1] They have been used to treat osteoporosis[2] and as radio-imaging agents[3] and have reduced metastases in breast cancer.[4]

The introduction of a bisphosphonate moiety into pharmacologically active molecules, like fluoroquinolone antibacterial drugs, may enhance their ability to bind to, concentrate in, and/or be retained by infected bone, a site often difficult to treat clinically.[5] Their excellent pharmacological distribution make the fluoroquinolones the drug of choice in treatment of many chronic infections. For example, ciprofloxacin's broad-spectrum bactericidal activity affects many pathogens, including methicillin-susceptible *Staphylococcus aureus* and *Pseudomonas aeruginosa,*[6] both associated with osteomyelitis.[5,7] In this study, the bisphosphonate derivatives of three fluoroquinolone antibacterials, norfloxacin[8] (compound **1**), enoxacin[9] (compound **2**), and ciprofloxacin[10] (compound **3**), were prepared.[11]

Results

The biological properties of modified fluoroquinolone antibacterials were investigated by comparing derivatives in which bisphosphonate and fluoroquinolone moieties were linked with or without an intermediate carbon, i.e., positioned between the nitrogen of the fluoroquinolone's piperazine group and the carbon of the bisphosphonate. For compounds linked by one carbon,

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Scheme 1. Synthesis of Bis-phosphono-ethyl Derivatives (**5**−**10**) of Fluoroquinolone Antibacterials: Norfloxacin (**1**), Enoxacin (**2**), and Ciprofloxacin (**3**)[a]

1   X= C, R= C$_2$H$_5$
2   X= N, R= C$_2$H$_5$
3   X= C, R= cyclopropyl

5   X= C, R= C$_2$H$_5$
6   X= N, R= C$_2$H$_5$
7   X= C, R= cyclopropyl

8   X= C, R= C$_2$H$_5$
9   X= N, R= C$_2$H$_5$
10 X= C, R= cyclopropyl

[a] Reaction conditions: (a) N(C$_2$H$_5$)$_3$; (b) (CH$_3$)$_3$SiBr; (c) H$_2$O.

compounds **1**, **2**, or **3** were allowed to react with tetraethyl ethenylidenebisphosphonate[12] (compound **4**) in dichloromethane in the presence of triethylamine resulting in bis-(diethoxy-phosphoryl)-ethyl derivatives **5**, **6**, or **7**, respectively (Scheme 1). The ester groupings were then removed by bromotrimethylsilane and subsequent hydrolysis,[13] giving rise to compound 8, 9, or 10, respectively, as hydrobromide salts. When the linkage between the bisphosphonate and the fluroquinolone lacked a carbon, a bis-phosphonomethyl moiety was introduced into compound **1**, **2**, and **3**. By allowing them to react with a mixture of triethyl orthoformate and diethyl phosphite at 145 °C,[14] compounds **11**, **12**, and **13**, respectively, were obtained (Scheme 2, Supporting Information). Bromotrimethylsilane-mediated hydrolysis of ester groups resulted in the free bisphos-

Table 1. Comparison of MIC Activity (μg/mL) of **3** (ciprofloxacin) and Its Bis-phosphonoethyl (**7** and **10**) and Bisphosphonomethyl Derivatives (**13** and **16**)

| bacterial strain	**3**	**7**	**10**	**13**	**16**
S. aureus ATCC 12598	2.0	1.0	8.0−16	nd	nd
S. aureus ATCC 25923	1.56	3.12	8.0−16	12.5	50.0
S. epidermidis ATCC 110001	0.78	0.78	25.0	6.25	25.0
S. faecalis OKI 80171	3.12	3.12	50.0	50.0	100
S. typhi OKI 10084	<0.39	<0.39	0.78	0.78	0.78
E. coli OKI 35034	<0.39	<0.39	0.78	0.78	<0.39
P. vulgaris OKI 60002	<0.39	<0.39	<0.39	25.0	6.25
Ps. aeruginosa ATCC 27853	3.12	3.12	25.0	>100	25.0
H. pylori HIH[a] 27604	0.78	0.78	3.12	25.0	12.5
H. pylori HIH[a] 27606	<0.39	<0.39	0.78	25.0	12.5
A. actinomycetemcommitans ATCC 29527	<0.25	<0.25	1.0−2.0	nd	nd

[a] Provided by the Hungarian Institutes of Health.

phonic acids derivatives, compounds **14**, **15**, or **16**, as hydrobromide salts. The structures of all compounds were confirmed by FAB or ESP mass spectrometry as well as ^{1}H and ^{13}C NMR spectra.

Effect of Conjugating Bisphosphonate to Fluoroquinolone Antibacterials. Scheme 1 outlines the synthesis employed to convert a fluoroquinolone to its bisphosphonate ethyl ester and free bisphosphonic acid derivatives. Table 1 compares the antibacterial activity of one such parent with that of its derivatives. Of the parent antibacterials, compounds **1**, **2**, and **3**, compound **3** (ciprofloxacin) was the most active against both Gram-positive and Gram-negative microorganisms. This pattern persisted with the bis-(diethoxy-phosphoryl)-ethyl derivatives, compounds **5**, **6**, and **7**, and the bis-(diethoxy-phosphoryl)-methyl derivatives, compounds **11**, **12**, and **13**. Furthermore, with one exception (compound **5** vs *Escherichia coli*), comparing the ester compounds **5**, **6**, and **7** to their respective parent compound and correcting for the increased molecular weight (approximately twice that of the parent), there was no diminution of activity of any of the bis-(diethoxy-phosphorly)-ethyl ester derivatives of compounds **1**, **2**, and **3**. The most active derivatives were compounds **7** and **10**. These analogues and their parent, compound **3**, were chosen for further biological characterization.

On a molar basis, direct linkage, i.e., without a carbon linker between the bisphosphonate and fluoroquinolone, resulted in an increase, a decrease in a few cases, or no change in the minimum inhibitory concentration (MIC).

Testing of Compounds 3, 7, and 10 versus Clinical Isolates. Methicillin-sensitive clinical isolates (MSSA, $n = 29$) of *S. aureus* had MIC values (median ± SEM) of 1.0 ± 0.1, 2.0 ± 0.3, and 32.0 ± 2.9 μg/mL for compounds **3**, **7**, and **10**, respectively ($p < 0.001$). In the case of methicillin-resistant strains (MRSA), $n = 16$, of *S. aureus*, all of them exhibited resistance against all three compounds (MIC = >8.0 μg/mL). The MIC$_{90}$ of compounds **3**, **7**, and **10** for MSSA were 1.65, 3.91, and 51.3 μg/mL, respectively. Minimum bactericidal activity (MBC) of all three compounds were found to be the same as the MIC. Among laboratory strains, resistance against compound **10** was identified among two of three Gram-positive strains, but only one of five Gram-negative strains ($p = 0.22$, Fisher's Exact). Seven of eight strains (two Gram-positives and five Gram-negatives) were similarly susceptible to compounds **3** and **7**, with MIC values below 2.0 μg/mL. Removal of the one carbon link between the bisphosphonate and the fluoroquinolone moieties did not improve the antibacterial properties of
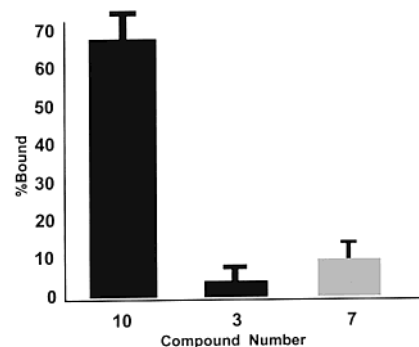

Figure 1. Compounds **3**, **7**, and **10** were added to porcine tibial bone powder, and percent binding was determined by subtracting % free cps (supernatant) from controls without bone (total cps).

conjugates. The free acid form of most compounds yielded less antibacterial activity.

MIC values for compounds **3**, **7**, and **10** against clinical isolates of *E. coli* ($n = 18$) were 0.03 ± 0.01, 0.06 ± 0.1, 1.0 ± 0.9 μg/mL, respectively. All 18 isolates were sensitive to compounds **3** and **7**, while 17 of 18 were sensitive to compound **10**. Compound **4** lacked antibacterial activity (128 ug/mL).

Affinity for Bone. Compound **10** bound strongly to porcine bone powder in slurries (Figure 1). Compounds **3** and **7** bound to bone poorly ($4 \pm 1\%$ and $10 \pm 3\%$, respectively, $p < 0.01$) and were similar to one another ($p = 0.51$). Binding of compound **10** to porcine bone was unaffected by lowering the pH from 8.5 to 7.2, by substituting autoclaved bone for nonautoclaved bone, by placement in rat plasma, and by removal of protein by TCA extraction before measuring drug-associated fluorescence. EDTA (100 mM) blocked the binding of compound **10** to porcine bone and was reversed by high levels of CaCl$_2$ (750 mM), H$_3$PO$_4$ (10 mM), and 3% trichloracaetic acid (data not shown). Compound **10** also bound to rat and human bone.

Effect on Bacterial Growth after Binding to Bone. An experiment was conducted to test the antibacterial activity of compound **10** after it bound to bone tissue. Compounds **3** and **10** at concentrations 2-fold above their respective MICs were incubated with bone. Following incubation, the bone slurries were washed, and *S. aureus* was added. Figure 2 shows that compound **3** only slowed bacterial growth, while compound **10**, which bound saturarly (Figure 3, Supporting Information), completely inhibited the growth of *S. aureus*.
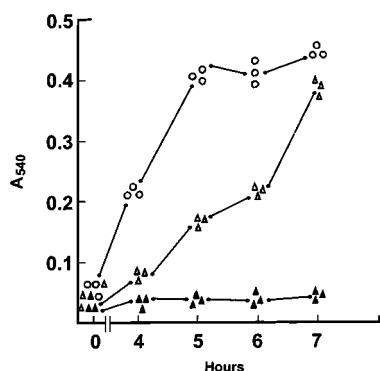
Figure 2. Compounds **3** or **10** was added to bone powder, vortexed, and washed three times. A culture of *S. aureus* was added and bacterial growth monitored turbidimetrically. Top, *S. aureus* only; middle, *S. aureus* plus compound **3**; bottom, *S. aureus* plus compound **10**.

Fluorescence Characterization. Equimolar amounts of compounds **3**, **7**, and **10** produced identical fluorescent spectra (Figure 4, Supporting Information).

Discussion

We have synthesized a new family of antibacterials in which bone-binding bisphosphonate groups were conjugated to potent antibacterial drugs. These compounds have the ability to bind to bone and to inhibit the growth of bacteria and may offer additional treatment options for high concentration targeting of an antibacterial to the site of a pathogen.[15]

With a single exception, despite an approximate doubling of the molecular weight relative to the parent, the molar-based antibacterial activity of those esterified conjugates linked by a carbon between the two moieties was unaffected. However, when de-esterfied, the MICs of these compounds against all Gram-positives were increased significantly, but against most Gram-negatives, the parent activity was retained.

The ability of compound **10** to bind to washed bone powders compensated for its reduced antibacterial activity and proved superior to its nonbinding parent, compound **3**, in inhibiting the growth of *S. aureus*. Recently, it was shown that the binding of compound **10** to calcium beads (Skelite, Millenium Biologix, Kingston, Ontario) was sensitive to acid pH.[16] Because local infections acidify the environment, it is likely that the growth of *S. aureus* was inhibited by compound **10** after its release from bone powder by an acid pH environment. Saturation data (Figure 3) showed that 80% or ca. 30 μg/100 mg powder of E-41 bound to bone powder (2-4 X MIC of E-41 vs *S. aureus*).

Experimental Section

Chemistry. Solvents were distilled before use. Organic extracts were dried over magnesium sulfate. Solutions were concentrated at 35−40 °C (bath) at ca. 17 mmHg. Melting points were determined in capillary tubes and are reported uncorrected. For thin-layer chromatography, precoated aluminum-backed plates (silica gel 60 F254, Merck, layer thickness: 0.2 mm) were used. Compounds were visualized by spraying with 7% ammonium molybdate in 5% sulfuric acid and heating. ^{1}H NMR spectra: Bruker WP 200 SY (200 MHz) and Bruker Avance DRX-500 (500 MHz) instruments; tetramethylsilane as internal standard. ^{13}C NMR spectra were recorded on Bruker WP 200 SY (50 MHz) and Bruker Avance DRX-500 (125 MHz) instruments. Fast atom bombardment

(FAB) mass spectra were measured on a VG-7070 spectrometer (matrix: glycerol; gas: xenon). Electrospray (ESP) mass spectrometric measurements (positive and negative ion detections) were run on a FINNIGAN TSQ 7000 triple quadrupole mass spectrometer equipped with API source; samples were introduced into 50% methanol solution containing 0.1% acetic acid.

General Method for Preparation of Compounds 5, 6, and 7. To a solution of compound **1**, **2**, or **3** (2 mmol) in dry dichloromethane (20 mL) were added triethylamine (2 mmol) and compound **4** (3 mmol). The reaction mixture was stirred for 3 h at room temperature then diluted with 20 mL of solvent, washed with water, dried, and evaporated to dryness. The residue was recrystallized from toluene, yielding compounds **5**, **6**, or **7**, respectively.

7-(4-(2,2-Bis-(diethoxy-phosphoryl)-ethyl)-piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (Compound 7): yield 75%; mp 117−118 °C; MS (FAB) m/z 632 (M+H)[+]; ^{1}H NMR (200 MHz, CDCl$_3$) δ 8.7 (1H, s, H-2), 7.95 (1H, d, H-5), 7.35 (1H, d, H-8), 4.15−4.35 (8H, m, -CH_2CH$_3$), 3.55 (1H, m, cyclopropyl-CH), 3.35 (4H, m, piperazine), 3.0 (2H, m, H-1″), 2.5−2.8 (5H, m, piperazine, H-2″), 1.4 (14H, t, -CH_2CH$_3$, cyclopropyl-CH_2), 1.2 (2H, m, cyclopropyl-CH_2); ^{13}C NMR (50 MHz, CDCl$_3$) δ 177.0 (C-4), 166.8 (COOH), 153.6 (d, C-6, $^1J_{C,F}$ = 250 Hz), 147.4 (C-2), 145.7 (d, C-7, $^2J_{C,F}$ = 11 Hz), 139.0 (C-8a), 119.7 (d, C-4a, $^3J_{C,F}$ = 8 Hz), 112.3 (d, C-5, $^2J_{C,F}$ = 23 Hz); 108.0 (C-3), 104.7 (C-8), 62.8, 62.7, and 62.5 (O-CH_2CH$_3$), 53.9, 52.5, 49.7, and 49.6 (piperazine, C-1″), 36.3 (t, C-2″, $^1J_{C,P}$ = 132 Hz), 35.3 (cyclopropyl-CH), 16.5 and 16.4 (O−CH$_2$$CH_3$); 8.2 (cyclopropyl-$CH_2$). Anal. Calcd. for C$_{27}H_{40}FN_3O_9P_2$ (631.574): C, 51.35%; H, 6.38%; N, 6.65%; P, 9.81%. Found: C, 50.90%; H, 6.37%; N, 6.32%; P, 9.53%.

General Procedure for Synthesis of Compounds 11, 12, and 13. A mixture of norfloxacin, enoxacin, or ciprofloxacin (2 mmol), triethyl orthoformate (4.4 mmol), and diethyl phosphite (8 mmol) was stirred at 145 °C in a flask equipped with a reflux condenser. The system was evacuated occasionally through the top of the condenser in order to remove low boiling side products. After 0.5 h the reaction mixture solidified. After cooling, the product was treated with dry ether, and the crystals of compounds **11**, **12**, or **13** were removed by filtration and were recrystallized from ethanol.

7-(4-(Bis-(diethoxyphosphoryl)-methyl)-piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (Compound 13): yield 67%; mp 213−215 °C; ^{1}H NMR (200 MHz, CDCl$_3$) δ 8.75 (1H, s, H-2), 8.05 (1H, d, H-5), 7.35 (1H, d, H-8), 4.2−4.4 (8H, m, -CH_2CH$_3$), 3.3−3.6 (10H, m, piperazine, H-5), 4.4 (2H, q, N-CH_2CH$_3$), 4.25 (8H, m, O-CH_2CH$_3$), 3.95 (4H, m, piperazine), 3.5 (1H, t, P-CH-P), 3.3 (4H, m, piperazine), 1.5 (3H, t, N-CH$_2$$CH_3$), cyclopropyl-$CH$, P-$CH$-P) 1.45 (14 H, m, -CH$_2$$CH_3$, cyclopropyl-$CH_2$), 1.2 (2H, m, cyclopropyl-CH_2). High-resolution mass spectrum: observed: 618.2147 (M + H)[+]. Calculated for C$_{26}$H$_{39}$FN$_3$O$_9$P$_2$: 618.2146.

Deesterification of Compounds 5, 6, 7, 11, 12, or 13. A solution of Compounds **5**, **6**, **7**, **11**, **12**, or **13** (l mmol) in dry dichloromethane (10 mL) was treated with bromotrimethylsilane (10 mmol), and the mixture was allowed to react for 3 days at room temperature. The solvent was evaporated, and then 20 mL of water was added. The mixture was stirred for 24 h and evaporated to dryness. The residue was agitated with dichloromethane (10 mL) for 6 h, the solvent was decanted, and the product was suspended in ether and filtered.

7-(4-(2,2-Bis-phosphono-ethyl)-piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (compound 10): yield 93%; mp 160 °C (dec); MS (ESI positive ion detection) m/z 520 (M+H)[+]; ^{1}H NMR (200 MHz, D$_2$O+K$_2$CO$_3$) δ 8.35 (1H, s, H-2), 7.5 (1H, d, H-5), 7.3 (1H, d, H-8), 3.3−3.6 (11H, m, piperazine, H-1″, cyclopropyl-CH), 2.35 (1H, tt, H-2″), 1.25 (2H, m, cyclopropyl-CH_2), 0.95 (2H, m, cyclopropyl-CH_2); ^{13}C NMR (50 MHz, D$_2$O + K$_2$CO$_3$) δ 175.2 (C-4), 172.5 (COOH), 153.0 (d, C-6, $^1J_{C,F}$ = 246 Hz), 146.9 (C-2), 142.8 (d, C-7, $^2J_{C,F}$ = 11 Hz), 138.4 (C-8a), 122.3 (d, C-4a,

$^3J_{C,F}$ = 7 Hz), 116.8 (C-3), 111.4 (d, C-5, $^2J_{C,F}$ = 23 Hz), 106.7 (C-8), 57.1 (C-1″), 50.7 and 48.0 (piperazine), 36.2 (t, C-2″, $^1J_{C,P}$ = 113 Hz), 34.8 (cyclopropyl-*CH*), 7.5 (cyclopropyl-*CH₂*). Anal. Calcd for $C_{19}H_{24}FN_3O_9P_2$·2HBr (681.184): C, 32.50%; H, 3.85%; Br, 23.46%; P, 9.09%. Found: C, 31.90%; H, 3.92%; Br, 24.00%; P, 9.24%.

In Vitro MIC: Measurement of Antibacterial Activity. Antibacterial activity was measured by the agar-dilution method. Most compounds were soluble in 0.2% $NaHCO_3$, and those that were not, were soluble in dimethyl sulfoxide. Antibacterials were added to molten agar (50 °C), and after the agar solidified, 0.25 mL of bacterial suspension was streaked onto the surface of the agar-compound mixture. MIC values were determined after 48 h of incubation at 37 °C. A limited number of MBC assays were conducted.

Antibacterial Activity of Compounds against Clinical Isolates. Antibacterials were diluted serially (256 μg to 0.5 μg/mL) in 96-well microtitration plate rows. Bacteria (A_{540} = 0.15 × 10⁷ cfu/mL, 10 mL log phase growth) were added and incubated at 37 °C for 18 h, and wells were inspected macroscopically for growth. The MIC value was defined as the lowest concentration capable of inhibiting bacterial growth. The MIC_{90} value was calculated from multiple isolates, using median MIC values ± 1 SD, and use of the *z* statistic.

Fluorescence. Fluorescence was determined using a FluoroMax-2 spectrofluorometer, with slit widths set at 10 and 20 nm (excitation and emission, respectively). Compounds **3**, **7**, and **10**, dissolved in AMPSO (3-[(1,1-dimethyl-2-hydroxyethyl)-amino]-2-hydroxypropanesulfonic acid, Sigma Chemicals) biological buffer (50 mM, pH 8.5) at concentrations of 2, 4, or 4 mg/mL respectively, were excited at 297 nm, and emission counts-per-second (cps) were monitored at 480 nm. A synchronous emission instrument mode (fixed interval of 55 nm, incremental excitation increase of 1 nm) was used when interfering substances were present.

Preparation of Bone Powder. Long bones of adolescent pigs and adult Sprague−Dawley male rats were collected after animal sacrifice. Human bone powder was obtained from the Virginia Tissue Bank (Richmond, VA). Scraped long bones were snap-frozen in liquid nitrogen. Bone powder was washed, autoclaved, and air-dried.

Bone Affinity. The ability of compounds **3**, **7**, and **10** to bind to bone was determined spectrofluorometrically by measuring compound-associated fluoresence of solutions before and after addition of insoluble bone powder. Antibacterials (4−95 μg/mL in 4 mL of AMPSO) were incubated with bone powder (10 mg/mL) for 18 h at 37 °C with mixing by inversion. Samples were then centrifuged (10 min, 1000*g*). Fluorescence of the supernatants were compared and calculated as follows: % Bound = (total − free)/total cps × 100%.

For the assessment of the ability to impede bacterial growth following compound binding to bone, antibacterials and bone powder were incubated overnight at 37 °C, with repeating tube inversion. The pellet was washed three times (final dilution = (2−8) × 10³) and then suspended in microbiologic growth medium (4% tryptic soy broth). *S. aureus* (10⁷ colony-forming units) was added and incubated at 37 °C with tube inversion for 24 h, and bacterial growth was monitored turbidimetrically (540 nm).

Supporting Information Available: Analytical data for compounds **5**, **6**, **8**, **9**, **11**, **12**, **14**, **15**, and **16**; saturation binding curve of compound **10** to bone powder including inset of Lineweaver-Burke plot (Figure 3); fluorescent spectra of compounds **3**, **7**, and **10** (Figure 4); Scheme 2 showing synthesis of bis-phosphonomethyl derivatives, and MIC tables comparing compounds **1** and **2** to their respective bis-phosphonomethyl derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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