Antirheumatic Agents. I. Novel Methotrexate Derivatives Bearing an Indoline Moiety

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Various novel methotrexate (MTX) derivatives bearing an indoline moiety were synthesized and tested for biological activities using human peripheral blood mononuclear cell (hPBMC) and human synovial cells (hSC) derived from patients with rheumatoid arthritis (RA). Compounds having potent activity *in vitro* were further evaluated using an adjuvant arthritis model *in vivo*. *N*-[1-(2,4-Diamino-6-pteridinylmethyl)indoline-5-carbonyl]-L-glutamic acid 2f showed more potent activities than MTX *in vitro* and *in vivo*, and *N*-[1-(2,4-diamino-6-pteridinylmethyl)-indoline-5-carbonyl]-L-2-aminoadipic acid 2d exhibited fairly good activities *in vitro* and considerable activity *in vivo*. Compound 2d was, as expected, not sensitive to folyl-polyglutamate synthetase (FPGS) and did not undergo polyglutamation, a process which may be responsible for a side-effect during MTX therapy.

Key words methotrexate; polyglutamation; antirheumatic activity

Methotrexate (MTX, Chart 1), synthesized about 50 years ago, is still in use as an antileukemia agent with high antifolate activity. It is also effective in the treatment of rheumatoid arthritis[1] (RA), psoriasis[2] and other diseases[3,4] on the basis of its biological profile. A double-blind test of RA with MTX in the U.S.A. has recently confirmed its high efficacy.[5] However, long-term MTX therapy can have serious side effects, such as hepatic disorder and lung fibrosis,[6] and the development of less toxic MTX derivatives is desirable. Therefore, we have designed and synthesized novel MTX derivatives (Chart 2) in order to develop a safer agent than MTX for use as an antirheumatic agent.

For the reduction of side effects caused by MTX, we have focused on the polyglutamation at the γ-carboxylic acid of the glutamic acid moiety in MTX.[7] Such polyglutamation is suggested to be responsible not only for the potentiation of efficacy, but also for the side effects during MTX therapy, because the accumulation of polyglutamated MTX catalyzed by folyl-polyglutamate synthetase (FPGS) causes cell death due to the depletion of reduced folates.[8] Therefore, we selected other amino acids such as ornithine, homoglutamic acid and homocysteic acid. These amino acids were not expected to be converted into polyglutamated metabolites because they have no carboxylic acid at their γ-position.

Oefner *et al.* analyzed the crystal structure of human dihydrofolate reductase (DHFR) with folate and developed a model for the binding of MTX to DHFR.[9] According to this model, there existed a hydrophobic open space between DHFR and the aminobenzoic acid part of MTX. We speculated that filling this space with a fixed hydrophobic substituent might cause tighter binding to DHFR and higher anti-DHFR activity than MTX. This hypothesis led us to design novel MTX derivatives bearing an indoline moiety in place of the aminobenzoic acid part.

Based on the above considerations, we have focused on the replacement of glutamic acid with other amino acids and the introduction of an indoline moiety in place of aminobenzoic acid. We report here the synthesis of novel MTX derivatives and their biological activities.

Chemistry

Compounds **2a—d, f** were synthesized by standard methods from **3** as shown in Chart 3. Indoline-5-carboxylic acid (**3**) was prepared according to Ikan and Rapaport's method.[10] The amino group of **3** was further protected with a carbobenzoxy group. The protected compound **4** was converted to the acid chloride by treatment with

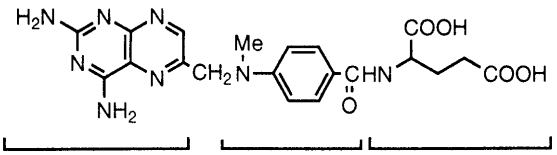

Chart 1

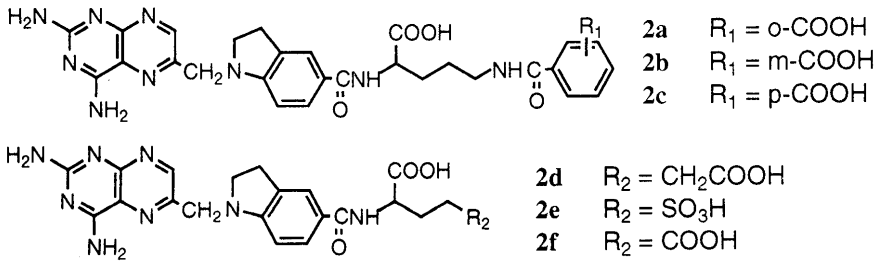

Chart 2

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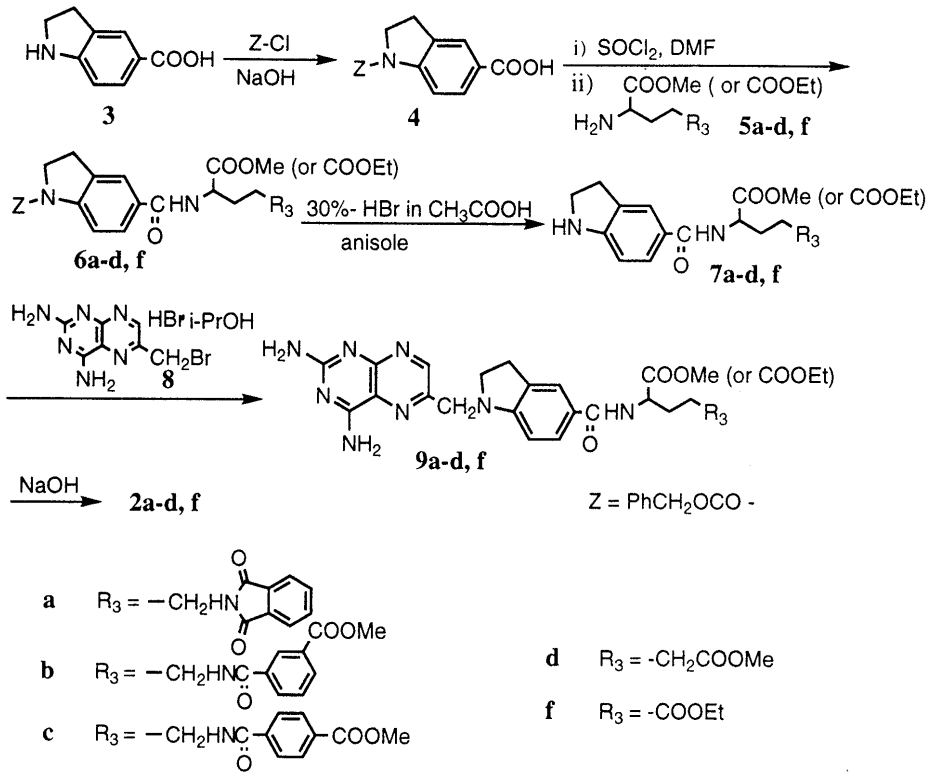

Chart 3

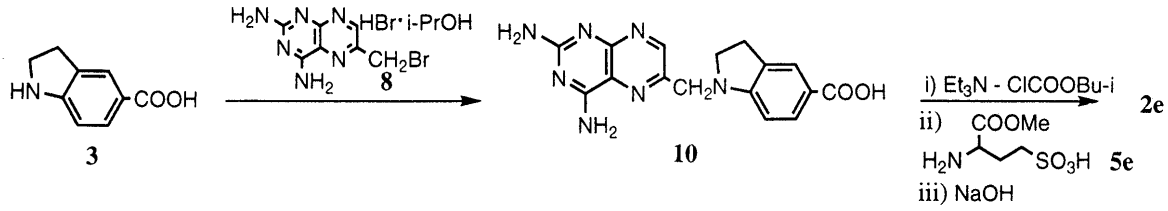

Chart 4

thionyl chloride, and subsequent coupling with amino esters **5a—d, f** was performed by the Schotten–Baumann procedure to give the amides **6a—d, f**. The carbobenzoxy group of **6a—d, f** was then removed with HBr–CH_3COOH or by palladium catalytic hydrogenation to give the deprotected amines **7a—d, f**. These amines were effectively alkylated with 6-bromomethyl-2,4-diaminopteridine (**8**)[11] to give compounds **9a—d, f**. These were hydrolyzed with 1 N NaOH to give the final compounds **2a—d, f**.

Compound **2e** was synthesized using DeGraw's method[12] as shown in Chart 4. Unlike **2a—d, f**, **3** was alkylated with **8** first to give compound **10**. The amidation of **10** with **5e** was performed by the mixed acid anhydride method and the resulting product (sulfonic acid methyl ester) was hydrolyzed with alkali to give **2e**.

Results and Discussion

The compounds synthesized in this study were tested *in vitro* on human peripheral blood mononuclear cells (hPBMC) and human synovial cells (hSC) derived from patients with RA. All tested compounds exhibited strong anti-proliferative activities in hPBMC assay as shown in Table 1. In particular, **2a** inhibited the hPBMC proliferation with the IC_{50} of 2.1 nM, which is 10 times less than that of the parent MTX. However, **2b** and **2c**, which are isomers of **2a**, exhibited less potent inhibitory activities with IC_{50} values of 10 and 71 nM, respectively, than **2a** in the hPBMC assay. Although **2a**, **2b** and **2c** have N^{δ}-(COOH-substituted benzoyl) ornithine in the place of the amino acid moiety, their IC_{50} values in the hPBMC assays varied depending on the position of the COOH group on

Table 1. Anti-proliferative Activities of Novel MTX Derivatives

Compound No.	IC_{50} (nM) value	
	Human PBMC[a]	Human SC[b]
2a	2.1	22
2b	10	110
2c	71	2600
2d	41	100
2e	22	150
2f	10	16
MTX	18	55

a) PBMC (1×10^5 cells) from several healthy donors were cultured with various concentrations of drugs and PHA (0.3 μg/ml) for 3 d. [^{3}H]UdR (1 μCi/well) was added to each well for the last 5 h of culture and the proliferation was assessed by determining [^{3}H]UdR uptake into the cells. The results are the mean of triplicate assays. *b*) SC (3×10^3 cells) from synovial membrane of RA patients were cultured with various concentrations of drugs for 5 d. [^{3}H]UdR was added for the last 2 d of culture and the proliferation was assessed by determining [^{3}H]UdR uptake into the cells. The results are the mean of triplicate assays.

the benzene ring. These findings suggest that each COOH group of **2a**, **2b** and **2c** interacts specifically with a target protein (presumably DHFR), and/or there is a critical size for the binding pocket. Compounds **2d** and **2e** having a similar amino acid to the parent MTX, inhibited hPBMC proliferation with the IC_{50} values of 41 and 22 nM, respectively, exhibiting somewhat weaker anti-proliferative activity than MTX. Compound **2f**, having glutamic acid, which is the same amino acid as that of MTX, exhibited a somewhat more potent inhibitory activity (IC_{50} of 10 nM) than that of MTX in the hPBMC assays. This result supports our hypothesis that compounds having a fixed hydrophobic substituent on the benzene ring of MTX bind more tightly to DHFR than MTX does.

In contrast with the results in the hPBMC assays, all tested compounds exhibited anti-proliferative activities to various extents in the hSC assays. The IC_{50} value of **2c** in the hSC assay was 2600 nM, 50 times less active than MTX, whereas the activity of **2c** in the hPBMC assay was only 3.5 times less than that of MTX. The IC_{50} values of the other compounds in the hSC assays ranged from 16 to 150 nM, and that of MTX was 55 nM.

To evaluate the antirheumatic activities of **2a**, **2d**, **2e** and **2f**, these compounds were tested for ability to suppress the development of the rat adjuvant arthritis model[13] *in vivo*. The results in Fig. 1 show that **2f** completely suppressed the development of arthritis at a dose of 0.25 mg/kg *p.o.* and that **2f** had a more potent activity than the parent MTX at the same dose. Also, as shown in Figs. 2 and 3, **2d** and **2e** suppressed the development of the arthritis in a dose-dependent manner in the range from 1.0 to 5.0 mg/kg *p.o.* On the other hand, **2a**, which exhibited the most potent anti-proliferative activity in the hPBMC assays, did not exhibit any suppressive activity in this arthritis model (data not shown).

We had postulated that MTX derivatives having glutamic acid, such as **2f**, could be polyglutaminated by FPGS, while MTX derivatives having another amino acid could not. Therefore, **2d** and **2f** were evaluated for activity as substrates of FPGS *in vitro* using Moran's method.[14] The results (Table 2) suggest that **2f** is a good substrate of FPGS, like MTX, but **2d** is not.

As previously mentioned, polyglutamation is thought to be responsible for the toxicities during MTX therapy as well as the potentiation of the efficacy of MTX. So it is important to know whether or not MTX derivatives that can not be metabolized to polyglutamate, can exhibit potent pharmacological effects *in vivo*. In this respect, **2d** is noteworthy, even though its activity *in vivo* is somewhat less than that of MTX. Although **2f** is rather more potent

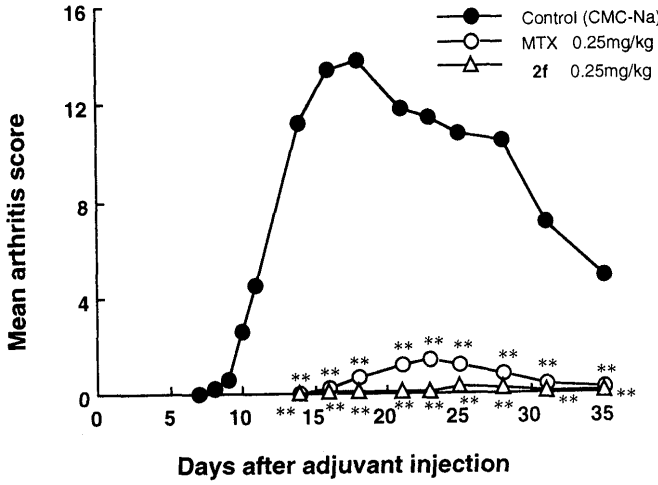

Fig. 1. Effect of MTX and **2f** on the Development of Adjuvant Arthritis in Rats

Drugs were suspended in 0.1% CMC-Na solution and administered orally 5 times a week for 2 weeks from the day of adjuvant injection. Each group consisted of 5 rats. Statistical significance of differences from the control was analyzed by means of Wilcoxon's rank sum test. $** p<0.02$.

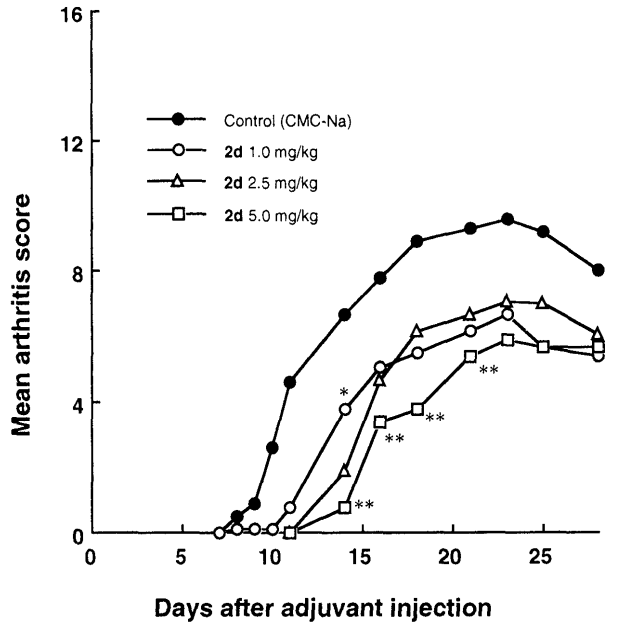

Fig. 2. Effect of **2d** on the Development of Adjuvant Arthritis in Rats

Drugs were suspended in 0.1% CMC-Na solution and administered orally 5 times a week for 2 weeks from the day of adjuvant injection. Each group consisted of 5 rats. Statistical significance of differences from the control was analyzed by means of Wilcoxon's rank sum test. $** p<0.02$; $* p<0.05$.

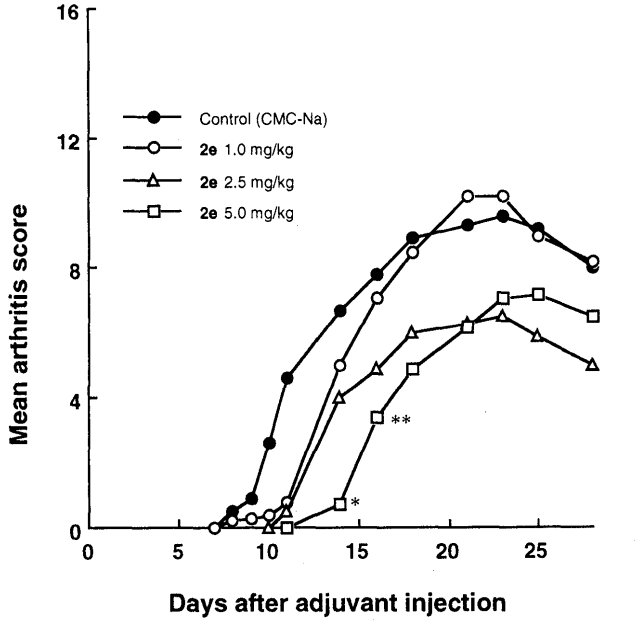

Fig. 3. Effect of **2e** on the Development of Adjuvant Arthritis in Rats

Drugs were suspended in 0.1% CMC-Na solution and administered orally 5 times a week for 2 weeks from the day of adjuvant injection. Each group consisted of 5 rats. Statistical significance of differences from the control was analyzed by means of Wilcoxon's rank sum test. $** p<0.02$; $* p<0.05$.

Table 2. Activities of MTX Derivatives as Substrates of FPGS

Compound No.	Drug concentration (μM)	FPGS activity (nmol/mg/h)
MTX	100	2.64
2f	100	1.26
2d	100	<0.01
2d	500	0.07

FPGS activity was determined as described in ref. 14, with partially purified enzyme from rat liver. The results are the mean of triplicate assays.

activity *in vivo* than MTX, it is of less interest because it does undergo polyglutamation.

In conclusion, we have found canditate antirheumatic agents **2d** and **2e** which are expected to be safer therapeutic drugs than MTX because they do not undergo polyglutamation. Furthermore, **2d** and **2e** were orally active in a dose-dependent manner in a rat adjuvant arthritis model. Such drugs could be of great benefit to RA patients who have to take drugs over a long period.

Experimental

NMR spectra were recorded on a JEOL Model JNM-FX200 NMR spectrometer with Me_4Si as the reference, infrared spectra were recorded on a Hitachi 270-3 Infrared Spectrometer, and mass spectra were recorded on a Shimadzu GCMS-QP1000. FAB and HR-FAB mass spectra were recorded on a VG Analytical VG11-250, and HR mass spectra were recorded on a Fisons OPUS-3100. TLC was routinely performed on Merck Kieselgel 60 F254. HPLC analysis was performed on a Hitachi L-3000 (detector) with a Hitachi L-6200 (pump), and the column was a YMC-Pack A-312 S-5 120A ODS. Melting points were taken with a Yanaco MP.

N^{δ}-(3-Methoxycarbonylbenzoyl)ornithine Methyl Ester (5b) A mixture of N^{α}-carbobenzoxyornithine (2.4 g), isophthalic acid monomethyl ester chloride (1.9 g) and potassium carbonate (4.0 g) in CH_2Cl_2–H_2O (1 : 1, 80 ml) was vigorously stirred overnight at room temperature, and concentrated under reduced pressure until the total volume was about 40 ml. The pH of the concentrate was adjusted to 3.0 with 1 N HCl, and the whole was extracted with AcOEt. The extract was dried over Na_2SO_4 and concentrated. The residue was dissolved with dimethylformamide (100 ml). To this solution was added potassium carbonate (2.5 g) and methyl iodide (5.0 ml), and then the resulting mixture was stirred overnight. It was then poured into water and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4 and filtered and the filtrate was concentrated. To a solution of the residue in MeOH (50 ml) was added 5%-Pd/C (100 mg), and this mixture was stirred overnight under a hydrogen atmosphere. It was filtered and the filtrate was concentrated. The residue was chromatographed on silica gel with $CHCl_3$–MeOH (20 : 1) as the eluent to give 360 mg (12%) of **5b** as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 1.6—2.1 (4H, m), 3.50 (3H, s), 3.72 (3H, s), 3.92 (3H, s), 7.49 (1H, t, J=7.8 Hz), 7.63 (1H, m), 8.0—8.2 (2H, m), 8.43 (s, 1H). IR (KBr) cm^{-1}: 3600—3100, 2950, 1730—1700, 1680—1640. MS m/z: 308 (M^+), 163. *Anal.* Calcd for $C_{15}H_{20}N_2O_5$: C, 58.43; H, 6.54; N, 9.09. Found: C, 58.20; H, 6.49; N, 9.07.

N^{δ}-(4-Methoxycarbonylbenzoyl)ornithine Methyl Ester (5c) Using the same procedure as described for the preparation of **5b**, compound **5c** was prepared from N^{α}-carbobenzoxyornithine and terephthalic acid monomethyl ester chloride. The yield of **5c** was 33.7%. White powder. ^{1}H-NMR ($CDCl_3$) δ: 1.6—2.0 (4H, m), 3.50 (3H, m), 3.72 (3H, s), 3.92 (3H, s), 7.49 (1H, t, J=7.8 Hz), 7.63 (1H, m), 8.0—8.2 (2H, m), 8.43 (1H, s). IR (KBr) cm^{-1}: 3600—3100, 2950, 1730—1710, 1680—1640. MS m/z: 308 (M^+), 163. *Anal.* Calcd for $C_{15}H_{20}N_2O_5$: C, 58.43; H, 6.54; N, 9.09. Found: C, 58.34; H, 6.48; N, 9.11.

1-Carbobenzoxyindoline-5-carboxylic Acid (4) To a mixture of indoline-5-carboxylic acid (**3**, 2.0 g) and NaOH (2.7 g) in ether (20 ml)–water (20 ml) was added carbobenzoxy chloride (2.6 g) at 0 °C with vigorous stirring. Stirring was continued at room temperature. The reaction mixture was adjusted to pH 2.0 with 1 N HCl, and the resulting precipitate was collected by filtration and dried under vacuum to give 2.8 g (77%) of **4** as a white powder. ^{1}H-NMR (DMSO-d_6) δ: 3.10 (2H, t, J=8.8 Hz), 4.03 (2H, t, J=8.8 Hz), 5.23 (2H, s), 7.2—8.0 (8H, m). IR (KBr) cm^{-1}. 1720, 1680, 1610, 1500. MS m/z: 297 (M^+), 91. *Anal.* Calcd for $C_{17}H_{15}NO_4$: C, 68.68; H, 5.09; N, 4.71. Found: C, 68.72; H, 4.96; N, 4.80.

N^{α}-(1-Carbobenzoxyindoline-5-carbonyl)-N^{δ}-phthaloylornithine Methyl Ester (6a) A mixture of compound **4** (297 mg) and a catalytic amount of dimethylformamide (DMF) in thionyl chloride (2.5 ml) was stirred for 2 h at room temperature, and concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (4 ml). To this solution was added a solution of **5a**[15] (250 mg) and potassium carbonate (640 mg), and the whole mixture was vigorously stirred overnight, then poured into water, and extracted with $CHCl_3$. The extract was dried over Na_2SO_4 and concentrated. The residue was chromatographed on silica gel with $CHCl_3$–MeOH (100 : 1) as the eluent to give 330 mg (59%) of **6a** as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 1.6—2.1 (4H, m), 3.12 (2H, t, J=8.8 Hz), 3.72 (2H, m), 3.76 (3H, s), 4.08 (2H, t, J=8.8 Hz), 4.84 (1H, m), 5.27 (2H, s), 6.80 (1H, d, J=7.8 Hz), 7.1—7.5 (6H, m), 7.5—7.9 (6H, m). IR (KBr) cm^{-1}: 3320, 2950, 1710. MS m/z: 555 (M^+), 91. HR-MS m/z: Calcd for $C_{31}H_{29}N_3O_4$: M, 555.2005. Found: 555.1977 (M^+).

N^{α}-(1-Carbobenzoxyindoline-5-carbonyl)-N^{δ}-(3-methoxycarbonylbenzoyl)ornithine Methyl Ester (6b) Using the same procedure as described for the preparation of **6a**, compound **6b** was prepared from **4** and **5b**. The yield of **6b** was 55%. Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 1.6—2.1 (4H, m), 3.08 (t, 2H, J=8.3 Hz), 3.52 (2H, m), 3.75 (3H, s), 3.88 (3H, s), 4.06 (2H, t, J=8.3 Hz), 4.78 (1H, m), 5.26 (2H, s), 7.16 (1H, d, J=7.3 Hz), 7.2—7.5 (7H, m), 7.65 (2H, m), 7.9—8.1 (2H,m), 8.43 (1H, s). IR (KBr) cm^{-1}: 3320, 2950, 1740, 1720, 1640. MS m/z: 587 (M^+), 91. HR-MS m/z: Calcd for $C_{32}H_{33}N_3O_8$: M, 587.2267. Found: 587.2256 (M^+).

N^{α}-(1-Carbobenzoxyindoline-5-carbonyl)-N^{δ}-(4-methoxycarbonylbenzoyl)ornithine Methyl Ester (6c) Using the same procedure as described for the preparation of **6a**, compound **6c** was prepared from **4** and **5c**. The yield of **6c** was 91%. White powder. ^{1}H-NMR ($CDCl_3$) δ: 1.7—2.2 (4H,m), 3.16 (2H, t, J=8.3 Hz), 3.61 (2H, m), 3.79 (3H, s), 3.94 (3H, s), 4.11 (2H, t, J=8.3 Hz), 4.83 (1H, m), 5.29 (2H, s), 6.89 (1H, d, J=7.3 Hz), 7.02 (1H, m), 7.40 (5H, m), 7.66 (2H, d, J=7.3 Hz), 7.90 (2H, d, J=8.8 Hz), 8.08 (2H, d, J=8.8 Hz). IR (KBr) cm^{-1}: 1750, 1710, 1630, 1610. MS m/z: 587 (M^+), 91. HR-MS m/z: Calcd for $C_{32}H_{33}N_3O_8$: M, 587.2267. Found: 587.2272 (M^+).

N-(1-Carbobenzoxyindoline-5-carbonyl)-L-2-aminoadipic Acid Dimethyl Ester (6d) Using the same procedure as described for the preparation of **6a**, compound **6d** was prepared from **4** and L-2-aminoadipic acid dimethyl ester hydrochloride. The yield of **6d** was 60%. White powder. ^{1}H-NMR ($CDCl_3$) δ: 1.6—2.1 (4H, m), 2.36 (2H, t, J=6.8 Hz), 3.13 (2H, m), 3.66 (3H, s), 3.77 (3H, s), 4.09 (2H, m), 4.78 (1H, m), 5.27 (2H, s), 6.80 (1H, d, J=7.8 Hz), 7.2—7.5 (6H, m), 7.63 (2H, m). IR (KBr) cm^{-1}: 3400—3300, 2950, 1750, 1720, 1650, 1610. MS m/z: 468 (M^+), 91. HR-MS m/z: Calcd for $C_{25}H_{28}N_2O_7$: M, 468.1896. Found: 468.1898 (M^+).

N-(1-Carbobenzoxyindoline-5-carbonyl)-L-glutamic Acid Diethyl Ester (6f) Using the same procedure as described for the preparation of **6a**, compound **6f** was prepared from **4** and L-glutamic acid diethyl ester hydrochloride. The yield of **6f** was 76%. White powder. ^{1}H-NMR ($CDCl_3$) δ: 1.19 (3H, t, J=7.8 Hz), 1.25 (3H, t, J=7.8 Hz), 2.1—2.6 (4H, m), 3.06 (2H, t, J=8.3 Hz), 3.8—4.3 (6H, m), 4.75 (1H, m), 5.20 (2H, s), 6.79 (1H, d, J=7.8 Hz), 7.2—7.7 (8H,m). IR (KBr) cm^{-1}: 3320, 1760, 1730, 1710, 1630. MS m/z: 482 (M^+), 91. HR-MS m/z: Calcd for $C_{26}H_{30}N_2O_7$: M, 482.2053. Found: 482.2042 (M^+).

N^{α}-(Indoline-5-carbonyl)-N^{δ}-phthaloylornithine Methyl Ester (7a) A mixture of **6a** (330 mg) and anisole (330 mg) in 30% HBr–CH_3COOH (8 ml) was stirred for 4 h at room temperature, and then poured into a large amount of ether (50 ml). The resulting precipitates were washed with ether by decantation, and the residual oil was suspended in $CHCl_3$. The $CHCl_3$ layer was shaken with aqueous $NaHCO_3$, then the organic layer was dried over Na_2SO_4, and concentrated. The residue was chromatographed on silica gel with $CHCl_3$–MeOH (100 : 3) as the eluent to give 147 mg (84%) of **7a** as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 1.6—2.1 (4H, m), 3.04 (2H, t, J=8.3 Hz), 3.62 (2H, t, J=8.3 Hz), 3.72 (2H, m), 3.75 (3H, s), 4.86 (1H, m), 6.5—6.7 (2H, m), 7.52 (2H, m), 7.68 (2H, m), 7.82 (2H, m). IR (KBr) cm^{-1}: 3500, 2960, 1780, 1740, 1710, 1640, 1620. MS m/z: 421 (M^+), 146.

N^{α}-(Indoline-5-carbonyl)-N^{δ}-(3-methoxycarbonylbenzoyl)ornithine Methyl Ester (7b) Using the same procedure as described for the preparation of **7a**, compound **7b** was prepared from **6b**. The yield of **7b**

was 65%. Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 1.6—2.1 (4H, m), 3.08 (2H, t, J=8.3Hz), 3.52 (2H, m), 3.67 (2H, t, J=8.3 Hz), 3.75 (3H, s), 3.89 (3H, s), 4.75 (1H, m), 6.73 (1H, d, J=7.8 Hz), 6.93 (1H, m), 7.16 (1H, d, J=7.8 Hz), 7.47 (1H, m), 7.55 (2H, m), 8.09 (3H, m), 8.45 (1H, s). IR (neat) cm^{-1}: 3500—3200, 2950, 1750—1710, 1670—1610. MS m/z: 453 (M^+), 146.

N^{α}-(Indoline-5-carbonyl)-N^{δ}-(4-methoxycarbonylbenzoyl)ornithine Methyl Ester (7c) Using the same procedure as described for the preparation of **7a**, compound **7c** was prepared from **6c**. The yield of **7c** was 60%. Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 1.6—2.1 (4H, m), 3.05 (2H, t, J=8.3 Hz), 3.23 (1H, br s), 3.48 (2H, m), 3.62 (2H, t, J=8.3 Hz), 3.76 (3H, s), 3.94 (3H, s), 4.75 (1H, m), 6.57 (1H, d, J=7.8 Hz), 7.19 (1H, d, J=7.8 Hz), 7.55 (2H, m), 7.89 (3H, m), 8.07 (2H, d, J=8.3 Hz). IR (neat) cm^{-1}: 3390, 3300, 2950, 1740, 1720. MS m/z: 453 (M^+), 146.

N-(Indoline-5-carbonyl)-L-2-aminoadipic Acid Dimethyl Ester (7d) Using the same procedure as described for the preparation of **7a**, compound **7d** was prepared from **6d**. The yield of **7d** was 60%. Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 1.6—2.1 (4H, m), 2.36 (2H, t, J=6.8 Hz), 3.07 (2H, m), 3.66 (2H, m), 3.66 (3H, s), 3.77 (3H, s), 4.18 (1H, m), 6.62 (2H, m), 7.54 (2H, m). IR (neat) cm^{-1}: 3400—3300, 2950, 1750, 1720, 1650. MS m/z: 334 (M^+), 146.

N-(Indoline-5-carbonyl)-L-glutamic Acid Diethyl Ester (7f) Using the same procedure as described for the preparation of **7a**, compound **7f** was prepared from **6f**. The yield of **7f** was 93%. White powder. ^{1}H-NMR ($CDCl_3$) δ: 1.21 (3H, t, J=7.8 Hz), 1.29 (3H, t, J=7.8 Hz), 2.0—2.6 (4H, m), 3.00 (2H, t, J=8.3 Hz), 3.59 (2H, t, J=8.3 Hz), 4.07 (2H, m), 4.19 (2H, m), 4.75 (1H, m), 6.47 (1H, d, J=8.8 Hz), 6.68 (1H, d, J=8.3 Hz), 6.72 (1H, br s), 7.45 (1H, d, J=8.8 Hz), 7.49 (1H, s). IR (KBr) cm^{-1}: 3400, 3300, 2980, 1740, 1720, 1630, 1610. MS m/z: 348 (M^+), 146. HR-MS m/z: Calcd for $C_{18}H_{24}N_2O_5$: M, 348.1685. Found: 348.1689 (M^+).

N^{α}-[1-(2,4-Diamino-6-pteridinylmethyl)indoline-5-carbonyl]-N^{δ}-phthaloylornithine Methyl Ester (9a) A suspension of **7a** (115 mg) and 6-bromomethyl-2,4-diamino-pteridine·HBr·iso-PrOH (**8**, 146 mg) in dimethylacetamide (DMA) (1 ml) was stirred for 5 h at 65 °C. The mixture was then poured into aquenous $NaHCO_3$, and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4, filtered and concentrated. The residue was chromatographed on silica gel with $CHCl_3$–MeOH (10 : 1) as an eluent to give 140 mg (75%) of **9a** as an orange powder. ^{1}H-NMR ($CDCl_3$—CD_3OD) δ: 1.7—2.2 (4H, m), 3.06 (2H, t, J=8.3 Hz), 3.56 (2H, t, J=8.3 Hz), 3.72 (2H, m), 3.76 (3H, s), 4.53 (2H, s), 4.88 (1H, m), 6.4—6.6 (2H, m), 7.56 (2H, m), 7.71 (2H, m), 7.84 (2H, m), 8.82 (1H, s). IR (KBr) cm^{-1}: 3350—3200, 1710, 1630, 1610, 1430. FAB-MS m/z: 596 (MH^+). HR-FAB-MS m/z: Calcd for $C_{30}H_{30}N_9O_5$: MH^+, 596.2373. Found: 596.2386 (MH^+).

N^{α}-[1-(2,4-Diamino-6-pteridinylmethyl)indoline-5-carbonyl]-N^{δ}-(3-methoxycarbonylbenzoyl)ornithine Methyl Ester (9b) Using the same procedure as described for the preparation of **9a**, compound **9b** was prepared from **7b**. The yield of **9b** was 45%. Orange powder. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 1.7—2.1 (4H, m), 3.06 (2H, t, J=8.3 Hz), 3.55 (4H, m), 3.78 (2H, s), 3.93 (3H, s), 4.52 (2H, s), 4.79 (1H, m), 6.51 (1H, d, J=7.8 Hz), 7.05 (1H, d, J=7.8 Hz), 7.4—7.7 (3H, m), 8.0—8.2 (2H, m), 8.45 (1H, m), 8.77 (1H, s). IR (KBr) cm^{-1}: 3500—3100, 2950, 1730, 1650, 1640, 1610. FAB-MS m/z: 628 (MH^+). HR-FAB-MS m/z: Calcd for $C_{31}H_{34}N_9O_6$: MH^+, 628.2635. Found: 628.2646 (MH^+).

N^{α}-[1-(2,4-Diamino-6-pteridinylmethyl)indoline-5-carbonyl]-N^{δ}-(4-methoxycarbonylbenzoyl)ornithine Methyl Ester (9c) Using the same procedure as described for the preparation of **9a**, compound **9c** was prepared from **7c**. The yield of **9c** was 93%. Orange powder. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 1.6—2.1 (4H, m), 3.08 (2H, t, J=8.8 Hz), 3.4—3.7 (4H, m), 3.79 (3H, s), 3.94 (3H, s), 4.54 (2H, s), 4.83 (1H, m), 6.52 (1H, d, J=8.3 Hz), 6.79 (1H, d, J=6.8 Hz), 7.17 (1H, m), 7.59 (2H, m), 7.92 (2H, m), 8.09 (2H, d, J=8.3 Hz), 8.81 (1H, s). IR (KBr) cm^{-1}: 3470—3200, 1720, 1610, 1540, 1500, 1440. FAB-MS m/z: 628 (MH^+). HR-FAB-MS m/z: Calcd for $C_{31}H_{34}N_9O_6$: MH^+, 628.2635. Found: 628.2624 (MH^+).

N-[1-(2,4-Diamino-6-pteridinylmethyl)indoline-5-carbonyl]-L-2-aminoadipic Acid Dimethyl Ester (9d) Using the same procedure as described for the preparation of **9a**, compound **9d** was prepared from **7d**. The yield of **9d** was 55%. Orange powder. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 1.6—2.1 (4H, m), 2.38 (2H, t, J=6.8 Hz), 3.07 (2H, m), 3.57 (2H, m), 3.67 (3H, s), 3.78 (3H, s), 4.53 (2H, s), 4.74 (1H, m), 6.52 (1H, d, J=8.3 Hz), 7.01 (1H, d, J=7.8 Hz), 7.57 (2H, m), 8.77 (1H, s). IR (KBr) cm^{-1}: 3500—3200, 1740, 1630, 1610, 1500. FAB-MS m/z: 509 (MH^+). HR-MS m/z: Calcd for $C_{24}H_{28}N_8O_5$: M, 508.2182. Found: 508.2207 (M^+).

N-[1-(2,4-Diamino-6-pteridinylmethyl)indoline-5-carbonyl]-L-glutamic Acid Diethyl Ester (9f) Using the same procedure as described for the preparation of **9a**, compound **9f** was prepared from **7f**. The yield of **9f** was 63%. Orange powder. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 1.22 (3H, t, J=7.8 Hz), 1.30 (3H, t, J=7.8 Hz), 2.0—2.5 (4H, m), 3.07 (2H, t, J=8.3 Hz), 3.57 (2H, t, J=8.3 Hz), 4.10 (2H, m), 4.23 (2H, m), 4.79 (1H, m), 5.24 (2H, s), 6.51 (1H, d, J=8.3 Hz), 6.76 (1H, d, J=7.8 Hz), 7.57 (2H, m), 8.82 (1H, s). IR (KBr) cm^{-1}: 3300—3200, 1730, 1630, 1610, 1590. FAB-MS m/z: 523 (MH^+). HR-MS m/z: Calcd for $C_{25}H_{30}N_8O_5$: M, 522.2339. Found: 522.2323 (M^+).

N^{α}-[1-(2,4-Diamino-6-pteridinylmethyl)indoline-5-carbonyl]-N^{δ}-hemiphthaloylornithine (2a) To a solution of **9a** (140 mg) in EtOH (10 ml) was added 1 N NaOH (0.75 ml) and the reaction mixture was stirred overnight at room temperature, then concentrated under reduced pressure. The residue was dissolved in water (10 ml), and the solution was acidified to pH 3.7 with 1 N HCl. The precipitate was collected by filtration and dried under vacuum to give 86.7 mg (63%) of **2a** as an orange powder. ^{1}H-NMR (DMSO-d_6) δ: 1.5—2.1 (4H, m), 3.14 (2H, t, J=8.3 Hz), 3.58 (2H, t, J=8.3 Hz), 4.38 (1H, m), 4.54 (2H, s), 6.71 (1H, m), 7.3—7.6 (4H, m), 7.6—7.8 (3H, m), 8.08 (1H, m), 8.71 (1H, s). IR (KBr) cm^{-1}: 3500—3400, 1640, 1620. FAB-MS m/z: 600 (MH^+). mp 195—199 °C (dec). $[\alpha]_D^{25}$ −2.00° (c=0.30, 1 N $NaHCO_3$). Analysis by HPLC (solvent, CH_3COOH–CH_3COONa, pH 5.4: MeOH = 7 : 3; flow rate 1.0 cm^3/min; detection, absorb. 254 nm) showed the purity to be at least 97% (retention time 18 min).

N^{α}-[1-(2,4-Diamino-6-pteridinylmethyl)indoline-5-carbonyl]-N^{δ}-isophthaloyl Ornithine (2b) Using the same procedure as described for the preparation of **2a**, compound **2b** was prepared from **9b**. The yield of **2b** was 75%. Orange powder. ^{1}H-NMR (DMSO-d_6) δ: 1.5—2.0 (4H, m), 3.00 (2H, t, J=8.8 Hz), 3.32 (2H, m), 3.59 (2H, t, J=8.8 Hz), 4.40 (1H, m), 4.56 (2H, s), 6.69 (1H, d, J=8.3 Hz), 7.56 (1H, m), 7.63 (2H, m), 8.0—8.2 (2H, m), 8.44 (1H, s), 8.65 (1H, m), 8.73 (1H, s). IR (KBr) cm^{-1}: 3600—3000, 2950, 1640, 1610. FAB-MS m/z: 600 (MH^+). mp 200—204 °C (dec.). $[\alpha]_D^{25}$ −7.00° (c=0.40, 1 N $NaHCO_3$). Analysis by HPLC (solvent, CH_3COOH–CH_3COONa, pH 5.4: MeOH = 7 : 3; flow rate, 1.0 cm^3/min; detection, 254 nm) showed the purity to be at least 98% (retention time 19 min).

N^{α}-[1-(2,4-Diamino-6-pteridinylmethyl)indoline-5-carbonyl]-N^{δ}-terephthaloyl Ornithine (2c) Using the same procedure as described for the preparation of **2a**, compound **2c** was prepared from **9c**. The yield of **2c** was 76%. Orange powder. ^{1}H-NMR (DMSO-d_6) δ: 1.5—2.0 (4H, m), 3.00 (2H, t, J=8.8 Hz), 3.30 (2H, m), 3.58 (2H, t, J=8.8 Hz), 4.36 (1H, m), 4.54 (2H, s), 6.68 (1H, d, J=8.8 Hz), 7.62 (2H, m), 7.91 (2H, d, J=8.3 Hz), 7.99 (2H, d, J=8.3 Hz), 8.10 (1H, d, J=7.8 Hz), 8.61 (1H, m), 8.71 (1H, s). IR (KBr) cm^{-1}: 3500—3300, 1640, 1610. FAB-MS m/z: 600 (MH^+). mp 215—220 °C (dec.). $[\alpha]_D^{25}$ −9.52° (c=0.42, 1 N $NaHCO_3$). Analysis by HPLC (solvent, CH_3COOH–CH_3COONa, pH 5.4: MeOH = 7 : 3; flow rate, 1.0 cm^3/min; detection, 254 nm) showed the purity to be at least 97% (retention time 15 min).

N-[1-(2,4-Diamino-6-pteridinylmethyl)indoline-5-carbonyl]-L-2-aminoadipic Acid (2d) Using the same procedure as described for the preparation of **2a**, compound **2d** was prepared from **9d**. The yield of **2d** was 84%. Orange powder. ^{1}H-NMR (DMSO-d_6) δ: 1.4—1.9 (4H, m), 2.23 (2H, t, J=6.8 Hz), 3.01 (2H, m), 3.58 (2H, m), 4.32 (1H, m), 4.55 (2H, s), 6.69 (1H, d, J=8.3 Hz), 7.63 (2H, m), 8.10 (1H, d, J=8.3 Hz), 8.72 (1H, s). IR (KBr) cm^{-1}: 3600—3100, 1650, 1620. FAB-MS m/z: 481 (MH^+). mp 192—196 °C (dec.). $[\alpha]_D^{25}$ +19.60° (c=0.50, 1 N $NaHCO_3$). Analysis by HPLC (solvent, CH_3COOH–CH_3COONa, pH 5.4: MeOH = 3 : 1; flow rate, 1.0 cm^3/min; detection, 254 nm) showed the purity to be at least 96% (retention time 17 min).

N-[1-(2,4-Diamino-6-pteridinylmethyl)indoline-5-carbonyl]-L-glutamic Acid (2f) Using the same procedure as described for the preparation of **2a**, compound **2f** was prepared from **9f**. The yield of **2f** was 81%. Orange powder. ^{1}H-NMR (DMSO-d_6) δ: 1.94 (2H, m), 2.32 (2H, m), 2.98 (2H, t, J=8.8 Hz), 3.56 (2H, t, J=8.8 Hz), 4.29 (1H, m), 4.53 (2H, s), 6.71 (1H, d, J=8.8 Hz), 7.57 (1H, s), 7.59 (1H, d, J=8.8 Hz), 8.72 (1H, s). IR (KBr) cm^{-1}: 3600—3300, 1670, 1640. FAB-MS m/z: 467 (MH^+). mp 201—204 °C (dec.). $[\alpha]_D^{25}$ +9.33° (c=0.30, 1 N $NaHCO_3$). Analysis by HPLC (solvent, CH_3COOH–CH_3COONa, pH 5.4: MeOH = 4 : 1; flow rate, 1.0 cm^3/min; detection, 254 nm) showed the purity to be at least 96% (retention time 22 min).

1-(2,4-Diamino-6-pteridinylmethyl)indoline-5-carboxylic Acid (10) A mixture of **3** (87 mg) and **8** (137 mg) in DMA (2 ml) was stirred for 4 h

at 55 °C, poured into water, and stored in a cold place overnight. The resulting precipitate was collected by filtration. The obtained solids were dissolved in a small amount of 1 N NaOH, and adjusted to pH 6.5 with 1 N HCl. The resulting brown precipitate was collected by filtration and dried under vacuum to give 56 mg (31%) of **10** as a brown powder. ^{1}H-NMR (DMSO-d_6) δ: 3.01 (2H, t, J=8.8 Hz), 3.64 (1H, t, J=8.8 Hz), 4.60 (2H, s), 6.68 (1H, d, J=8.3 Hz), 7.16 (2H, s), 7.58 (1H, s), 7.65 (1H, d, J=8.3 Hz), 8.09 (1H, s), 8.25 (1H, s), 8.77 (1H, s). IR (KBr) cm^{-1}: 3400—3100, 1640, 1280. FAB-MS m/z: 338 (MH^+). HR-MS m/z: Calcd for $C_{16}H_{15}N_7O_2$: M 337.1287. Found: 337.1288 (M^+).

***N*-[1-(2,4-Diamino-6-pteridinylmethyl)indoline-5-carbonyl]-L-homocysteic Acid Monoammonium Salt (2e)** To a solution of compound **10** (188 mg) in DMA (20 ml) were added triethylamine (210 μl) and iso-BuOCOCl (65 μl) at room temperature under a nitrogen atmosphere and the mixture was stirred for 10 min. To this mixture were added homocysteic acid methyl ester hydrobromide **5e** (117 mg), additional triethylamine (126 μl) and iso-BuOCOCl (49 μl). More **5e** (71 mg) was added 10 min later. The reaction mixture was stirred for 30 min, and concentrated under reduced pressure. The residue was dissolved in 1 N NaOH, and the solution was adjusted to pH 8.0 with NH_4HCO_3. The resulting solution was concentrated under reduced pressure, and the residue was chromatographed on DEAE-cellulose with 3% $(NH_4)_2CO_3$ as an eluent to give 82 mg (58%) of **2e** as a yellow powder. ^{1}H-NMR (D_2O) δ: 2.0—2.4 (2H, m), 3.15 (4H, m), 3.49 (2H, m), 4.34 (1H, m), 4.49 (2H, s), 6.66 (1H, m), 7.55 (2H, m), 8.69 (1H, s). IR (KBr) cm^{-1}: 3500—3300, 1640, 1610. mp >300 °C. $[\alpha]_D^{25}$ +6.19° (c=0.50, H_2O). *Anal.* Calcd for $C_{20}H_{25}N_9O_6S \cdot 2H_2O$: C, 43.24; H, 5.26; N, 22.69; S,5.77. Found: C, 43.59; H, 5.23; N, 22.73; S, 5.93.

Peripheral Blood Mononuclear Cell Culture Peripheral blood mononuclear cells from a healthy donor were separated by centrifugation on Ficoll–Paque (Pharmacia, Uppsala, Sweden). Cells were resuspended in RPMI 1640 medium containing 5% fetal bovine serum (FBS: Hyclone Laboratories Inc., Logan, UT), glutamine, penicillin G and streptomycin. Cells (1×10^5 cells/well) were cultured in 0.2 ml in 96-well microtiter plates (Corning #25870) with phytohemagglutinin (0.3 μg/ml) (PHA: Welcome Foundation Ltd., Dartford, UK) for 3 d. [^{3}H]Deoxyuridine (UdR: 1 μCi/well) (Amersham International plc, Buckinghamshire, UK) was added to each well for the last 5 h of the culture and the proliferation was assessed by determining [^{3}H]UdR uptake into the cells.

Synovial Fibroblastic Cell Culture Synovial tissues were obtained from RA patients at the time of joint surgery. The tissue was minced and enzymatically dissociated with 5 mg/ml of collagenase (type I: Sigma Chemical Co.) and 0.15 mg/ml of deoxyribonuclease (DNase) (from bovine pancreas; Sigma Chemical Co.) in Iscove's modified Dulbecco's medium (IMDM: Gibco) for 1 h at 37 °C. The resulting cells were plated in a culture flask and allowed to adhere, and the nonadherent cells were then removed. Synovial fibroblastic cells (SC) were used for proliferation assay in the third to sixth passages. SC were resuspended in IMDM medium containing 5% FBS, supplemented with penicillin G and streptomycin. Cells (3×10^3 cells/well) were cultured in 0.2 ml in 96-well microtiter plates (Falcon #3072) for 5 d. [^{3}H]UdR (1 μCi/well) was added for the last 2 d of the culture and the proliferation was assessed by determining [^{3}H]UdR uptake.

Induction of Adjuvant Arthritis Induction of adjuvant arthritis was done as previously reported.[13] Briefly, male rats (6-week-old) were inoculated into the base of the tail with 50 μl of liquid paraffin containing 35 μg of heat-killed *Mycobacterium tuberculosis* H37 Ra (Difco Laboratories, Detroit, MI). The system described by Trentham *et al.*[16] was used to assess the severity of the arthritis. Each paw was graded from 0 to 4 based on erythema, swelling, and deformity of the joint.

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