Thiazolidine-Diones

BIOCHEMICAL AND BIOLOGICAL ACTIVITY OF A NOVEL CLASS OF TYROSINE PROTEIN KINASE INHIBITORS

(Received for publication, July 2, 1990)

Johanna F. Geissler, Peter Traxler, Urs Regenass, Brendan J. Murray, Johannes L. Roesel, Thomas Meyer, Elaine McGlynn, Angelo Storni, and Nicholas B. Lydon

From the Oncology and Virology Research Department, Pharmaceuticals Division, Ciba-Geigy Ltd., CH-4002 Basel, Switzerland

Various derivatives of thiazolidine-diones have been identified as tyrosine protein kinase inhibitors. The epidermal growth factor (EGF) receptor kinase and c-src kinase were inhibited in vitro with IC_{50} values in the range of 1-7 \mu M. The v-abl tyrosine protein kinase was not inhibited by thiazolidine-diones. Inhibition was found to be specific for tyrosine protein kinases. Inhibition of serine/threonine protein kinases was not observed. The active derivatives were shown to inhibit EGF-induced receptor autophosphorylation, either in vitro or in intact cells, and were also found to inhibit growth of the EGF-dependent BALB/MK and A431 cell lines (IC_{50} 1-3 \mu M). Growth of the interleukin-3-dependent myeloid cell line FDC-P1 was inhibited with equal efficiency. Thus, in these cell lines, members of the c-src kinase family are also potential targets for inhibition by the compounds.

Gene products involved in transducing growth stimuli in response to growth factors are frequently associated with malignant cellular proliferation. Such gene products thus represent attractive targets for chemotherapeutic intervention and interruption of signal transduction pathways.

The EGF receptor is a transmembrane glycoprotein which transduces mitogenic signals generated by binding of EGF or transforming growth factor \( \alpha \) (1). The receptor is composed of an extracellular hormone-binding domain and an intracellular tyrosine protein kinase domain which catalyzes receptor autophosphorylation and phosphorylation of cellular substrates upon stimulation (reviewed in Refs. 1-6). The growth factor-dependent tyrosine kinase activity of the cytoplasmic domain is regarded as the primary mechanism for the generation of intracellular signals that initiate the multiple cellular responses which culminate in DNA synthesis and cell proliferation. The dependence of this pathway on tyrosine kinase activity has been confirmed by site-directed mutagenesis. Mutations within the kinase domain which eliminate enzyme activity have been shown to abolish signal transduction via the EGF receptor (7, 8).

Comparison of the primary amino acid sequence within the kinase domain of members of the tyrosine protein kinase gene family have revealed a high degree of homology due to conservation of residues essential for catalytic function, especially within the nucleotide-binding site (9). However, since tyrosine protein kinases show distinct substrate specificities (10, 11) the design of selective enzyme inhibitors should be possible. Several classes of compounds have been reported to inhibit tyrosine protein kinases. These compounds include halo-methyl ketones (12, 13), amiloride (14), the microbial alkaloid staurosporine (15-17), and flavonoids such as genistein and quercetin (18-20). Although potent inhibitors, these compounds lack selectivity with respect to other kinases (e.g. serine/threonine protein kinases). Various classes of selective inhibitors of tyrosine protein kinases have been reported. These include (hydroxyphenyl) carboxylates (21), the hydroquinone erstatins (22-24), 4-hydroxycinnamamide derivatives (25, 26), and a group of cinnamic acid derivatives (27-29) referred to as tyrphostins. These compounds were found to inhibit the enzymes by competition with the phosphoryl acceptor substrate.

During a search for selective EGF receptor kinase inhibitors, we have identified a group of thiazolidine-dione derivatives which inhibit enzymes within the tyrosine protein kinase family. The present paper reports enzymatic and cellular activities of this new class of inhibitors.

EXPERIMENTAL PROCEDURES

Materials—The peptides angiotensin II, [Val²]angiotensin II, EGF, and random polymer Gln,Tyr (4:1) were from Sigma. Leupeptin and aprotinin were obtained from Boehringer Mannheim. [γ-\(^{32}\)P]ATP was from Amersham Corp. Rabbit antisemur against the EGF receptor was from Cambridge Research Biochemicals (Cambridge, United Kingdom (U.K.)). CAMP-dependent protein kinase from rabbit muscle was a gift from Dr. B. Hemmings, Friedrich Miescher Institute, Basel, Switzerland. SF9 cells were obtained from Dr. M. Summers (Texas A&M University). A431 human squamous carcinoma cells (33) were obtained from the European Collection of Animal Cell Cultures (ECACC, Porton Down, U.K.). The EGF-dependent BALB/MK mouse keratinocyte cell line was kindly provided by Dr. S. Aaronson (34). FDC-P1 mouse bone marrow-derived myeloid precursor cell line (35) was obtained from Dr. K. Ballmer (Friedrich Miescher Institute, Basel). A recombinant baculovirus carrying the c-src gene and monoclonal antibody 4G10 against phosphotyrosine were provided by Dr. T. Roberts (Dana Farber Cancer Institute, Boston). Vectors avide/Biotin Complex kits with alkaline phosphatase as enzyme marker and alkaline phosphatase substrates were obtained from Vector Laboratories, Burlingame, CA. Sodium orthovanadate was from Fisher. Cell culture reagents and materials were from Gibco/Bethesda Research Laboratories, and tissue culture plates were from Falcon.

Synthesis of Thiazolidine-Diones—The thiazolidine-dione-azines of general type I (Fig. 1) were synthesized by cyclization of a thiazoline-dione-thiosemicarbazone with appropriately substituted \( \alpha \)-brominated carboxylic acids (\( R_1 = H, CH_3, OH, CH_2OH, CH_3COOH, CH_3OCH_2 \), see Fig. 2). The thiazolidine-dione-thiosemicarbazones
with $R_1 = \text{CH}_2 = \text{CH} = \text{CH}_2$ and $R_1 = \text{CH}_2 = \text{C} \cdot \text{CH}_3 = \text{CH}_2$ were obtained in three steps starting from 1-acetyl-4-methyl-thiosemicarbazide. For the synthesis of derivatives with an exocyclic double bond (compounds 1 and 2, see Fig. 1), 2-bromo-3-methoxy-propanic acid ($R_2 = \text{CH}_2 = \text{CH} = \text{CH}_2$) was used for the condensation with the corresponding thiazolidine-dione-thiosemicarbazide as the methylenetri tert-butyliodide was cleaved with BB$_3$ to the bromomethyl derivative ($R_2 = \text{CH}_2 \text{Br}$), followed by elimination of HI. Addition of dimethylamine to the $\alpha,\beta$-unsaturated double bond in compound 2 yielded compound 3 with $R_3 = \text{CH}_2 \cdot \text{N} \cdot \text{CH}_3$.

Separation of compounds—Partially purified EGF receptor kinase was prepared from A431 cells as previously described (30). Recombinant v-abl kinase was expressed in Escherichia coli using vector pablHP. The protein A-fusion product encoded by this vector was affinity purified on IgG-Affi-Gel 10 as previously described (31). c-src kinase was obtained from S99 cells that were infected at mid-log phase with recombinant c-src baculovirus as a standard. The c-src kinase was purified to homogeneity. The specific activity of c-src kinase measured at 20°C using 10$\mu$M ATP and random polymer Glu,Tyr (4:1) as substrate was 54 nmol/min/mg. Protein kinase C from porcine brain was purified as described previously (17).

Kinase Assays—Determination of EGF receptor kinase activity was performed as described (17) using A431 membranes as the enzyme source and angiotensin II (1 mg/ml) as substrate. All compounds were dissolved in dimethyl sulfoxide giving a final concentration of 5%. EGF receptor autophosphorylation assays were performed essentially as for the EGF receptor kinase assay, but in the absence of exogenous peptide substrate, with 2$pM$ ATP (1$\mu$Ci assayed ATP; 10$\mu$M [3H]ATP (3000–5000 cpm/pmol), 10$pM$ of enzyme (~50 ng of protein), and test compounds as indicated. Reactions were terminated with 30$pM$ of Laemmli sample buffer, the mixtures were boiled for 5 min and analyzed by SDS-PAGE (32) followed by autoradiography. IC$_{50}$ values for inhibition of EGF-R autophosphorylation were estimated by laser densitometry of x-ray films. v-abl kinase was assayed essentially as described for the v-abl kinase, using the random polymer Glu,Tyr (1:4) as substrate (0.125 mg/ml) (10). Reactions were terminated with 10$\mu$M of 0.5 M EDTA, pH 8.0. Aliquots of the reaction mixtures were spotted onto glass fiber filters (Whatman), washed with 30% ice-cold trichloroacetic acid, and washed in 6% acetic acid to remove non-precipitated material. Activity determinations of protein kinase C using histone H1 (0.2 mg/ml) as substrate, and of CAMP-dependent protein kinase using Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) (0.1 mg/ml) as substrate, were performed as described previously (17).

Cell Culture—S99 cells and were grown in medium TC 100, supplemented with 10% FCS, A431 cells were cultured in DMEM, supplemented with 10% FCS, and BALB/MK cells were grown in calcium-free DMEM and Ham's F-12 medium 3:1 (v/v), supplemented with 5% FCS, insulin (5$\mu$g/ml), transferrin (5$\mu$g/ml), sodium selenate (5$\mu$g/ml), and EGF (10$\mu$g/ml) (51). FDC-P1 cells were grown in DMEM supplemented with 10% FCS, 50$\mu$M mercaptoethanol and 2% Wehi 3b conditioned medium (36).

Antiproliferative Assays—Assays were performed essentially as described previously (17). Cells (BALB/MK cells, 10,000; FDC-P1, 1,500; A431, 2,500 cells/well, respectively) were seeded into 96-well microtiter plates and incubated overnight. Drugs dissolved in dimethyl sulfoxide were added in serial dilutions (the final dimethyl sulfoxide concentrations in all assays did not exceed 1%). After addition, the plates were incubated for 3 days (MK cells) or 4 days (A431 and FDC-P1 cells) which allowed control cultures to undergo at least three cell divisions. Growth of MK or A431 cells was monitored using methylene blue staining (17). The colorimetric MTT assay was used for determining FDC-P1 cell proliferation essentially as described (37). Formazan crystals were dissolved in 10% Triton X-100 in acidic isopropyl alcohol prior to optical determination (38). IC$_{50}$ values were defined as the drug concentrations which resulted in a 50% decrease in cell number as compared with the control cultures in the absence of inhibitor. IC$_{50}$ values represent the mean and standard deviations of two to three independent experiments.

Immunoblot Analysis—A431 cells were seeded in T-25 tissue culture flasks at 10$^3$ cells/flask in DMEM with 10% FCS, grown to 80% confluence and then starved for 24 h in DMEM containing 0.5% FCS. Compounds were added and incubated with cells for the indicated pretreatment times. EGF (400 ng/ml) was added to cultures which were then incubated for another 10 min. Cells were scraped off the dish surface into the culture medium, centrifuged at 1000$\times g$ for 5 min and washed with 10 ml of phosphate-buffered saline. Cells were lysed in SDS-PAGE sample buffer and boiled for 5 min. Following SDS-PAGE (8% gels), proteins were transferred onto Immobilon membranes (Millipore, Bedford, MA) by semidyosity blotting. Membranes were blocked overnight at 4°C with 3% bovine serum albumin/phosphate-buffered saline and then incubated with primary antibody (1$\mu$g/ml in 3% bovine serum albumin/phosphate-buffered saline) for 2 h. Biotinylated secondary antibody and Avidin/Biotin complex with alkaline phosphatase were used for development according to the manufacturer (Vector). IC$_{50}$ values for phosphotyrosine blots were estimated by laser densitometry.

Protein Determination—Protein concentrations were determined according to the method of Bradford (39), using bovine serum albumin as a standard.

RESULTS

In Vitro Activity of Compounds as Inhibitors of Protein Kinases—A series of thiazolidine-diones of the general formula I (Fig. 1) were synthesized as described (Fig. 2) and tested in vitro for inhibition of the EGF receptor tyrosine protein kinase (Table I). Compounds 1–3, which have either a $\text{CH}_3$ or $\text{CH}_2 \cdot \text{N} \cdot \text{CH}_3$ group at $R_2$, were found to be potent inhibitors of the EGF receptor kinase with IC$_{50}$ values of 6, 1, and 2$\mu$M, respectively, when assayed in vitro using an exogenous peptide substrate (angiotensin II). The compounds were further tested for selectivity within the tyrosine kinase enzyme family using recombinant c-src and v-abl kinases. The inhibition profiles of compounds 1–10 with respect to c-src kinase were essentially the same as for the EGF receptor kinase (i.e. compounds 1–3 were inhibitors of the c-src kinase with IC$_{50}$ values of 4, 3, and 7$\mu$M, respectively), while compounds 4–10 were inactive. In marked contrast, the v-abl kinase was not inhibited by compounds 1–10 (IC$_{50} > 100$ µM).

Only compound 2 was found to have marginal inhibitory
Thiazolidine-Diones as Inhibitors of Tyrosine Protein Kinases

0 s

CHSN -; NHNHCOCH₃ 8rcH*COOH . t ,CNHCOCH,

PM

coMPOUND2 o 0

8 ' .- EGF . . f + * *

1 HCI

R₂ = H, CH₂, OH, CH*OH, CH₂COOH R₁ = CH₂CH=CH,

( compounds 4,5,6,7,8,9, CH₂C = CH,

RP = CH₂OCH₃ LH₃

1. Em,

2. -Hr3,

( compounds 1,2, (compound 3)

FIG. 2. Synthesis of thiazolidine-diones. Details of the chemical synthesis of the compounds are described under “Experimental Procedures.”

TABLE I

Inhibition of protein kinases by thiazolidine-diones

The abbreviations used are: PKC, protein kinase C; PKA, c-AMP-dependent protein kinase; ND, not determined.

| Compound | IC₅₀ (µM) | v-abl IC₅₀ (µM) | c-src IC₅₀ (µM) | PKC IC₅₀ (µM) | PKA IC₅₀ (µM) |
|----------|----------|----------------|----------------|----------------|----------------|
| 1        | 6        | >100           | 4              | >500           | >600           |
| 2        | 1        | 1              | 3              | >100           | >600           |
| 3        | 2        | >100           | 7              | 350            | >600           |
| 4        | >60      | >100           | >100           | ND             | ND             |
| 5        | >80      | >100           | >100           | ND             | ND             |
| 6        | >60      | >100           | >100           | ND             | ND             |
| 7        | >100     | >100           | >100           | ND             | ND             |
| 8        | >80      | >100           | >100           | ND             | ND             |
| 9        | >90      | >100           | >100           | ND             | ND             |
| 10       | ~100     | >100           | >100           | >100           | >100           |
| Genistein| 1        | 39             | >50            | 15             | >100           |

activity against the v-abl kinase (IC₅₀ ~ 100 µM). The compounds were also tested against serine/threonine protein kinases. No inhibition of either protein kinase C or cAMP-dependent protein kinase was found in the concentration range where tyrosine protein kinases were inhibited. The thiazolidine-diones thus show higher selectivity than the reference inhibitor genistein (Table I).

Inhibition of Autophosphorylation of EGF Receptor and v-abl Kinase in Vitro—EGF receptor autophosphorylation, which occurs under physiological conditions as a result of ligand stimulation, was investigated using a purified plasma membrane preparation from A431 cells. EGF receptor autophosphorylation in vitro was inhibited by compound 2 with an IC₅₀ value of ~5 µM (Fig. 3A). The IC₅₀ value is similar to that obtained using the non-physiological exogenous peptide substrate angiotensin II (Table I). The effect of compound 2 on v-abl kinase autophosphorylation was tested (Fig. 3B). Only at the highest concentration of drug (100 µM) was inhibition of autophosphorylation observed (Fig. 3B, lane 2).

Kinetics of Inhibition of Exogenous Substrate Phosphorylation—It is generally assumed that competitive inhibitors of ATP are likely to lack specificity among ATP-dependent enzymes. Competition experiments with compound 2 were performed to determine the kinetic nature of inhibition by thiazolidine-diones relative to ATP. The c-src kinase was used as the target enzyme in kinetic studies due to the higher quality of results obtained with pure enzyme. Using the random polymer Glu,Tyr (4:1) as exogenous substrate, Concentrations of compound 2 were 0 (■), 3.1 µM (▲), and 6.2 µM (○). Points represent the mean of quadruplicate determinations. Curves were calculated using the Enzfitter program (40) with simple weighting. The Lineweaver-Burk representation is shown as an inset.

Inhibition of EGF-induced Cell Proliferation—BALB/MK mouse epidermal keratinocytes have a strong dependence on

FIG. 3. Effect of compound 2 on autophosphorylation of tyrosine kinases in vitro. A, inhibition of EGF receptor autophosphorylation. A431 membranes were incubated for 10 min with EGF (lanes 2–7) before addition of the indicated concentrations of compound 2 (lanes 3–7). Autophosphorylation reactions were started by addition of radiolabeled ATP and terminated by the addition of SDS-PAGE sample buffer. An autoradiogram of the dried SDS gel (8%) after 2 h of exposure is shown. B, effect of compound 2 on v-abl autophosphorylation. Recombinant v-abl kinase was assayed using 2 µM radiolabeled ATP. Compound 2 (lanes 2–7) and compound 6 (lane 1) were added at the indicated concentrations. After stopping reactions with sample buffer, the mixture was subjected to SDS-PAGE (10% gel). An autoradiogram of the dried gel is shown.

FIG. 4. Kinetics of c-src kinase inhibition by compound 2. c-src kinase was assayed as described under “Experimental Procedures” using 0.125 mg/ml Glu,Tyr (4:1) as exogenous substrate. Concentrations of compound 2 were 0 (■), 3.1 µM (▲), and 6.2 µM (○). Points represent the mean of quadruplicate determinations. Curves were calculated using the Enzfitter program (40) with simple weighting. The Lineweaver-Burk representation is shown as an inset.
Thiazolidine-Diones as Inhibitors of Tyrosine Protein Kinases

EGF for proliferation (34; Fig. 5, panel B). Thiazolidine-dione analogues were tested for inhibition of EGF-stimulated cellular proliferation of BALB/MK cells. Compounds 1–3, which were potent inhibitors of the EGF receptor kinase in vitro, showed potent antiproliferative activity with IC_{50} values of ~1 μM (Fig. 5, panel A). Enzymatically inactive analogues were without antiproliferative activity, Table II. In addition, compounds were tested for antiproliferative activity using the A431 cell line, which is known to express high levels of the EGF receptor (41). The thiazolidine-diones were found to have an essentially identical antiproliferative activity profile as compared to that obtained using the BALB/MK cell line (active compounds having IC_{50} values in the range 1–2 μM (Table II). Compounds were then tested for antiproliferative activity using the FDC-P1 cell line which lacks EGF receptor expression. FDC-P1 is a myeloid, bone marrow-derived cell line (35), which requires either IL-3 or granulocyte macrophage colony stimulating factor for growth. Growth of this cell line was also inhibited by compounds 1–3 (Table II) with virtually the same efficiency as were the BALB/MK and A431 cell lines.

Effect of Thiazolidine-Diones on EGF-induced EGF Receptor Autophosphorylation in Intact Cells—The effect of thiazolidine-diones on EGF-induced tyrosine phosphorylation was studied using the A431 cell line. EGF was added to serum starved cells which had been pretreated with inhibitors. EGF-dependent cellular tyrosine phosphorylation and EGF receptor expression was monitored by immunoblotting with phosphotyrosine and EGF receptor-specific antibodies. Compounds 1–3, which inhibited the EGF receptor kinase in vitro,
Thiazolidine-Diones as Inhibitors of Tyrosine Protein Kinases

**TABLE III**

*In vitro potency and selectivity of tyrosine protein kinase inhibitors*

Only data on inhibition of the epidermal growth factor-R (EGF-R), insulin receptor (IR), v-src, c-src, v-abl, PKA (cAMP-dependent protein kinase) and PKC (protein kinase C) protein kinases have been included. Inhibitors have been limited to the best characterized members of each structural class. To enable comparison with thiazolidine-diones (Table I), we have included our own unpublished results where available. The following abbreviations have been used: AII, angiotensin II; [Val']angiotensin II; polyGAT, polymer Glu,Ala,Tyr(6:3:1); polyGT, polymer Glu,Tyr(4:1); src-peptide, src autophosphorylation site peptide.

| Compound        | Kinase | IC₅₀ (µM) | Substrate                        | Ref. |
|-----------------|--------|-----------|----------------------------------|------|
| Genistein       | EGF-R  | 2.6       | Autophosphorylation               | 18   |
|                 |        | 22        | Histone 2B                       | 18   |
|                 |        | 1.0       | AI                               | This paper |
|                 | v-src  | 26        | Casein                           | 18   |
|                 | c-src  | >50       | polyGT                           | This paper |
|                 | c-src  | 30        | [Val']AII                         | This paper |
|                 | v-abl  | >100      | Kemptide                         | This paper |
|                 | PKA    | >100      | Histone H1                       | This paper |
|                 | PKC    | 45        | Histone H1                       | 15   |
| Staurosporine   | EGF-R  | 0.6       | src peptide                      | 16   |
|                 |        | 0.028     | AI                               | 17   |
|                 | IR     | 0.06      | src peptide                      | 16   |
|                 | v-src  | 0.006     | Autophosphorylation               | 15   |
|                 | c-src  | 0.01      | polyGT                           | Unpublished* |
|                 | v-abl  | 0.08      | [Val']AII                         | Unpublished* |
|                 | PKC    | 0.006     | Histone H1                       | 17   |
|                 | PKA    | 0.015     | Kemptide                         | 17   |
| Erastatin       | EGF-R  | 1.4       | Autophosphorylation               | 22   |
|                 |        | 6.0 (K₅) | src peptide                      | 23   |
|                 |        | 12.7      | AI                               | Unpublished |
|                 | v-abl  | >100      | [Val']AII                         | Unpublished |
|                 | PKA    | >100      | Kemptide                         | Unpublished |
| Hydroxy-cinnamamide (ST036) | EGF-R  | 1         | Casein                           | 26   |
|                 |        | 2.1 (K₅) | Casein                           | 26   |
|                 |        | 9         | AI                               | Unpublished |
|                 | c-src  | 18        | Immunocomplex                     | 26   |
|                 | v-src  | 87        | Immunocomplex                     | 26   |
|                 | v-abl  | 39        | [Val']AII                         | Unpublished |
|                 | PKA    | >100      | Histone H1                       | 25   |
|                 | PKC    | >100      | Histone H1                       | 25   |
| Tyrphostins RG50864 | EGF-R  | 2.4 (K₅, 0.85) | polyGAT                        | 27, 28, 29 |
|                 | IR     | 640 (K₅) | polyGAT                          | 27, 28, 29 |
| RG50810         | EGF-R  | 35 (K₅, 11) | polyGAT                        | 27, 28, 29 |
|                 | IR     | 42        | polyGAT                          | 27, 28, 29 |
|                 | PKA    | 1200 (K₅) | Not specified                    | 27, 28, 29 |
|                 | PKC    | >1000     | Not specified                    | 27, 28, 29 |

*J. F. Geissler, P. Traxler, J. L. Roesel, T. Meyer, N. B. Lydon, unpublished results.

A number of tyrosine protein kinase inhibitors of different structural classes have previously been reported. For comparative purposes a selection of the best characterized examples are shown in Table III. The thiazolidine-diones are a new class of protein kinase inhibitors with specificity for tyrosine protein kinases. With the serine/threonine protein kinases tested, no inhibition was observed. Within the family of tyrosine protein kinases, which are highly homologous in their kinase domains, inhibitors of the thiazolidine-dione class exhibit partially selective enzyme inhibition. While both the EGF receptor kinase and the c-src kinase were inhibited with equal efficiency, the same compounds were inactive (IC₅₀ > 100 µM) on the v-abl kinase. These results were surprising as showed clear inhibition of EGF receptor autophosphorylation in A431 cells (Fig. 6A, lanes 3–5). In contrast, the negative control compound 6, which was inactive as an inhibitor in vitro, had no effect on cellular EGF receptor autophosphorylation (lane 6). Drug treatment was found to have no significant effect on EGF receptor expression when compared with untreated control cells (Fig. 6B). The inhibitory effect of compound 2 on EGF receptor autophosphorylation was dependent on the drug concentration and treatment time. Following a 2-h preincubation period with compound 2, an IC₅₀ value in the range of 12.5–25 µM was observed (Fig. 7). Similar results were obtained for compounds 1 and 3 (results not shown). The inhibitory effect of the compounds increased with increasing preincubation time from 2 to 10 h, while the level of EGF receptor expression was essentially constant (data not shown). Following treatment with high concentrations of compounds 1–3, A431 cells detached from the culture dish surface in extended sheets (50% detachment with 100 µM of compounds 1–3 after 1 h of treatment). This effect was not observed with the enzymatically inactive analogues (compounds 4–10).

**DISCUSSION**

A number of tyrosine protein kinase inhibitors of different structural classes have previously been reported. For comparative purposes a selection of the best characterized examples are shown in Table III. The thiazolidine-diones are a new class of protein kinase inhibitors with specificity for tyrosine protein kinases. With the serine/threonine protein kinases tested, no inhibition was observed. Within the family of tyrosine protein kinases, which are highly homologous in their kinase domains, inhibitors of the thiazolidine-dione class exhibit partially selective enzyme inhibition. While both the EGF receptor kinase and the c-src kinase were inhibited with equal efficiency, the same compounds were inactive (IC₅₀ > 100 µM) on the v-abl kinase. These results were surprising as
the c-src and v-abl kinases are viewed as more closely related to each other than to the EGF receptor kinase when compared at the level of sequence homology within their kinase domains (9). However, in terms of substrate preference in vitro, c-src kinase appears to be more similar to the EGF receptor kinase. Both kinases accept acidic random amino acid polymers, which are poor substrates for the v-abl kinase (10). From their kinetic behavior, thiazolidine-diones were not competitive with ATP and thus do not behave as ATP analogues. However, as mixed type inhibitors they may discriminate between members of the tyrosine protein kinase family with different substrate preferences.

Autophosphorylation of the EGF receptor is considered to be the first enzymatic event following ligand binding (1-6, 42). The physiological function of this event is incompletely understood. However, it is generally considered as a regulatory mechanism which results in the relief of an inhibitory constraint (2). EGF receptor autophosphorylation in vitro was inhibited by compound 2 with an IC50 similar to that for inhibition of exogenous substrate phosphorylation. Since autophosphorylation occurs either by an intramolecular reaction (43) or by trans-phosphorylation within EGF receptor dimers (44, 42), the local substrate concentration cannot be accurately determined, but may be in the molar range due to proximity effects (45). Thus, thiazolidine-diones are efficient inhibitors of both exogenous substrate phosphorylation and EGF receptor autophosphorylation in vitro.

From the immunoblot analysis of EGF receptor autophosphorylation in A431 cells, it was found that compounds 1-3 were able to inhibit EGF receptor autophosphorylation within intact cells without affecting the level of EGF receptor expression. From the time dependence of the inhibitory effect, it can be concluded that permeation or equilibration of the compounds within the cell is a relatively slow process occurring over a period of hours. Following a 2-h preincubation period, the IC50 for compound 2 was in the range of 12.5-25 μM.

Since compounds were shown to exert their inhibitory effect on EGF receptor in vitro as well as in intact cells, they were tested for inhibition of the EGF-dependent BALB/MK cell line ELM, i.e. -10-fold lower concentrations than inhibition of exogenous substrate phosphorylation in intact cells. However, in antiproliferative assays, cells were incubated with drugs over a period of several days. This long term treatment might explain the low drug concentrations required for inhibition of cellular growth by compounds 1-3. It is also possible that the threshold drug concentration for inhibition of proliferation is less than the concentration required for complete inhibition of receptor autophosphorylation. Additionally, phosphorylation of other cellular substrates of the EGF receptor kinase might be more sensitive to inhibition than those substrates tested. The possibility cannot be excluded that effects unrelated to inhibition of EGF receptor kinase activity were responsible for inhibition of cell proliferation. However, an effect completely unrelated to kinase inhibition appears unlikely, since inhibition of EGF dependent growth was only observed with thiazolidine-dione analogues which were active as inhibitors in vitro.

The proliferation of the EGF-independent FDC-P1 cell line was equally well inhibited by compounds 1-3 as were the EGF-dependent BALB/MK and A431 cell lines. The finding that the c-src kinase was inhibited in vitro by thiazolidine-diones with the same efficiency as the EGF receptor kinase might explain the broad spectrum of antiproliferative effects of these compounds. In the case of FDC-P1, possible targets are the c-src kinase or other members of the c-src enzyme family (9). Evidence is accumulating, that products of the c-src gene family may play an important role in the mitogenic response of hematopoietic cells to proliferation signals. In FDC-P1 cells, either of the mitogenic factors granulocyte macrophage colony stimulating factor or IL-3 have been shown to activate c-src kinase (46). Tyrosine phosphorylation also occurs in response to IL-3 treatment in other myeloid cells (47-49). Thus, it is likely that the inhibition of IL-3-dependent FDC-P1 cell proliferation is due to inhibition of a tyrosine kinase activity required for growth factor-dependent proliferation. Recently, it has been suggested that phosphorylation of c-src on mitosis-specific sites may influence mitotic events, presumably by regulating c-src kinase activity (50, 51). Compounds that inhibit c-src kinase might thus be general inhibitors of cell growth due to interference with events in the M-phase of the cell cycle. In this respect the detachment of A431 cells from the culture dish surface upon treatment with active thiazolidine-diones was of interest. It has been suggested that phosphorylation of mitosis-specific sites of c-src might influence the cytoskeleton, bringing about shape changes during mitosis (50, 51). Such effects of c-src kinase on cell morphology and cell to cell communication are well documented (52). However, due to other possible targets, including other members of the protein kinase family, the antiproliferative effects of thiazolidine-diones cannot at present be unambiguously linked to inhibition of a single target enzyme.

REFERENCES

1. Carpenter, G. (1987) Annu. Rev. Biochem. 56, 881-914
2. Bertics, P. J., and Gill, G. N. (1986) J. Biol. Chem. 261, 14642-
3. Hunter, T., and Cooper, J. A. (1985) Annu. Rev. Biochem. 54, 897-930
4. Schlessinger, J. (1988) Biochemistry 27, 3119-3123
5. Yarden, Y., and Ullrich, A. (1988) Annu. Rev. Biochem. 57, 443-
6. Richert, N., and Kung, H.-J. (1988) Biochim. Biophys. Acta 948, 287-304
7. Chen, W. S., Lazar, C. S., Poenie, M., Tsien, R. Y., Gill, G. N., and Rosenfeld, M. G. (1987) Nature 328, 820-823
8. Honegger, A. M., Szapary, D., Schmidt, A., Lyall, R., Van Ob-
9. Hanks, S. K., Quinn, A. M., and Hunter, T. (1986) Science 41,
10. Braun, S., Raymon, W. E., and Racker, E. (1984) J. Biol. Chem. 259, 2051-2064
11. Sahal, D., Ramachandran, J., and Fujita-Yamaguchi, Y. (1988) Arch. Biochem. Biophys. 260, 416-426
12. Richert, N., Davies, P., Jay, G., and Pastan, I. (1979) Cell 18,
13. Navarro, J., Abdel Ghanhy, M., and Racker, R. (1989) Biochemistry 28, 6138-6144
14. Davies, R. J., and Czech, M. P. (1985) J. Biol. Chem. 260, 2543-
15. Nakano, H., Kobayashi, E., Takahashi, I., Tamaoki, T., Kuzuru,
16. Meyer, T., Regenass, U., Fabbro, D., Alteri, E., Roesel, J., Mueller,
17. Akiyama, T., Ishida, J., Nakagawa, S., Osawara, H., Watanabe,
18. Graziani, Y., Eriksson, E., and Eriksson, R. L. (1983) Eur. J. Biochem. 135, 583-589
19. Glossmann, H., Presek, P., and Eigenbrodt, E. (1981) Naunyn-
Schmiedeberg's Arch. Pharmakol. 317, 100–102
21. Schachter, Y., Yaish, P., Chorow, M., Gilon, C., Braun, S., and Levitzki, A. (1989) EMBO J. 8, 1671–1676
22. Umezawa, H., Imoto, M., Sawa, T., Ishiki, K., Matsuda, N., Uchida, T., Inuma, H., Hamada, M., and Takeuchi, T. (1986) J. Antibiotics 39, 170–173
23. Imoto, M., Umezawa, K., Ishiki, K., Kunimoto, S., Sawa, T., Takeuchi, T., and Umezawa, H. (1987) J. Antibiot. 40, 1471–1473
24. Ishiki, K., Imoto, M., Sawa, T., Umezawa, K., Takeuchi, T., Umezawa, H., Tauchida, Y., Yoshioka, T., and Tatsuta, K. (1987) J. Antibiot. 40, 1209–1210
25. Shiraishi, T., Domoto, T., Imai, N., Shimada, Y., and Watanabe, K. (1987) Biochem. Biophys. Res. Commun. 147, 322–328
26. Shiraishi, T., Owada, M. K., Tatsukawa, Y., Yamashita, T., Watanabe, K., and Kagamagawa, T. (1989) Cancer Res. 49, 2374–2378
27. Yaish, P., Gazit, A., Gilon, C., and Levitzki, A. (1989) J. Med. Chem. 32, 2344–2352
28. Lyall, R. M., Zilberstein, A., Gazit, A., Gilon, C., Levitzki, A., and Schlessinger, J. (1989) J. Biol. Chem. 264, 14503–14509
29. Carpenter, G., King, L., Jr., and Cohen, S. (1979) J. Biol. Chem. 254, 4884–4891
30. Lydon, N., Adams, B., Poschet, J. F., Gutzwiller, A., and Matter, A. (1990) Oncogene Res. 5, 161–173
31. Laemmli, U. K. (1970) Nature 227, 680–685
32. Giard, D. J., Aaronson, S. A., Todaro, G. J., Arrostek, P., Kersey, J. H., Dosik, H., and Parks, W. P. (1973) J. Natl. Cancer Inst. 51, 1417–1422
33. Weissmann, B. E., and Aaronson, S. A. (1983) Cell 32, 599–606
34. Dexter, T. M., Garland, J., Scott, D., Scolnick, E., and Metcalf, D. (1980) J. Exp. Med. 152, 1036–1047
35. Palacios, R., Fernandez, C., and Sideras, P. (1982) Eur. J. Immunol. 12, 777–782
36. Mosman, T. (1983) J. Immunol. Methods 65, 55–63
37. Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Horelwijn, P., Demyter, J., and De Clercq, K. (1988) J. Virol. Methods 20, 305–321
38. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
39. Leatherbarrow, R. J. (1987) Enzyme, Elsevier Bioskot, Hills Road, Cambridge, United Kingdom
40. Ginsburg, E., and Vonderhaar, B. K. (1985) Cancer Lett. 38, 143–159
41. Schlessinger, J., Ullrich, A., Honegger, A. M., and Moolenaar, W. H. (1988) Cold Spring Harbor Symp. Quant. Biol. 53, 515–519
42. Weber, W., Bertics, P. J., and Gill, G. N. (1984) J. Biol. Chem. 259, 14631–14636
43. Yarden, Y., and Schlessinger, J. (1987) Biochemistry 26, 1434–1442
44. Lehnninger, A. (1982) Principles of Biochemistry, p. 225, Worth, New York
45. Mueer, J., Kaeck, S., Moroni, C., and Ballmer-Hofer, K. (1989) Oncogene 4, 1433–1439
46. Sorensen, P., Mui, A. L.-F., and Krystal, G. (1989) J. Biol. Chem. 264, 19253–19258
47. Morita, A. O., Schreurs, J., Miyajima, A., and Wang, J. Y. L. (1988) Mol. Cell. Biol. 8, 2714–2718
48. Koyasu, S., Tojo, A., Miyajima, A., Akiyama, Y., Kasuga, M., Urabe, A., Schreurs, J., Arai, K., Takaku, F., and Yahara, I. (1987) EMBO J. 6, 3979–3984
49. Morgen, D. O., Kaplan, J. M., Bishop, J. M., and Varmus, H. E. (1989) Cell 57, 775–786
50. Shenoy, S., Choi, J. K., Bogodina, S., Copeland, T. D., Maier, J. L., and Shalloway, D. (1989) Cell 57, 763–774
51. Parsons, J. T., and Weber, M. J. (1989) Curr. Top. Microbiol. Immunol. 147, 79–127