Tyrphostin AG 494 blocks Cdk2 activation

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Abstract We have previously shown that the EGFR kinase selective tyrphostin AG 494 fails to inhibit EGFR kinase in intact cells. Yet, AG 494 proved to inhibit EGF- or serum-induced cell proliferation (Osherov et al., *J. Biol. Chem.* 268 (1993) 11134–11142). In this preliminary communication we show that AG 494 as well as its close analogs AG 490 and AG 555 block Cdk2 activation. In contrast, AG 1478, a more selective EGFR kinase blocker which is also active as EGFR kinase blocker in intact cells, fails to do so. AG 494 exerts its full inhibitory activity on Cdk2 activation even when added 20 h subsequent to EGF addition when Cdk2 activation is maximal. The inhibitory activity on Cdk2 activation parallels its DNA synthesis inhibitory activity, strongly suggesting that its target is one of the molecular mechanisms involved in Cdk2 activation. AG 494 and its analogs may become useful lead compounds for the development of drugs aimed at the cell cycle machinery.
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Key words: Cdk2; Cell cycle; Tyrosine kinase; Tyrphostin

1. Introduction

We have recently developed a group protein tyrosine kinase (PTK) inhibitors (tyrphostins) represented by AG 494, capable of selectively inhibiting the EGF receptor (EGFR) kinase activity in the cell-free kinase assay. This compound and its close analogs were found to poorly inhibit other protein tyrosine kinases, even the closely related HER-2 kinase [1,2]. Surprisingly, these compounds had no effect on tyrosine phosphorylation in intact cells but nevertheless were found to exhibit potent antimitogenic activity.

We were able to show that these inhibitors are competitive with ATP and that the loss of inhibition of the EGF receptor kinase activity in the intact cells is due to high intracellular levels of ATP, which prevents inhibitor binding to the kinase site of the receptor [2]. Since the anti-mitogenic activity of AG 494 is independent of the high ATP concentration, we decided to explore in more detail its mode of action.

In this study, we have analyzed the mode of anti-mitogenic action of AG 494 in intact cells, as compared to AG 1478, a potent EGFR tyrosine kinase inhibitor which is active in cell-free as well as intact cells [3,4]. We show that AG 494 in contrast to AG 1478, inhibits signaling downstream of the EGFR and blocks Cdk2 activation.

2. Material and methods

2.1. Synthetic methods

The synthesis of AG 494 and AG 1478 is described elsewhere [1,4].

2.2. Cell culture

Cells termed DHER-14 were prepared by transfecting NIH-3T3 cells (clone 2.2) which lack endogenous EGF receptors with wild-type HER-1/EGF receptor and is the same used in our previous study [2]. The cell line was a generous gift of Drs. Asher Zilberstein, Joseph Schlessinger and Axel Ullrich. Cells were grown in DMEM supplemented with 10% calf serum (Gibco).

2.3. Preparation of crude membrane extract

Cells were lysed in hypotonic lysis buffer (1×10^6 cells/ml lysis buffer) containing 20 mM HEPES (pH 7.4), 10 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 µg/ml aprotinin, 5 µg/ml leupeptin, and homogenized in a tight-fit homogenizer. Nuclei and debris were pelleted at $600 \times g$ for 10 min, and discarded. The supernatant was centrifuged at $100\,000 \times g$ for 30 min and the resulting crude membrane pellet was resuspended in 125 mM NaCl, 50 mM HEPES (pH 7.4), 10% glycerol and frozen in liquid nitrogen.

2.4. Inhibition of autophosphorylation

Membranes (2 µg/assay) were pre-incubated with EGF (50 nM) in 50 mM HEPES (pH 7.4), 125 mM NaCl, for 15 min at 4°C. The assay was initiated by addition of 8 µl membranes to 12 µl reaction mixture containing 50 mM HEPES (pH 7.4), 125 mM NaCl, 2 µM ATP ($= 2\ K_m$ final), 1 µCi [^{32}P]γ-ATP, 4 mM MnCl$_2$, 24 mM MgCl$_2$, and 4 µl tyrphostin dissolved in 10% Me$_2$SO/45% ethanol/45% DDW (double-distilled water). The assay was conducted at 4°C and terminated after 30 s by the addition of 8 µl boiling SDS-PAGE sample buffer. Proteins were separated on 6% SDS-PAGE, and autoradiography performed. A single EGF inducible ^{32}P-labeled band comigrating in the molecular weight of the receptor could then be detected. This band was scanned and quantified by densitometry (Zeineh soft laser, Biomed). IC$_{50}$ values were calculated using the E-Z fit program (Perrella, Du-Pont).

All reactions were performed rapidly in V-shaped 96-well plates using a multipipette. Reactions were all linear for 30 s. The exposure times of the autoradiograms were controlled so that film exposure was linear. Basal EGF-independent activity did not exceed 5% of the EGF-activated autophosphorylation of HER-1/EGF receptors. Inhibition of pp60^{c-src} activity was essentially performed as described by using the DHER-14 cells as described in detail previously, using denatured enolase as a substrate [3]. Inhibition of autophosphorylation of wheat-germ agglutinin (WGA) purified insulin-receptor was performed as described by Schechter et al. [6]. Inhibition of autophosphorylation of HER-2/Neu receptor was performed using membranes prepared from NIH-3T3 cells using published procedures [1,2] transfected with the human HER-2/Neu receptor as described for HER-1 [1].

2.5. Inhibition of [^{3}H]thymidine uptake

Cells were seeded at 7000 cells/well in 96-well Costar dishes pre-coated with 1 µg/well human fibronectin (Boehringer-Mannheim). The cells were grown to confluence for 2 days. The medium was changed to DMEM containing 0.25% calf serum for 48 h and the cells were then incubated 16 h with either 20 nM EGF (Serotec), 50 µM lyso-phosphatidic acid or 50 ng/ml TPA (phorbol ester). After 16 h, [^{3}H]thymidine 0.5 µCi/ml was added for 4 h. Different concentrations of tyrphostins dissolved at $\times 100$ concentration in 10% Me$_2$SO were added either 30 min prior to the addition of mitogens, or during the last 4 h along with [^{3}H]thymidine addition. The trichloroacetic acid-precipitable material was quantified by scintillation counting. Basal EGF-independent activity did not exceed 5% of mitogen-dependent [^{3}H]thymidine uptake activity of DHER-14 cells. Tyrphostins were added either 30 min prior to EGF addition or at the time of [^{3}H]thymidine addition. Thus AG 494 and AG 1478 were present either for 20 or 4 h.

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2.6. Immunoprecipitation and immunoblot analysis

Cells were plated at 2×10^5 cells/5 ml/well in Costar 6-well dishes, grown to confluence for 2 days and then starved in 2 ml/well DMEM containing 0.25% calf serum for 48 h. Tyrphostins dissolved at ×100 concentration in 10% Me_2SO were added during the last 16 h or the last 2 h of starvation. EGF (20 nM) was then added and the plates were incubated for 2 min at 4°C with EGF (20 nM), during which time receptor autophosphorylation is linear [7]. The reaction was terminated by addition of 1 ml/well stop lysis buffer containing 20 mM HEPES (pH 7.4), 125 mM NaCl, 1% Triton X-100, 5 mM NaF, 100 μM $NaVO_3$, 200 μM $ZnCl_2$, 1 mM EDTA, 2 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, 5 μg/ml leupeptin. Immunoprecipitation using monoclonal antibody 108 was performed as described above. The immunocomplex was released in boiling sample buffer, and then samples were run on SDS-PAGE 5–15% gradient gels, transferred to nitrocellulose (ABN semi-dry blot), blocked for 30 min in TBST (Tris buffer saline (pH 7.4), 1% Tween-20, 5% bovine serum albumin), then probed with monoclonal anti-phosphotyrosine antibodies PT66 (Sigma-Biomakor) for 3 h at 40°C in TBST, washed 4 times in TBS, and then reproved with [^{125}I]goat anti-mouse antibodies (1 μCi/ml). The blots were then washed 4 times in TBS, air dried and analyzed by autoradiography.

2.7. Immunoprecipitation and Cdk2 kinase assays

DHER-14 cells were grown to confluence for 48 h in Costar 6-well dishes in DMEM +10% calf serum as described. Cells were starved for 3 days. During the last 24 h, cells were activated by various mitogens (20 nM EGF; 50 ng/ml TPA phorbol ester) or 50 μM lysophosphatidic acid (LPA) for various time periods. Cells were incubated with tyrphostins as described for each experiment. Cells were washed once in PBS, then lysed on ice using 1 ml/well cold IP buffer (50 mM HEPES, 250 mM NaCl, 5 mM DTT, 0.25% NP-40, 10 mM NaF, and protease inhibitors). Lysates were incubated on ice for 30 min, then centrifuged $14000 \times g$ for 15 min in a microfuge. Supernatants were transferred, and incubated for 2 h in the presence of a saturating concentration of 1 μg/eppendorf anti-Cdk2 antibodies. The immunocomplex was collected on protein A agarose beads (7.5 μl packed volume per eppendorf) and washed 3 times in IP buffer. The pellet was then washed a fourth time in kinase buffer (50 mM HEPES, 10 mM MgCl, 1 mM DTT, 1 μM cold ATP). All buffer was aspirated from the beads with a fine bore Hamilton syringe, and the kinase reaction initiated by the addition of 40 μl/eppendorf reaction mixture containing kinase buffer and 1 μCi/assay [γ-^{32}P]ATP and 2.5 μg Histone H1 (freshly prepared) per assay.

The reaction was performed for 20 min at 30°C, then stopped by addition of 12.5 μl/assay SDS-PAGE sample buffer ×4. Samples were separated on 10% SDS PAGE gels and exposed to film for ≈2 h at −70°C. Quantitation was performed by densitometry.

3. Results

The tyrphostin AG 494 and the quinazoline AG 1478 have been shown to selectively inhibit EGF receptor tyrosine kinase activity in vitro as compared to a variety of tyrosine kinases, including the closely related HER-2/Neu receptor (Table 1) [1–4].
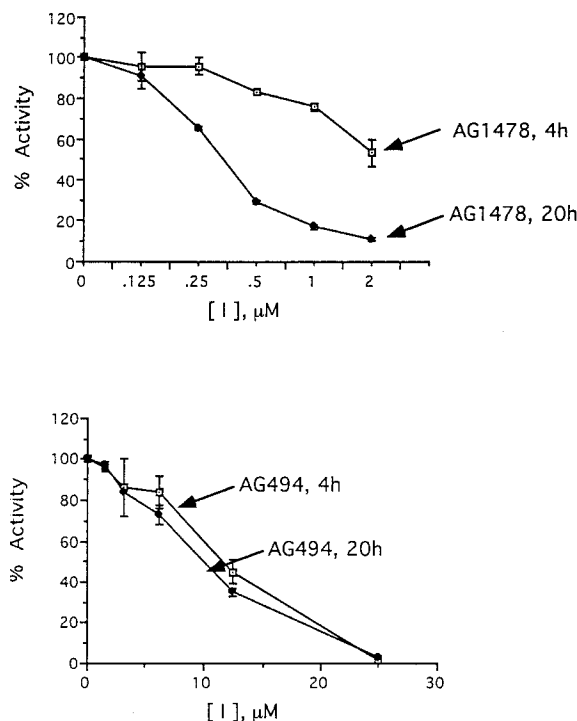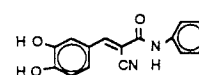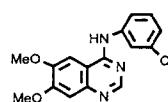

Fig. 1. Inhibition of EGF-induced [^{3}H]thymidine uptake by AG 1478 and AG 494. DHER-14 cells were serum starved for 48 h at 0.25% serum. Then EGF (20 μg/ml) was added for 20 h. [^{3}H]Thymidine was added after 16 h and tyrphostins were added either just prior to EGF addition or at 16 h. Other details are given in Section 2. 100% activity is 98000 dpm.

We have previously shown that AG 494 does not inhibit EGF receptor autophosphorylation and phosphorylation of endogenous substrates in intact DHER-14 cells [2]. In contrast, AG 1478, inhibits this activity effectively at doses similar to those shown to inhibit receptor activity in vitro [3,4].

3.1. Inhibition of EGF-dependent DNA synthesis

Growth-arrested DHER-14 cells were activated with EGF, TPA and LPA, and the extent of DNA synthesis measured by [^{3}H]thymidine uptake. AG 494 inhibits 50% of TPA- and LPA-dependent [^{3}H]thymidine uptake at ≈5 μM, whereas EGF-dependent [^{3}H]thymidine uptake is inhibited at ≈10.5 μM (Figs. 1 and 2). In contrast, and as expected for the more selective EGF receptor inhibitor, AG 1478, inhibits 50% of the EGF-dependent [^{3}H]thymidine uptake at ≈0.3 μM, whereas LPA- and TPA-dependent [^{3}H]thymidine uptake are inhibited at ≈8-fold higher concentrations (Figs. 1 and 2).

Table 1
Inhibition of autophosphorylation of various tyrosine kinases by AG 1478 and AG 494

Tyrosine kinase	Preparation	AG 1478 IC$_{50}$ (μM)	AG 494 IC$_{50}$ (μM)
EGFR/HER-1	membranes	0.0020	1.1 ± 0.24
HER-2/neu	membranes	2.6	39 ± 13
Insulin-R	WGA-purif.	>100	>100
c-src	immunoppt	>100	>100

Experimental details on the methodology for the determination of the IC$_{50}$ are given in Section 2.
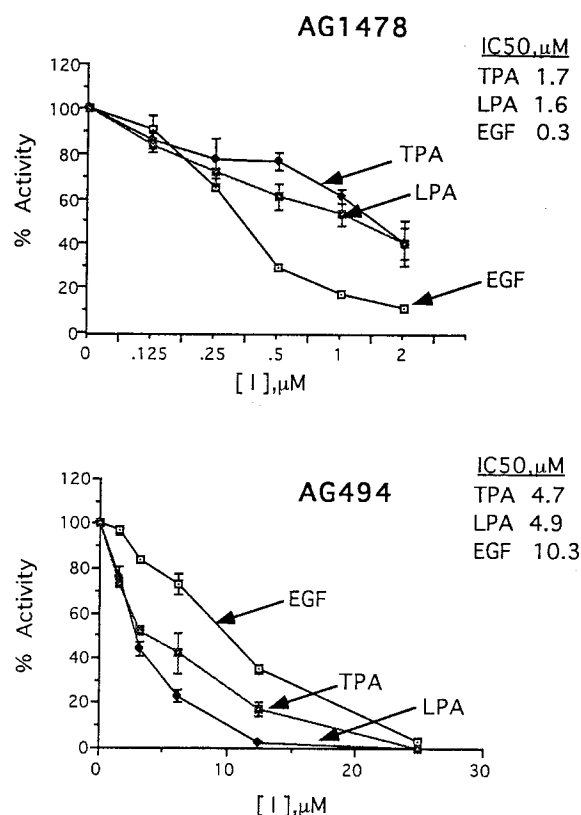
Fig. 2. Inhibition of [^{3}H]thymidine uptake induced by EGF, TPA and LPA by AG 1478 and AG 494. 100% is 96000 dpm for EGF, 85000 with TPA and 88000 with LPA. 10% serum under identical conditions yields 138000 dpm.

These results imply that AG 494 exerts its antimitogenic effects by inhibiting a signal transduction element acting downstream of the EGF receptor and which is common to EGF, TPA, LPA (Fig. 2) and serum [2].

AG 494 inhibits EGF-dependent [^{3}H]thymidine uptake with equal potency when added either just prior to EGF activation, or 16 h later, upon [^{3}H]thymidine addition when the cells are mostly in late G1/early S (Fig. 1). In contrast, AG 1478, as expected for a more selective EGFR kinase inhibitor, inhibits EGF-dependent [^{3}H]thymidine uptake far more potently when added just prior to EGF activation (Fig. 1). This result further supports the notion that AG 494 exerts its antimitogenic effect by inhibiting a signal transduction element downstream of the EGFR. Since we found that AG 494 does not inhibit directly MAPK ([8], data not shown) or the DNA replication machinery as measured on isolated drosophila nuclei ([8], data not shown), we hypothesized that AG 494 may block the cell cycle machinery governing G1 to S transition. Another reason for testing the cell cycle machinery was because AG 494 inhibits mitogenesis induced by EGF also by other mitogens such as TPA and LPA (Fig. 1) as well as serum [2]. Because AG 494 was found to block DNA synthesis even at 16 h after stimulation (Fig. 1) we decided to focus on Cdk2.

3.2. EGF-dependent activation of Cdk2 kinase in DHER-14 cells

Growth-arrested DHER-14 cells were stimulated by EGF for various time periods, and Cdk2 kinase activity was measured by histone H1 phosphorylation (Fig. 3A). Maximal stim-

ulation was attained after 24 h of stimulation and decreased within the next 24 h (48 h total incubation time). Activation was completely abolished by preblocking the antibodies with the cognate peptide (not shown). TPA and LPA stimulation followed a very similar time course of activation (not shown).

3.3. Inhibition of Cdk2 activation in DHER-14 cells by tyrphostins

Tyrphostins of the AG 494 group were added during the last 4 h of stimulation by EGF. AG 494, AG 490, AG 555 at a concentration of 30 μM, ~2.5-fold the IC$_{50}$ for thymidine uptake inhibition (Fig. 1) inhibit >80% of Cdk2 activity (Fig. 3A). AG 1478 at a concentration of 2 μM, ~7.5-fold concentration of that of IC$_{50}$ for thymidine uptake inhibition (Fig. 1) has only a small effect (Fig. 3B).

AG 494 inhibits in a dose-dependent manner of Cdk2 EGF-dependent activation with an IC$_{50}$ ~15 μM (Fig. 3B) very similar to the IC$_{50}$ value required to inhibit thymidine uptake (10.3–12 μM; Figs. 1 and 2). AG 1478 was only slightly inhibitory and induced only 31% at 2 μM when added at 20–24 h (Fig. 3B) similar to its DNA inhibitory effect when added late (Fig. 1). These results also support the more selective nature of AG 1478 whose inhibitory activity is most effective when added early upon EGF stimulation (Fig. 1). Maximal inhibition of Cdk2 activity is attained after 4 h of incubation of cells with 30 μM AG 494 where incubation of less than 1 h was markedly less effective (Fig. 3C). Since the kinetics of AG 494 cell entry has a half-life of ~10 min [2,8], it seems that the onset of the process of inhibition of Cdk2 activation has a half-life of ~1 h.

4. Discussion

Over the past decade a large number of PTK inhibitors have been synthesized, among them the tyrphostins [9]. Although, some tyrphostins like AG 494 were shown to be selective against EGFR kinase in cell-free assay they failed to retain this property in intact cells [2]. Yet, in spite of the failure of AG 494 to retain its EGFR kinase inhibitory activity in intact cells, it retained its anti-proliferative activity [2] (Figs. 1 and 2). It was found that AG 494 retains its full inhibitory activity on EGF-dependent DNA synthesis measured by [^{3}H]thymidine uptake, even when added 20 h subsequent to EGF addition (Fig. 1). Since AG 494 inhibits not only EGF- but also serum-dependent cell growth [2] and LPA- or TPA-dependent DNA synthesis (Fig. 2) it is apparent that the target of AG 494 action in intact cells is downstream to the EGFR kinase, as compared to the more selective EGFR kinase inhibitor AG 1478 which is also active as a selective EGFR kinase inhibitor in intact cells [3,8]. In this study we show that AG 494 and its analogs AG 490 and AG 555 block the activation of Cdk2 (Fig. 3B), a property not shared by AG 1478 (Fig. 3C). Activation of Cdk2 depends on a number of molecular events such as binding of cyclins, the dephosphorylation of the Thr[14] and Tyr[15] as well as the activating phosphorylation of Thr[161] [10]. Our results also do not rule out an effect of AG 494 on Cdk 4 which is activated in early G1 [10]. These and other molecular parameters are currently under investigation. Upon the hopeful identification of the molecular target(s) for AG 494 and its analogs it will be possible to design more potent inhibitors targeted to the inhibition of Cdk2 activation. Hopefully, iden-
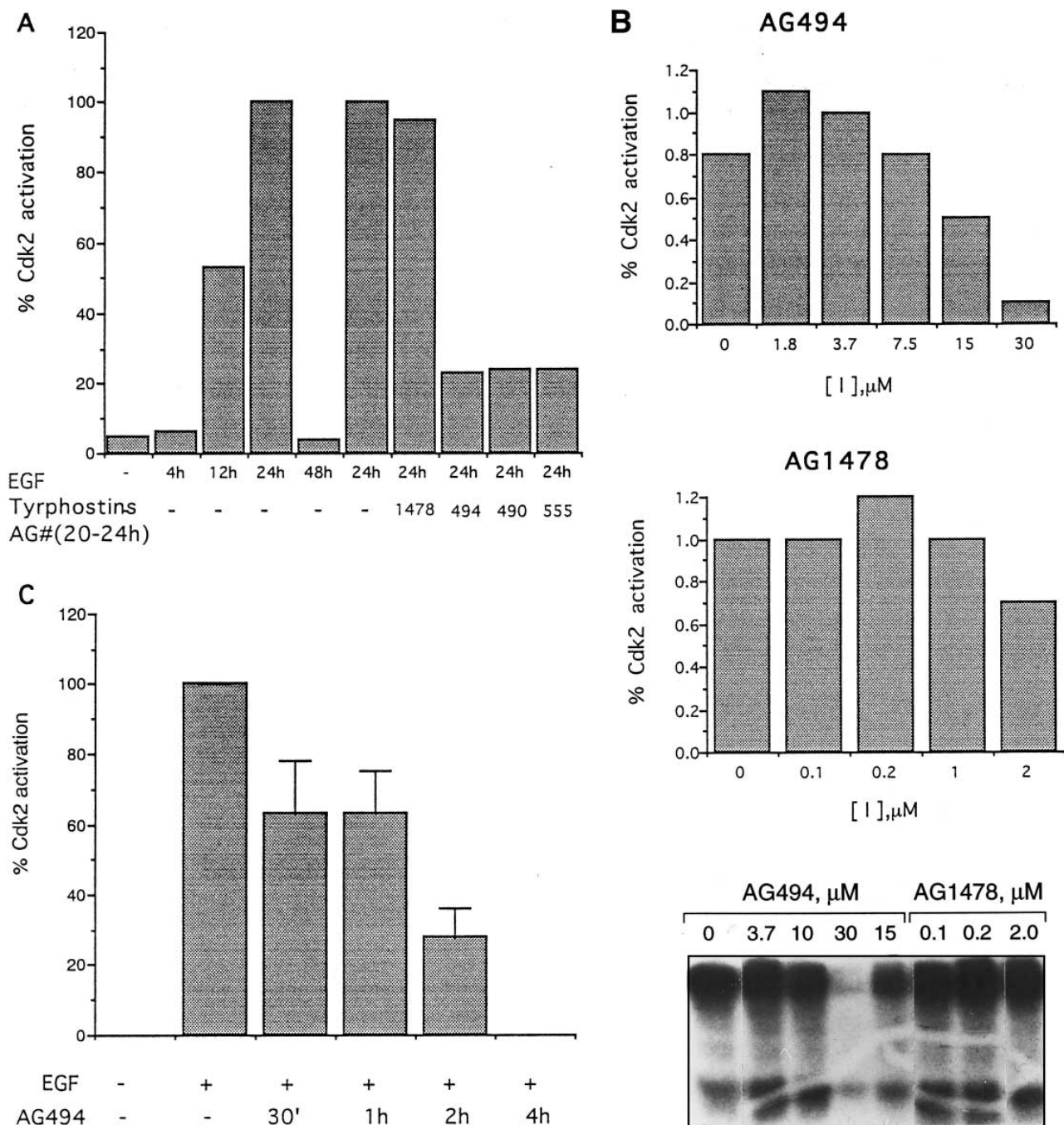
Fig. 3. Cdk2 activation and its inhibition by tyrphostins. Growth-arrested DHER-14 cells were activated by EGF (20 ng/ml). At various time points Cdk2 was immunoprecipitated and its activity using H1 histone was measured. Tyrphostins were added between 20-24 h. Other details are given in Section 2. A: Kinetics of Cdk2 activation and its inhibition by members of AG 494. B: Dose-dependent inhibition of Cdk2 activation by AG 494 and AG 1478. Bottom: a typical H1 phosphorylation assay and its inhibition. C: Kinetics of onset of Cdk2 kinase inhibition. As indicated AG 494 was added 20 h subsequent to EGF addition. Samples were removed, Cdk2 immunoprecipitated and its activity measured.

tification of such a target may also help in designing agents which block Cdk4 activation. Such inhibitors have the potential to become useful anti-proliferative agents.

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