A NEW METHOD FOR SYNTHESIS AND ANGIOGENIC EVALUATION OF LETEPRINIM POTASSIUM AND ITS NOVEL ANALOGS

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Abstract – We developed a novel pathway for the successful synthesis of leteprinim potassium 1, which is one of the candidate substances for treating Alzheimer’s disease, and subsequently synthesized 4 types of corresponding novel derivatives 2–5 that have hypoxanthine or 2-chloro-6-aminopurine as the nucleobase. We then determined the angiogenic activity of these compounds by using human umbilical vein endothelial cells. Compounds 1–4 showed no angiogenic potencies judging from statistical analysis, student’s t-test.

Leteprinim potassium (1, Neotrofin®), which shows neuroprotective activity and a nootropic effect by antagonizing glutamic acid, 1a was a candidate therapeutic agent for the treatment of neurodegenerative disorders such as Alzheimer’s disease. 1b,c Liu and co-workers reported the preparation of leteprinim potassium 1 by the reaction of a 4-aminobenzoate ester with acryloyl chloride, followed by coupling of the product with adenine and subsequent diazotization. 2 However, because of the high toxicity of acryloyl chloride and for the preparation of leteprinim potassium analogs with a modified 4-aminobenzoate moiety, we synthesized 1 by using a de novo synthetic pathway. This method includes the introduction of a propionate at the 9-position of the hypoxanthine skeleton and subsequent condensation of the product with a 4-aminobenzoate ester. We also used this approach to synthesize the analogs 2–5 of compound 1 and evaluated the angiogenic activities of all the five compounds. Based on the discovery of the bioactive substance 2-chlorocarbocyclic oxetanocin-A (6, 2-Cl-C.OXT-A, COA-Cl), which is a novel nucleoside analog that significantly stimulates tube formation in human umbilical endothelial cells (HUVEC) 3 and induces neurite outgrowth in PC12 cells (unpublished data), we synthesized analog 2, in which the
nucleobase of 1 is replaced by 2-chloroadenine of COA-Cl (Figure 1). We also synthesized letprinim potassium derivatives—$N^1$-methylletprinim potassium 3, which mimics the effects of caffeine 7, and pentose-bound letprinim potassium analogs 4 and 5, which mimic DNA or RNA compounds, by substitution of the pentose ring of the ethylene chain bound to the 9-position in the hypoxanthine skeleton of 1 (Figure 1). In particular, fluorine-substituted 2′,3′-dideoxynucleosides have long been known to show not only several bioactivities but also high stability under acidic conditions because of the binding of the fluorine atom to the pentose ring; therefore, we developed a method for the synthesis of compound 5.

The synthesis of 1 and its $N^1$-methylated analog 3 began with alkylation at the 9-position of the purine skeleton of the readily available adenine 8 (Scheme 1). First, 8 was allowed to react with ethyl 3-bromopropionate to give the 9-alkylated product 9 in 73% yield, which was treated with sodium nitrite in the presence of acetic acid to obtain hypoxanthine derivative 10 in 94% yield. Deamination at the 6-position of adenine was performed at this stage; this was because condensation of the 6-amino derivative with a 4-aminobenzoate ester in the subsequent steps could cause the 6-amino group in the adenine skeleton to react with the carboxyl group of the other 6-amino analog in the same manner as the amino group in the 4-aminobenzoate reagent. Ethyl ester 10 was hydrolyzed with a 1.0 M aqueous sodium hydroxide solution to give the corresponding carboxylic acid 11 in 96% yield. Subsequently, 11 was condensed with ethyl $p$-aminobenzoate in the presence of thionyl chloride at room temperature, and
Reagents and conditions: i, ethyl 3-bromopropionate, K$_2$CO$_3$, DMF, 50 °C, 3 h; ii, NaN$_2$, AcOH, rt, 16 h; iii, 1.0 M NaOH, 50 °C, 1 h; iv, ethyl 4-aminobenzoate, SOCl$_2$, MeCN, rt, 16 h; v, 1.0 M K$_2$CO$_3$, MeOH, 50 °C, 16 h; vi, Mel, K$_2$CO$_3$, DMF, 35 °C, 6 h; vii, 1.0 M K$_2$CO$_3$, MeOH, 50 °C, 16 h; viii, Amberlite IR-120H (K$^+$) ion-exchange resin

**Scheme 1.** Synthesis of leteprinim potassium 1 and $N^1$-methylleteprinim potassium 3

Reagents and conditions: i, NH$_3$, MeOH, 50 °C, 16 h; ii, ethyl 3-bromopropionate, K$_2$CO$_3$, DMF, 50 °C, 16 h; iii, 1.0 M NaOH, 50 °C, 1 h; iv, ethyl 4-aminobenzoate, SOCl$_2$, MeCN, rt, 16 h; v, 1.0 M K$_2$CO$_3$, MeOH, 50 °C, 16 h; vi, Amberlite IR-120H (K$^+$) ion-exchange resin

**Scheme 2.** Synthesis of compound 2

the condensation product 12 was obtained in 77% yield. Next, 12 was hydrolyzed with potassium carbonate in MeOH to obtain free leteprinim 13 in 93% yield, which was subsequently converted into 1 using a cation-exchange resin (K$^+$-type). On the other hand, the reaction of 13 with methyl iodide afforded 14 in 53% yield, which was hydrolyzed with 1.0 M potassium carbonate solution to obtain the corresponding carboxylic acid 15 in 59% yield. Compound 15 was subsequently converted into $N^1$-methylleteprinim potassium 3 in 87% yield using a cation-exchange resin.
Reagents and conditions: i, acetone, $\rho$-TsOH, 2,2-dimethoxypropane, rt, 10 h; ii, TEMPO, iodobenzene diacetate, H$_2$O, MeCN, rt, 5 h; iii, ethyl 4-aminobenzoate, SOCl$_2$, MeCN, rt, 9 h; iv, 90% CF$_3$CO$_2$H $aq$, rt, 30 min; v, 1 M K$_2$CO$_3$ $aq$, MeOH, 50 °C, 6 h; vi, Amberlite IR-120H (K$^+$) ion-exchange resin.

Scheme 3. Synthesis of compound 4

Reagents and conditions: i, HOCH$_2$CH$_2$CN, NaH, THF, 5 h, 45 °C; ii, MeOH, conc. HCl, rt, 2 h; iii, TEMPO, iodobenzene diacetate, H$_2$O, MeCN, rt, 3 h; iv, ethyl 4-aminobenzoate, SOCl$_2$, MeCN, 40 °C, 2 days; v, 1 M K$_2$CO$_3$ $aq$, MeOH, 50 °C, 10 h

Scheme 4. Synthesis of compound 5

COA-Cl derivative 2 was synthesized via a synthetic route similar to that employed for the preparation of 1 in scheme 1 (Scheme 2). 2,6-Dichloropurine 16 was treated with methanolic ammonia solution at 50 °C to afford the target material 2-chloroadenine 17 in 97% yield. The starting material 17 was reacted with ethyl 3-bromopropionate to give the corresponding compound 18, followed by hydrolysis to produce the carboxylic acid 19, which was then treated with ethyl 4-aminobenzoate to obtain 20; subsequent hydrolysis of 20 afforded the corresponding free acid 21. Finally, 21 was converted into the potassium salt, and subsequently, 2 was produced in 26% overall yield from 16.

To obtain the pentose-substituted analog of leteprinim potassium 4, inosine 22 was treated with 2,2-dimethoxypropane and $\rho$-toluenesulfonylic acid in acetone solution to give 23, protected as the 2',3'-O-acetonide,$^5$ in 68% yield (Scheme 3). Next, the 5'-hydroxymethyl group was oxidized using
TEMPO and iodobenzene diacetate to produce carboxylic acid 24.\textsuperscript{6} As described above, 24 was allowed to react with ethyl 4-aminobenzoate to afford the corresponding compound 25 in 95\% yield; subsequent deprotection of the 2′,3′-O-acetonide gave 2′,3′-diol 26.\textsuperscript{2} Furthermore, similar to the synthetic pathways in schemes 1 and 2, product 26 was used for hydrolysis of the ethyl ester with potassium carbonate in MeOH solution, and the resulting free carboxylic acid 27 was converted into the corresponding potassium salt, which finally yielded the target material 4.

As reported in our previous study, compound 29 was obtained from 6-chloro-3′-deoxypurine riboside 28 in 3 steps,\textsuperscript{8} as shown in scheme 4. Next, the 6-chloro group in the purine skeleton of 29 was reacted with sodium hydride and 2-cyanoethanol to produce the hypoxanthine derivative 30.\textsuperscript{9} Detritylation at the 5′-position of 30 was performed to obtain alcohol 31, followed by oxidation at the 5′-position to produce carboxylic acid 32.\textsuperscript{6} Then, 32 was condensed with ethyl 4-aminobenzoate and hydrolyzed with potassium carbonate/MeOH solution to afford carboxylic acid 5. Unfortunately, conversion of the free carboxylic acid at the 5′-position of the pentose skeleton into the potassium salt was unsuccessful.

We determined the activities of the analogs 1–4 using a well-established tube formation assay.\textsuperscript{3a,10} However, compound 5 could not assayed because of its insolubility in water. After an incubation period of 10 days with co-cultured fibroblast and additives, human umbilical vein endothelial cells (HUVECs) were stained using Tubule Staining Kit for CD31. Representative images are shown in Figure 2. VEGF was used as a positive control. The area of the formed tube was represented as a relative value to that formed in the well without any additive. Compounds 1, 2, and 3 apparently showed no effect on angiogenic potencies, but derivative 4 at a concentration of 100 \textmu M slightly stimulated tube formation, with 1.38 ± 0.36 (n = 5, Figure 3). However, student’s t-test revealed no significant difference between saline and compound 4 (p > 0.05, 100 \textmu M). Moreover, as shown in Figure 4, we performed a proliferation assay because angiogenesis is intimately associated with complex cellular processes, including proliferation of endothelial cells,\textsuperscript{10b} which also showed that compound 4 did not stimulate the proliferation of HUVECs (e.g., 100 \textmu M: 1.28 ± 0.18, n = 5, p > 0.05).

![Figure 2. Typical pictures of stimulated tube formation by compound 4. Additives are shown under the pictures.](image-url)
In summary, on the basis of the potent neuroprotective activity of leteprinim potassium 1 and the angiogenesis promoter COA-Cl, novel derivatives 2–5 with hypoxanthine or 2-chloro-6-aminopurine as the nucleobase were synthesized via 3-(9H-purinyl)propanoic acid derivatives 11 and 19 or 5′-oxoinosine derivatives 24 and 32 as the key intermediates, and their angiogenic activity was evaluated using HUVECs. However, none of these compounds showed angiogenic potencies judging from statistical analysis, student’s t-test. This result indicates that the structure of the 2-chloropurine skeleton and the cyclobutane ring moiety in 6 is essential to exert angiogenesis activity.

Figure 3. Tube formation assay of compounds 1–4. The tube formation assay was performed using an Angiogenesis Kit and the estimation was performed 10 days after the incubation with various additives (10-100 µM). The area of the formed tube was represented as a relative value to that formed in the control well with no additive. The effect of the solvent, 10% saline as well as a positive control, VEGF (10 ng/mL) were shown together. Results were expressed as mean ± SE of more than five individual experiments.

Figure 4. Effect of compound 4 on the proliferation. 48 h after the addition of additives, the cell viability was measured with a Cell Counting Kit-8. Cell viabilities were represented as relative values to that of the control well with no additive. The effect of the solvent, 10% saline as well as a positive control, VEGF (10 ng/mL) were shown together. Results were expressed as mean ± SE of more than five individual experiments.
EXPERIMENTAL

Instrumentation

$^1$H NMR and $^{13}$C NMR spectra were taken with a Ultrashield™ 400 Plus FT NMR System (BRUKER). Chemical shifts and coupling constants ($J$) were given in $\delta$ and Hz, respectively. Melting points were determined on a Yanaco MP-500D. Elementary analyses were determined by a Perkin Elmer Series II CHNS/O Analyzer 2400. High-resolution mass spectrometry was performed on a APEX IV mass spectrometer (BRUKER) with electrospray ionization mass spectroscopy (ESI-MS). UV spectrum was obtained on a Perkin Elmer Lambda 35 UV/VIS Spectrometer in EtOH solution. Angiogenesis Kit, Tubule Staining Kit for CD31, HUVEC, fetal bovine serum (FBS), VEGF, HuMedia EG2, and HuMedia EB2 were purchased from Kurabo Co. (Osaka, Japan). Cell Counting Kit 8 was supplied by Dojindo Molecular Technologies (Kumamoto, Japan).

Ethyl 3-((6-amino-9H-purin-9-yl)propanate (9)

Adenine (8) (3.5 g, 25.9 mmol) was dissolved in DMF (190.0 mL), and sodium carbonate (7.20 g, 51.8 mmol), ethyl 3-bromopropionate (6.66 mL, 51.8 mmol) were added to the solution, and then stirred for 3 h at 50 °C. The mixture was quenched with acetic acid (3.3 mL), and was extracted with benzene, and washed with saturated aqueous sodium chloride solution, and dried with sodium sulfate, and then evaporated. The residue was recrystallized with EtOH to give white crystals 9 (4.42 g, 18.8 mmol, 73%).

$^1$H NMR (CDCl$_3$): $\delta$ 8.36 (1 H, s, H-2), 7.90 (1 H, s, H-8), 5.51 (1 H, s, 6 -NH$_2$), 4.50 (2 H, t, $J$ 6.4, $-$NCH$_2$CH$_2$CO-$)$, 4.13 (2 H, q, $J$ 7.2, $-$OEt), 2.93 (2 H, t, $J$ 6.4, $-$NCH$_2$CH$_2$CO-$)$, 1.22 (3 H, t, $J$ 7.2, $-$OEt); HRMS (ESI) Calcd for C$_{10}$H$_{13}$N$_5$O$_2$Na: [M+Na]$^+$: 258.0962. Found 258.0953; Anal. Cald for C$_{10}$H$_{13}$N$_5$O$_2$: C; 51.06, H; 5.57, N; 29.77. Found C; 51.15, H; 5.59, N; 29.73; Mp 169.1-170.4 °C; UV: max 259.90nm, 209.7nm (sh) (MeOH).

Ethyl 3-((1,6-dihydro-6-oxo-9H-purin-9-yl)propanate (10)

Compound 9 (3.80 g, 16.2 mmol) was dissolved in acetic acid (200.0 mL), and sodium nitrite (15.0 g, 217.4 mmol) was added to the solution bit by bit, and then stirred for 16 h at room temperature. The mixture was evaporated, and extracted with CHCl$_3$ and H$_2$O, and washed with saturated sodium chloride solution, and dried with sodium sulfate, and then evaporated. The resultant residue was recrystallized with MeOH to give white crystals 10 (3.57 g, 15.11 mmol, 94%). $^1$H NMR (CDCl$_3$): $\delta$ 7.99 (1 H, s, H-8), 7.88 (1 H, s, H-2), 4.49 (2 H, t, $J$ 6.4, $-$NCH$_2$CH$_2$CO-$)$, 4.15 (2 H, q, $J$ 7.2, $-$OEt), 2.91 (2 H, t, $J$ 6.4, $-$NCH$_2$CH$_2$CO-$)$, 1.23 (3 H, t, $J$ 7.2, $-$CH$_2$CH$_3$); HRMS (ESI) Calcd for C$_{10}$H$_{13}$N$_5$NaO$_3$: [M+Na]$^+$: 259.0802. Found 259.0797; Anal. Cald for C$_{10}$H$_{13}$N$_5$O$_3$: C; 51.06, H; 5.57, N; 29.77. Found C; 51.15, H; 5.59, N; 29.73; Mp 169.1-170.4 °C; UV: max 259.90nm, 209.7nm (sh) (MeOH).

3-(1,6-Dihydro-6-oxo-9H-purin-9-yl)propanoic acid (11)

Compound 10 (3.17 g, 13.4 mmol) was dissolved in H$_2$O (50.0 mL), and 1.0 M NaOH $aq.$ (30.0 mL) was
added to the solution, and then stirred for 1 h at 50 °C. The mixture was quenched with 1.0 M HCl \(aq\). (30.0 mL), and pH was adjusted at 3. Resultant deposited crystal was filtrated to give white crystals 11 (2.68 g, 12.85 mmol, 96%). \(^1\)H NMR (DMSO-\(d_6\)): \(\delta 12.26\) (1 H, s, H-1), 8.04 (2 H, s, H-2 and H-8), 4.33 (2 H, t, \(J 6.8\), -NCH\(_2\)CH\(_2\)CO-), 2.81 (2 H, t, \(J 6.8\), -NCH\(_2\)CH\(_2\)CO-); Anal. Cald for C\(_8\)H\(_8\)N\(_4\)O\(_3\): C; 45.37, H; 4.00, N; 26.46. Found C; 45.41, H; 4.02, N; 26.39. Ms m/z 208 \([\text{M}^+\text{]}\) Mp 271.9-274.7 °C. UV: max 250.60 nm, 208.30 nm (sh) (MeOH) max 255.10 nm, 227.90 nm, 226.40 nm, 224.2 nm, 222.6 nm, 220.3 nm, 207.8 nm (sh) (NaOH).

Ethyl 4-[(3-(1,6-dihydro-6-oxo-9\(H\)-purin-9-yl)-1-oxopropyl]amino]benzoate (12)

Compound 11 (2.68 g, 12.85 mmol) was dissolved in MeCN (150.0 mL), and ethyl 4-aminobenzoate (4.67 g, 28.27 mmol), thionyl chloride (6.0 mL, 82.24 mmol) were added to the solution, and then stirred for 16 h at room temperature. The mixture was cooled down with ice bath, and saturated aqueous sodium bicarbonate solution (30.0 mL) was added, and pH was adjusted at 10. Resultant deposited crystal was filtrated to give white crystals 12 (3.50 g, 9.85 mmol, 77%). \(^1\)H NMR (DMSO-\(d_6\)): \(\delta 10.34\) (1 H, s, -CONH-), 8.03 (2 H, s, H-2 and H-8), 7.90 (2 H, d, \(J 9.2\), Ar), 7.68 (2 H, d, \(J 9.2\), Ar), 4.45 (2 H, t, \(J 6.8\), -NCH\(_2\)CH\(_2\)CO-), 4.28 (2 H, q, \(J 7.0\), -OEt), 2.99 (2 H, t, \(J 6.8\), -NCH\(_2\)CH\(_2\)CO-), 1.31 (3 H, t, \(J 7.2\), -OEt); HRMS (ESI) Calcd for C\(_{17}\)H\(_{17}\)N\(_5\)NaO\(_4\) \([\text{M+Na}^+\text{]}\): 378.1173. Found 378.1171. Mp 273.1-276.0 °C.

4-[(3-(1,6-Dihydro-6-oxo-9\(H\)-purin-9-yl)-1-oxopropanoyl]amino]benzoic acid (13)

Compound 12 (3.50 g, 9.85 mmol) was dissolved in MeOH (150.0 mL), and 1.0 M potassium carbonate \(aq\). (50.0 mL) was added to the solution, and then stirred for 16 h at 50 °C. The mixture was cooled down with ice bath, and acidified with acetic acid (ca. 6 mL) at pH 3. Resultant deposited crystal was filtrated to give white crystals 13 (2.99 g, 9.14 mmol, 93%). \(^1\)H NMR (D\(_2\)O): \(\delta 8.11\) (1 H, s, H-2), 8.03 (1 H, s, H-8), 7.26 (2 H, d, H3+H5 of –C\(_6\)H\(_4\)), 4.60 (2 H, t, \(J 6.4\), -NCH\(_2\)CH\(_2\)CO-), 2.95 (2 H, t, J 6.4, -NCH\(_2\)CH\(_2\)CO-); HRMS (ESI) Calcd for C\(_{15}\)H\(_{13}\)N\(_3\)NaO\(_4\) \([\text{M+Na}^+\text{]}\): 350.0860. Found 350.0857. Mp 250 °C. (dec.).

Potassium 4-[(3-(1,6-dihydro-6-oxo-9\(H\)-purin-9-yl)-1-oxopropyl]amino]benzoate (leteprinim potassium, 1)

Compound 13 (51.9 mg, 0.16 mmol) was dissolved in H\(_2\)O (5.0 mL), and 25% ammonia \(aq\). (1.0 mL) was added to the solution, and then evaporated by half in volume. The mixture was eluted with H\(_2\)O by using Amberlite IR-120H (K\(^+\)) ion exchange resin, and resultant solvent was evaporated. The residue was recrystallized with EtOH to give white crystals, leteprinim potassium (1) (32.8 mg, 0.09 mmol, 57%). \(^1\)H NMR (D\(_2\)O): \(\delta 8.04\), 8.03 (each 1H, s, H2 or H8 hypoxanthine), 7.76 (2H, d, H2+H6 of –C\(_6\)H\(_4\), J 8.8), 7.25 (2 H, d, H3+H5 of –C\(_6\)H\(_4\), J 8.8), 4.57 (2H, t, -NCH\(_2\)CH\(_2\)CO-, J 6.4), 2.94 (2 H, t, -NCH\(_2\)CH\(_2\)CO-, J
6.4; $^{13}$C NMR (100MHz, D$_2$O): δ 174.9, 171.5, 167.0, 152.9, 149.5, 140.4, 139.0, 132.8, 129.8, 123.1, 120.6, 40.2, 36.8; HRMS (ESI) Calcd for C$_{15}$H$_{12}$KN$_5$NaO$_4$ [M+Na]$^+$: 388.0419. Found 388.0419. Mp > 300 °C.

Methyl 4-[[3-(6-hydro-1-methyl-6-oxo-9H-purin-9-yl)-1-oxopropyl]amino]benzoate (14)

Compound 13 (407.5 mg, 1.25 mmol) was dissolved in DMF (50.0 mL), and sodium carbonate (518.0 mg, 3.75 mmol), methyl iodide (311 μl, 5.00 mmol) was added to the solution, and then stirred for 6 h at 50 °C. The mixture was quenched with acetic acid (10.0 mL), and the residue was recrystallized with H$_2$O to give white crystals 14 (214.2 mg, 0.60 mmol, 53%). $^1$H NMR (DMSO-d$_6$): δ 10.03 (1 H, s, -CONH-), 8.32 (1 H, s, H-2), 7.98 (1 H, s, H-8), 7.82 (2 H, d, J 8.8, Ar), 7.60 (2 H, d, J 8.8, Ar), 4.38 (2 H, t, J 6.8, -NCH$_2$CH$_2$CO-), 3.74 (3 H, s, 1-NCH$_3$), 3.43 (3 H, s, -OMe), 2.92 (2 H, t, J 6.8, -NCH$_2$CH$_2$CO-); HRMS (ESI) Calcd for C$_{17}$H$_{17}$N$_5$NaO$_4$[M+Na]$^+$: 378.1173. Found 378.1177. Mp > 300 °C.

4-[[3-(6-Hydro-1-methyl-6-oxo-9H-purin-9-yl)-1-oxopropyl]amino]benzoic acid (15)

Compound 14 (214.2 mg, 0.60 mmol) was dissolved in MeOH (20.0 mL), and 1.0 M potassium carbonate aq. (15.0 mL) was added to the solution, and then stirred for 16 h at 50 °C. The mixture was cooled down with ice bath, and acidified with acetic acid (ca. 10 mL) at pH 3. The reaction mixture was evaporated, and the resultant residue was recrystallized to give white crystals 15 (120.4 mg, 0.35 mmol, 59%). $^1$H NMR (DMSO-d$_6$): δ 12.70 (1 H, s, -COOH), 10.31 (1 H, s, -CONH-), 8.39 (1 H, s, H-2), 8.05 (1 H, s, H-8), 7.64 (2 H, d, J 8.8, Ar), 4.45 (2 H, t, J 6.8, -NCH$_2$CH$_2$CO-), 3.51 (3 H, s, 1-NCH$_3$), 2.99 (2 H, t, J 6.8, -NCH$_2$CH$_2$CO-); HRMS (ESI) Calcd for C$_{16}$H$_{15}$N$_5$NaO$_4$[M+Na]$^+$: 364.1016. Found 364.1014. Mp 283.5-289.4 °C.

Potassium 4-[[3-(6-hydro-1-methyl-6-oxo-9H-purin-9-yl)-1-oxopropyl]amino]benzoate (3)

Compound 15 (77.9 mg, 0.23 mmol) was dissolved in H$_2$O (5.0 mL), and 25% ammonia aq. (1.5 mL) was added to the solution, and then evaporated by half in volume. The mixture was eluted with H$_2$O by using Amberlite IR-120H (K$^+$) ion exchange resin, and resultant solvent was evaporated. The resultant residue was recrystallized with EtOH to give white crystals 3 (75.5 mg, 19.9 mmol, 87%). $^1$H NMR (DMSO-d$_6$): δ 9.94 (1 H, s, -CONH-), 8.40 (1 H, s, H-2), 8.05 (1 H, s, H-8), 7.71 (2 H, d, J 8.8, Ar), 7.37 (2 H, d, J 8.8, Ar), 4.44 (2 H, t, J 6.8, -NCH$_2$CH$_2$CO-), 3.51 (3 H, s, 1-NCH$_3$), 2.92 (2 H, t, J 6.8, -NCH$_2$CH$_2$CO-); $^{13}$C NMR (100MHz, DMSO-d$_6$): δ 168.2, 168.0, 156.4, 148.4, 147.7, 140.9, 138.7, 137.0, 129.4, 123.1, 117.5, 42.0, 36.1, 33.4; HRMS (ESI) Calcd for C$_{16}$H$_{14}$KN$_5$NaO$_4$[M+Na]$^+$: 402.0575. Found 402.0581. Mp 277.4 °C. (dec.).

2-Chloroadenine (17)

2,6-Dichloropurine (16) (5.0 g, 26.5 mmol) was dissolved in MeOH (100.0 mL), and liquid ammonia (20.0 mL) was added to the solution, and then sealed and stirred for 16 h at 100 °C. The mixture was
 evaporated, and the resultant residue was recrystallized with H2O to give white crystals 2-chloroadenine (17) (4.36 g, 23.1 mmol, 97%). 1H NMR (CDCl3): δ 12.72 (1 H, s, H-9), 8.11 (1 H, s, H-8), 7.57 (2 H, s, 6-NH2); Mp > 300 °C.

**Ethyl 3-(6-amino-2-chloro-9H-purine-9-yl)propanate (18)**

2-Chloroadenine (17) (945.8 mg, 5.58 mmol) was dissolved in DMF (70.0 mL), and sodium carbonate (1.93 g, 14.0 mmol), ethyl 3-bromopropionate (1.43 mL, 11.16 mmol) were added to the solution, and then stirred for 16 h at 50 °C. The mixture was quenched with acetic acid (1.6 mL), and was extracted with CHCl3, and washed with saturated aqueous sodium chloride solution, and dried with sodium sulfate, and then evaporated. The residue was purified by silica gel column chromatography (10% MeOH in CHCl3) to give crystals 18 (730.1 mg, 2.71 mmol, 49%). 1H NMR (CDCl3): δ 7.88 (1 H, s, H-8), 5.73 (1 H, s, 6 -NH2), 4.46 (2 H, t, J 6.4 , -NCH2CH2CO-), 4.14 (2 H, q, J 7.2, -OEt), 2.90 (2 H, t, J 6.4 , -NCH2CH2CO-), 1.23 (3 H, t, J 7.2, -OEt); HRMS (ESI) Calcd for C10H12ClN5NaO4 [M+Na]+: 292.0572. Found 292.0565. Mp 162.3-162.6 °C.

**3-(6-Amino-2-chloro-9H-purin-9-yl)propanoic acid (19)**

Compound 18 (1.40 g, 5.19 mmol) was dissolved in H2O (20.0 mL), and 1.0 M NaOH aq. (14.0 mL) was added to the solution, and then stirred for 1 h at 50 °C. The mixture was quenched with 1.0 M HCl aq. (14.0 mL), and pH was adjusted at 3. Resultant deposited crystal was filtrated to give white crystals 19 (1.18 g, 4.89 mmol, 95%). 1H NMR (DMSO-d6): δ 12.50 (1 H, s, -COOH), 8.09 (1 H, s, H-8), 7.72 (1 H, s, 6-NH2), 4.28 (2 H, t, J 6.8, -NCH2CH2CO-), 2.83 (2H, t, J 6.8, -NCH2CH2CO-); HRMS (ESI) Calcd for C8H8ClN5NaO4 [M+Na]+: 264.0259. Found 264.0262. Mp 247.5-249.5 °C.

**Ethyl 4-[[3-(2-chloro-6-amino-9H-purin-9-yl)-1-oxopropyl]amino]benzoate (20)**

Compound 19 (1.08 g, 4.47 mmol) was dissolved in MeCN (60.0 mL), and ethyl 4-aminobenzoate (1.62 g, 9.83 mmol), thionyl chloride (4.2 mL, 57.22 mmol) were added to the solution, and then stirred for 16 h at room temperature. The mixture was cooled down with ice bath, and saturated aqueous sodium bicarbonate solution (130.0 mL) was added, and pH was adjusted at 10. Resultant deposited crystal was filtrated, and recrystallized with EtOH/H2O system to give white crystals 20 (1.32 g, 3.39 mmol, 76%). 1H NMR (DMSO-d6): δ 10.30 (1 H, s, -CONH-), 8.08 (1 H, s, H-8), 7.90 (2 H, d, J 8.8, Ar), 7.68 (2 H, d, J 8.8, Ar), 7.64 (1 H, s, 6-NH2), 4.42 (2 H, t, J 6.8, -NCH2CH2CO-), 4.29 (2 H, q, J 7.2, -OEt), 2.98 (2 H, t, J 6.8, -NCH2CH2CO-), 1.32 (3 H, t, J 7.2, -OEt); HRMS (ESI) Calcd for C17H17ClN6NaO3 [M+Na]+: 411.0943. Found 411.0940. Mp 223.8-224.8 °C.

**4-[[3-(2-chloro-6-amino-9H-purin-9-yl)-1-oxopropyl]amino]benzoic acid (21)**

Compound 20 (355.0 mg, 0.91 mmol) was dissolved in MeOH (30.0 mL), and 1.0 M potassium carbonate aq. (10.0 mL) was added to the solution, and then stirred for 16 h at 50 °C. The mixture was cooled down
with ice bath, and acidified with acetic acid (ca. 10 mL) at pH 3. Resultant deposited crystal was filtrated to give white crystals 21 (283 mg, 0.79 mmol, 86%). $^1$H NMR (DMSO-d$_6$): $\delta$ 10.22 (1 H, s, -CONH-), 8.07 (1 H, s, H-8), 7.86 (2 H, d, J 8.8, Ar), 7.61-7.64 (4 H, m, Ar and 6-NH$_2$), 6.63 (2 H, t, J 6.8, -NCH$_2$CH$_2$CO-), 2.97 (2 H, t, J 6.8, -NCH$_2$CH$_2$CO-); HRMS (ESI) Calcd for C$_{15}$H$_{13}$ClN$_6$NaO$_3$ [M+Na]$^+$: 383.0630. Found 383.0630. Mp 257.3-260.0 °C.

Potassium 4-[[3-(2-chloro-6-amino-9H-purin-9-yl)-1-oxopropyl]amino]benzoate (2)

Compound 21 (55.7 mg, 0.15 mmol) was dissolved in H$_2$O (5.0 mL), and 25% ammonia aq. (1.0 mL) was added to the solution, and then evaporated by half in volume. The mixture was eluted with H$_2$O by using Amberlite IR-120H (K$^+$) ion exchange resin, and resultant solvent was evaporated. The residue was recrystallized with EtOH to give white crystals 2 (46.8 mg, 0.12 mmol, 76%). $^1$H NMR (DMSO-d$_6$): $\delta$ 9.91 (1 H, s, -CONH-), 8.07 (1 H, s, H-8), 7.73 (2 H, d, J 8.8, Ar), 7.64 (1 H, s, 6-NH$_2$), 7.38 (2 H, d, J 8.8, Ar), 4.41 (2 H, t, J 6.8, -NCH$_2$CH$_2$CO-), 2.91 (2 H, t, J 6.8, -NCH$_2$CH$_2$CO-); $^{13}$C NMR (100MHz, DMSO-d$_6$): $\delta$ 168.7, 168.2, 156.7, 152.8, 150.5, 141.7, 139.0, 136.5, 129.4, 117.7, 117.6, 53.0, 35.8; HRMS (ESI) Calcd for C$_{15}$H$_{12}$ClKN$_6$NaO$_3$ [M+Na]$^+$: 421.0189. Found 421.0196. Mp > 300 °C.

2',3'-O-Isopropyrideneinosine (23)

Inosine (5) (27.7 g, 103.5 mmol) was dissolved in acetone (1600.0 mL), and 2,2-dimethoxypropane (80.0 mL), p-toluenesulfonic acid (18.45 g, 97.02 mmol) were added to the solution, and then stirred for 14 h at room temperature. The mixture was evaporated, and the residue H$_2$O (375.0 mL) and 25% ammonia aq. (6.3 mL) was added to the residue, and recrystallized at 4 °C to give crystals 23 (21.58 g, 70.0 mmol, 68%). $^1$H NMR (400MHz, DMSO-d$_6$): $\delta$ 12.40 (1 H, s, H-1), 8.30 (1 H, s, H-2), 8.09 (1 H, s, H-8), 6.10 (1 H, d, J 2.8, H-1'), 5.27 (1 H, dd, J 6.0 and 3.2, H-2'), 5.12 (1 H, m, 5'-OH), 4.93 (1 H, dd, J 6.0 and 2.4, H-3'), 4.22 (1 H, m, H-4'), 3.53 (2 H, m, H-5'ab), 1.54 (3 H, s, -CH$_3$), 1.32 (3 H, s, -CH$_3$).

2',3'-O-Isopropyridene-5'-oxoinosine (24)

Acetonide (23) (5.04 g, 16.4 mmol), TEMPO (510.0 mg, 3.26 mmol), and iodobenzene diacetate (11.62 g, 36.0 mmol) were dissolved in MeCN/H$_2$O (1:1, 65.4 mL) and stirred, with the exclusion of light, for 5 h. The solvents were carefully evaporated from the resultant suspension, and the reaction residue was sequentially triturated with acetone and Et$_2$O to yield the acid 24 (4.42 g, 13.8 mmol, 84%). $^1$H NMR (400MHz, DMSO-d$_6$): $\delta$ 12.89 (1 H, s, CO$_2$H), 12.36 (1 H, s, H-1), 8.20 (1 H, s, H-2), 8.01 (1 H, s, H-8), 6.32 (1 H, m, H-1'), 5.47 (1 H, dd, J 6.0 and 2.0, H-2'), 5.42 (1 H, m, H-3'), 4.72 (1 H, m, H-4'), 1.52 (3 H, s, -CH$_3$), 1.33 (3 H, s, -CH$_3$).

Ethyl 4-[[3-(1,6-dihydro-6-oxo-9H-purin-9-yl)-1-deoxy-2,3-O-(1-methylethylidene)-β-D-ribofuranuronyl]amino]benzoate (25)

Compound 24 (644.5 mg, 2.0 mmol) was dissolved in MeCN (24.0 mL), and ethyl 4-aminobenzoate
(660.8 mg, 4.0 mmol), thionyl chloride (1.6 mL, 21.93 mmol) were added to the solution, and then stirred for 10 h at room temperature. The mixture was extracted with AcOEt, and neutralized with saturated aqueous sodium bicarbonate solution, and washed with saturated sodium chloride solution, and dried with sodium sulfate, and then evaporated. The residue was purified by silica gel column chromatography (10% MeOH in CHCl₃) to give crystals 25 (887.5 mg, 1.90 mmol, 95%). ¹H NMR (400MHz, CDCl₃): δ 8.38 (1 H, s, H-1), 7.94 (1 H, s, H-2), 7.92 (1 H, s, H-8), 7.87 (2 H, d, J 8.4, Ar), 7.41 (2 H, d, J 8.4, Ar), 6.18 (1 H, d, J 2.0, H-1'), 5.58 (1 H, dd, J 6.0 and 2.0, H-2'), 5.41 (1 H, dd, J 6.0 and 2.4, H-3'), 4.89 (1 H, d, J 2.4, H-4'), 4.22 (2 H, m, -OEt), 1.66 (3 H, s, -CH₃), 1.43 (3 H, s, -CH₃), 1.31 (1 H, t, J 6.8, -OEt).

Ethyl 4-[[3-(1,6-dihydro-6-oxo-9H-purin-9-yl)-1-deoxy-β-D-ribofuranuronoyl]amino]benzoate (26)
Compound 25 (5.92 g, 12.6 mmol) was dissolved in 90% trifluoroacetic acid aq. (44.0 mL), and stirred for 30 min at room temperature. The mixture was evaporated, and the resulting residue was purified by silica gel column chromatography (30% MeOH in CHCl₃) to give crystals 26 (3.95 g, 9.20 mmol, 73%). ¹H NMR (400MHz, DMSO-d₆): δ 12.35 (1 H, s, -NHCO-), 10.42 (1 H, s, H-1), 8.44 (1 H, s, H-2), 7.96 (1 H, s, H-8), 7.88 (2 H, d, J 8.8, Ar), 7.73 (2 H, d, J 8.8, Ar), 5.99 (1 H, d, J 6.0, H-1'), 5.75 (1 H, d, J 4.8, -OH), 5.66 (1 H, d, J 6.0, -OH), 4.58 (1 H, m, H-2'), 4.54 (1 H, d, J 2.8, H-4'), 4.32 (1 H, m, H-3'), 4.23 (2 H, q, J 7.2, -OEt), 1.26 (1 H, t, J 7.2, -OEt); HRMS (ESI) Calcd for C₁₉H₁₉N₅NaO₇ [M+Na]⁺: 452.11767. Found 452.11765.

4-[[3-(1,6-Dihydro-6-oxo-9H-purin-9-yl)-1-deoxy-β-D-ribofuranuronoyl]amino]benzoic acid (27)
Compound 26 (860.0 mg, 1.88 mmol) was dissolved in MeOH (30.0 mL), and 1.0 M potassium carbonate aq. (10.0 mL) was added to the solution, and then stirred for 6 h at 50 °C. The mixture was cooled down with ice bath, and acidified with acetic acid (ca. 2.4 mL) at pH 3. The reaction mixture was evaporated, and the resultant residue was recrystallized with MeOH to give white crystals 27 (550.4 mg, 1.39 mmol, 74%). ¹H NMR (400MHz, DMSO-d₆): δ 10.62 (1 H, s, H-1), 8.49 (1 H, s, H-2), 8.04 (1 H, s, H-8), 7.84 (2 H, d, J 8.4, Ar), 7.57 (2 H, d, J 8.4, Ar), 6.03 (1 H, d, J 6.8, H-1'), 4.65 (1 H, d, J 2.0, H-4'), 4.58 (1 H, dd, J 6.8 and 4.8, Ar), 4.32 (1 H, dd, J 4.8 and 2.0, H-3'); HRMS (ESI) Calcd for C₁₇H₁₅N₅NaO₇ [M+Na]⁺: 424.08637. Found 424.08566.

Potassium 4-[[3-(1,6-dihydro-6-oxo-9H-purin-9-yl)-1-deoxy-β-D-ribofuranuronoyl]amino]benzoate (4)
Compound 27 (50.0 mg, 0.13 mmol) was dissolved in H₂O (5.0 mL), and 25% ammonia aq. (0.5 mL) was added to the solution, and then evaporated by half in volume. The mixture was eluted with H₂O by using Amberlite IR-120H (K⁺) ion exchange resin, and resultant solvent was evaporated. The resultant residue was recrystallized with EtOH to give white crystals 4 (54.7 mg, 0.13 mmol, 100%). ¹H NMR (400MHz, D₂O): δ 10.62 (1 H, s, H-1), 8.49 (1 H, s, H-2), 8.04 (1 H, s, H-8), 7.84 (2 H, d, J 8.4, Ar), 7.57
(2 H, d, J 8.4, Ar), 6.03 (1 H, d, J 6.8, H-1'), 4.65 (1 H, d, J 2.0, H-4'), 4.58 (1 H, dd, J 6.8 and 4.8, H-2'), 4.32 (1 H, dd, J 4.8 and 2.0, H-3'); \(^{13}\)C NMR (100MHz, DMSO-\(d_6\)): \(\delta\) 168.8, 168.5, 148.7, 147.0, 138.8, 138.8, 137.0, 129.7, 124.6, 122.8, 87.8, 84.6, 73.7, 73.5; HRMS (ESI) Calcd for C\(_{17}\)H\(_{14}\)KN\(_{5}\)NaO\(_7\) [M+Na\(^+\): 462.04225. Found 462.04135.

9-[2,3-Dideoxy-2-fluoro-5-\(\beta\)-D-threo-pentofuranosyl]-1,6-dihydro-6-oxo-9\(H\)-purine (30)

Compound 29 (4.63 g, 9.0 mmol) was treated for 1 h at 45 °C with sodium 2-cyanoethoxide, which was prepared from 3-hydroxypropionitrile (2.04 mL, 30.0 mmol) and sodium hydride (909.0 mg, 27.3 mmol) in THF (150.0 mL). The reaction was quenched with acetic acid (3.0 mL) and the mixture was recrystallized with AcOEt/hexane to give crystals 30 (3.95 g, 7.92 mmol, 88%). \(^1\)H NMR (400MHz, DMSO-\(d_6\)): \(\delta\) 12.45 (1 H, s, H-1), 8.08 (1 H, d, J 2.4, H-8), 7.93 (1 H, s, H-2), 6.33 (1 H, dd, J 16.8 and 4.0, H-1'), 5.30-5.50 (1 H, m, H-2'), 4.38 (1 H, m, H-4'), 3.31 (1 H, m, H-5'), 3.15 (1 H, dd, J 10.4 and 3.2, H-5'b); HRMS (ESI) Calcd for C\(_{29}\)H\(_{25}\)FN\(_{4}\)NaO\(_3\) [M+Na\(^+\): 519.18029. Found 519.17744.

9-[2,3-Dideoxy-2-fluoro-\(\beta\)-D-threo-pentofuranosyl]-1,6-dihydro-6-oxo-9\(H\)-purine (31)

Compound 30 (3.56 g, 7.16 mmol) was dissolved in MeOH (100.0 mL), and conc. HCl (4.0 mL) was added to the solution by dropwise, and then stirred for 2 h at room temperature. The mixture was quenched with saturated aqueous sodium bicarbonate solution (30 mL) for neutralization, and then evaporated. The residue was purified by silica gel column chromatography (30% MeOH in CHCl\(_3\)) to give crystals 31 (1.82 g, 7.16 mmol, 100%). \(^1\)H NMR (400MHz, DMSO-\(d_6\)): \(\delta\) 12.32 (1 H, s, H-1), 8.24 (1 H, d, J 2.4, H-8), 8.08 (1 H, s, H-2), 6.39 (1 H, dd, J 15.2 and 4.0, H-1'), 5.35-5.56 (1 H, m, H-2'), 5.04 (1 H, m, 5'-OH), 4.18 (1 H, m, H-4'), 3.61 (2 H, m, H-5'ab), 2.57 (1 H, m, H-3'a), 2.26 (1 H, m, H-3'b); HRMS (ESI) Calcd for C\(_{10}\)H\(_9\)FN\(_{4}\)NaO\(_4\) [M+Na\(^+\): 277.07074. Found 277.06581.

9-[2,3-Dideoxy-2-fluoro-\(\beta\)-D-threo-pentofuranosyl]-1,6-dihydro-6-oxo-9\(H\)-purine (32)

Compound 31 (1.80 g, 7.08 mmol), TEMPO (300.0 mg, 1.92 mmol), and iodo benzene diacetate (5.50 g, 17.1 mmol) were dissolved in MeCN/H\(_2\)O (1:1, 24.0 mL) and stirred, with the exclusion of light, for 3 h. The solvents were carefully evaporated from the resultant suspension, and the reaction residue was sequentially triturated with acetone and Et\(_2\)O to yield the acid 32 (1.77 g, 6.58 mmol, 93%). \(^1\)H NMR (400MHz, DMSO-\(d_6\)): \(\delta\) 12.40 (1 H, s, H-1), 8.35 (1 H, d, J 2.4, H-8), 8.08 (1 H, s, H-2), 6.46 (1 H, dd, J 14.0 and 3.2, H-1'), 5.25-5.48 (1 H, m, H-2'), 4.78 (1 H, J 9.6 and 2.4, H-4'), 2.85 (1 H, m, H-3'a), 2.61 (1 H, m, H-3'b); HRMS (ESI) Calcd for C\(_{10}\)H\(_9\)FN\(_{4}\)NaO\(_4\) [M+Na\(^+\): 291.05000. Found 291.05005.

Ethyl 4-[[3-(1,6-dihydro-6-oxo-9\(H\)-purin-9-yl)-1-deoxy-2,3-dideoxy-2-fluoro-\(\beta\)-D-threo-pentofuranosyl]amino]benzoate (33)
Compound 32 (600.0 mg, 2.2 mmol) was dissolved in MeCN (150.0 mL), and ethyl 4-aminobenzoate (1834.0 mg, 11.1 mmol), thionyl chloride (10.0 mL, 137.1 mmol) were added to the solution, and then stirred for 46 h at 40 °C. The mixture was extracted with AcOEt, and neutralized with saturated aqueous sodium bicarbonate solution, and washed with saturated aqueous sodium chloride solution, and dried with sodium sulfate, and then evaporated. The residue was purified by silica gel column chromatography (20% MeOH in CHCl₃) to give crystals 33 (860.0 mg, 2.05 mmol, 93%). ¹H NMR (400MHz, DMSO-d₆): δ 12.40 (1 H, s, -CONH-), 10.46 (1 H, s, H-1), 8.41 (1 H, d, J 2.4, H-8), 8.10 (1 H, s, H-2), 7.95 (2 H, d, J 8.8, Ar), 7.88 (2 H, d, J 8.8, Ar), 6.52 (1 H, dd, J 19.6 and 3.2, H-1'), 5.37-5.54 (1 H, m, H-2'), 4.87 (1 H, dd, J 9.2 and 3.6, H-4'), 4.30 (2 H, q, J 7.2, -OEt), 2.98 (1 H, m, H-3'a), 2.69 (1 H, m, H-3'b), 1.32 (1 H, t, J 7.2, -OEt); HRMS (ESI) Calcd for C₁₉H₁₈FN₅NaO₅ [M+Na]⁺: 438.11842. Found 438.11782.

4-[[3-(1,6-Dihydro-6-oxo-9H-purin-9-yl)-1-deoxy-2,3-dideoxy-2-fluoro-β-D-threo-pentofuranosyl]-amino]benzoic acid (5)

Compound 33 (53.3 mg, 0.13 mmol) was dissolved in MeOH (3.0 mL), and 1.0 M potassium carbonate aq. (1.0 mL) was added to the solution, and then stirred for 5 h at 50 °C. The mixture was cooled down with ice bath, and acidified with acetic acid (ca. 120 µL) at pH 3. The reaction mixture was evaporated, and the resultant residue was recrystallized with MeOH to give white crystals 5 (49.7 mg, 0.13 mmol, 100%). ¹H NMR (400MHz, DMSO-d₆): δ 10.28 (1 H, s, H-1), 8.41 (1 H, d, J 2.4, H-8), 8.08 (1 H, s, H-2), 7.89 (2 H, d, J 8.4, Ar), 7.69 (2 H, d, J 8.4, Ar), 6.50 (1 H, dd, J 19.6 and 3.6, H-1'), 5.38-5.56 (1 H, m, H-2'), 4.85 (1 H, dd, J 9.2 and 4.0, H-4'), 2.98 (1 H, m, H-3'a), 2.68 (1 H, m, H-3'b); ¹³C NMR (100MHz, CD₃OD): δ 171.9, 171.6, 159.5, 149.8, 147.4, 141.1, 132.3, 131.3, 125.1, 120.9, 114.7, 93.1, 87.5, 78.2, 36.7; HRMS (ESI) Calcd for C₁₇H₁₄FN₅NaO₅ [M+Na]⁺: 410.08712. Found 410.08754.

Cell culture

A co-culture system of HUVEC and human fibroblasts (Angiogenesis Kit) was supplied in 24-well plates by Kurabo. Cells were incubated for 10 days prior to analysis with 450 µL of the culture medium and a 50 µL of saline that includes various additives. Culture medium was changed every 3 days, each time including freshly prepared additives.

Tube formation assay

Ten days following incubation periods with co-cultured fibroblasts and substrates (1–4), HUVEC were stained using Tubule Staining Kit for CD31. The area of the formed tube was measured by the ImageJ program. Two pictures from each well were provided for the estimation. VEGF (10 ng/mL) was used as a positive control.
Proliferation assay
HUVEC were seeded on gelatin coated 96-well plates, typically at 3000 cells/well in 100 µL of maintenance medium. After seeding, the plates were incubated for 24 h to permit anchorage, and then the culture medium was changed for the assay medium, consisting of 90 µL of HuMedia EB2 with 2% heat-inactivated FBS, and 10 µL of saline containing additives. The contents of HuMedia EB2 are almost identical with those of HuMedia EG2, except for the fact that the former does not include FBS, growth factor or antibiotics. The proliferation assay was performed using a Cell Counting Kit-8 48 h after the addition of compound 4.

Statistics
All experiments was performed at least 5 times. Mean values and standard error (SE) mean were shown. Statistical differences between groups were analyzed by Student’s t-test and Dunnett’s multiple comparison test using Microsoft Excel, and ANOVA followed by Scheffe’s F test using STAT VIEW II (Abacus Concepts). A P value less than 0.05 was considered to be statistically significant.

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