

Inhibitory effect and its kinetic analysis of tyrphostin AG1478 on recombinant human protein kinase CK2 holoenzyme¹

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KEY WORDS caseins; protein kinases; recombinant proteins; holoenzymes; tyrphostins; kinetics

ABSTRACT

AIM: To study the direct effect of tyrphostin AG1478 [4-(3-chloroanilino)-6, 7-dimethoxyquinazoline] on recombinant human protein kinase CK2 holoenzyme and its kinetics. **METHODS:** Recombinant human protein kinase CK2 α and β subunits were mixed at equal molar ratio and CK2 holoenzyme were reconstituted. The CK2 activity was assayed by detecting incorporation of [γ -³²P]ATP or [γ -³²P]GTP into substrates in various conditions. **RESULTS:** These results demonstrated that the recombinant human CK2 was a second messengers (Ca^{2+} , cAMP, and cGMP)-independent protein kinase, the characterization and function of the reconstituted holoenzyme were consistent with those of native CK2. AG1478 strongly inhibited the holoenzyme activity of recombinant human protein kinase CK2 with IC_{50} of 25.9 $\mu\text{mol/L}$, the inhibition is very close to that of *N*-(2-aminoethyl)-5-chloronaphthalene-1-sulfonamide (A3), but less potent than that of 5, 6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), known as CK2 special inhibitors with IC_{50} of 25.5 $\mu\text{mol/L}$ and 10.4 $\mu\text{mol/L}$ respectively. Kinetic studies of AG1478 on recombinant human CK2 showed that inhibitions were competitive with both GTP and casein, thus AG1478 was as bisubstrate inhibitor. **CONCLUSION:** The present study indicates that AG1478 is not only an effective inhibitor of protein tyrosine kinases of epidermal growth factor receptor (EGFR), but also a novel potent inhibitor

of protein kinase CK2. The recombinant human protein kinase CK2 might be used as a molecular target for simpler screening and development of more effective inhibitors of CK2.

INTRODUCTION

Protein kinase CK2 (an acronym derived from the misnomer "casein kinase II or 2") is a ubiquitous eukaryotic second messenger independent heterotetrameric serine/threonine protein kinase which is made by the association of two catalytic subunits (α and/or α') and two β regulatory subunits to generate native structures exhibiting the stoichiometries $\alpha_2\beta_2$, $\alpha'\beta_2$, and $\alpha\alpha'\beta_2$ ^[1-3]. CK2 three subunits are highly conserved in biological evolution, indicating a vital cellular role of CK2. Protein kinase CK2 has two important features^[1-3]; first, this enzyme, which has been studied since 1954, is able to phosphorylate some 200 protein substrates known to date, many of which play important roles in DNA replication and transcription, RNA processing and translation, cellular metabolism and movement, signal transduction and processing, cell proliferation and differentiation, regulation of oncogene and suppressor gene, etc, although its precise physiological functions of this protein kinase remain poorly understood; second, CK2 belongs to one of few protein kinases possessing dual-cosubstrate specificity, which accepts both ATP and GTP as phosphoryl donors. The activity of CK2 was remarkably elevated in proliferating and transformed cells, as well as neoplastic cells, compared with quiescent and normal cells^[1-3]. Some reports^[4,5] indicated that CK2 α or α' gene might be a proto-oncogene. Recently, it was pointed out that CK2 might become one of molecular targets of potential neoplastic and AIDS therapy, specific inhibitors of CK2 might have therapeutic potential^[1-3,6].

Gazit and others developed a family of TPK inhibitors called tyrphostins (also known as AG compounds), designed to mimic the tyrosine sub-

¹ Project supported by the Natural Science Foundation of Guangdong Province (011766), Medical Science Foundations of Health Department of Guangdong Province (A2000487), Key Achievement Supporting Foundation of Guangdong Medical College (XK0002).

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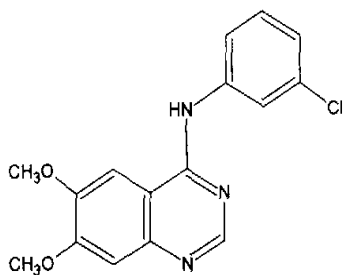
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Received 2001-09-03

Accepted 2002-04-19

strates^[7]. Tyrphostin AG1478 [4-(3-chloroanilino)-6,7-dimethoxyquinazoline], one of synthetic compounds with small molecular weight, was a highly potent specific inhibitor of epidermal growth factor receptor (EGFR) kinase^[7].

It is very difficult to get enough amount of CK2 for laboratory research because of its low concentration in tissues and cells. Moreover, CK2 is a tetramer in eukaryotic cells and the holoenzyme can only be disassociated into its subunits under denaturing conditions. We gained enough pure recombinant proteins of human CK2 α and β subunits through molecular cloning, prokaryotic expression, and purification, and performed some researches on their biological pharmacology^[8-11]. Because CK2 activity relates with cell growth, the inhibition of cell proliferation might affect the activity of this enzyme, thus we investigated the direct effect of tyrphostin AG1478 on recombinant human CK2 holoenzyme and its kinetics.



[4-(3-Chloroanilino)-6,7-dimethoxyquinazoline]
(Tyrphostin AG1478)

MATERIALS AND METHODS

Materials Tyrphostin AG1478 was synthesized^[7] and presented kindly by Prof Gazit A, Department of Organic Chemistry, The Hebrew University of Jerusalem, Israel. 5, 6-Dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) and N-(2-aminoethyl)-5-chloronaphthalene-1-sulfonamide (A3) were from CalBiochem. Heparin, spermine, ATP, and GTP were obtained from Sigma. P81 phosphocellulose filter paper was the product of Whatman. [γ -³²P]ATP or [γ -³²P]GTP (18.5 kBq, specific activity 1.85×10^{17} Bq/mol) were purchased from Yahui Biochemical Technology Co Ltd, Beijing. All other chemicals were of analytical grade.

Cloning and sequencing of cDNA encoding human protein kinase CK2 α and β subunits

Methods were carried out as described previously^[8,9].

Prokaryotic expression, purification, and reconstitution of recombinant human CK2 α and β subunits Human CK2 α and β subunits were expressed in a bacterial expression system [pT7-7/BL21 (DE3)] and purified to homogeneity as we described previously^[10,11], after equalmolar subunits were mixed, CK2 holoenzyme was spontaneously reconstituted.

Protein assay Protein concentration in the samples was determined by the method of staining with Coomassie brilliant blue R-250, using bovine serum albumin (BSA) as the standard.

Protein kinase CK2 activity assay^[10,11] Partially dephosphorylated casein was prepared by incubating casein 5 g in 50 mL of Tris-HCl 50 mmol/L (pH 9.5) at 100 °C for 10 min and dialyzing against buffer containing Tris-HCl 50 mmol/L (pH 7.5) and edetic acid 50 mmol/L (pH 7.5). CK2 assay was performed at 30 °C in a final volume of 35 μ L of buffer containing Tris-HCl 50 mmol/L (pH 7.2), KCl 150 mmol/L, MgCl₂ 10 mmol/L, ATP 50 μ mol/L, [γ -³²P]ATP, or [γ -³²P]GTP 18.5 kBq (specific activity 1.85×10^{17} Bq/mol), and dephosphorylated casein 2 g/L. Reactions were started by the addition of 15 μ L recombinant CK2 holoenzyme and terminated after 10 min by spotting 30 μ L of the reaction mixture onto 2 cm diameter P81 phosphocellulose paper. After the filter papers were washed thoroughly with phosphoric acid 85 mmol/L by occasional stirring, washed one time with acetone finally, and dried in oven at 80 °C, the radioactivity was measured in a LS6000C (Beckman) scintillation counter. One unit of protein kinase CK2 activity is defined as the amount that catalyses the transfer of 1 pmol of phosphate from [γ -³²P]ATP or [γ -³²P]GTP to casein per min at 30 °C.

Direct effect of tyrphostin AG1478 on recombinant human CK2 holoenzyme and calculation of semi-inhibitory concentration (IC₅₀) Calculation of IC₅₀ was performed according to semi-effect-probit method^[12]. In brief, X-coordinate and Y-coordinate represents logarithm of AG1478 concentration and probit of inhibitory rate to its corresponding concentration, respectively. With experiment data above, equation of linear regression was elicited and IC₅₀ was calculated (probit is 5 in 50 % inhibitory rate).

Enzyme kinetics according to the IC₅₀ result above, CK2 kinetic analysis was carried out in the condition of three concentrations of AG1478 (0, 20, and 80

$\mu\text{mol/L}$). In the condition in which casein was fixed at concentration (2 g/L) and GTP was changed at various concentrations (6.25–50 $\mu\text{mol/L}$), or GTP was fixed at 12.5 $\mu\text{mol/L}$ and casein was at different concentrations (1–8 g/L), protein kinase CK2 activity was assayed, which equaled to reaction velocity. Each experiment for a sample was triplicate. The kinetic parameters of apparent K_m and apparent V_{max} were calculated by Lineweaver-Burk plot, and the types of inhibitory effect of AG1478 on recombinant human protein kinase CK2 holoenzyme were identified.

Statistic analysis All values were shown as $\bar{x} \pm s$, and statistical significance was analyzed by paired t test.

RESULTS

Characterization of recombinant human CK2 holoenzyme

The results indicated that reconstituted CK2 holoenzyme possessed the same properties as natural CK2; it used casein as its substrate; its activity was decreased when using basic protein Histone III S and the substrate of protein tyrosine kinase (TPK) — poly (Glu:Tyr) 4:1 as its substrates; its activity was depressed with heparin and CK2 specific inhibitor DRB, but stimulated by spermine; the second messengers molecules cAMP, cGMP, and Ca^{2+} had no effect on its activity (Tab 1).

Tab 1. Characterization of recombinant human CK2 holoenzyme. $n=3$. $\bar{x} \pm s$. $^c P < 0.01$ vs control.

| Treatment | CK2 activity/ mU | Percent of control/% |
|---|---------------------|-------------------------|
| 1 Control | 1265 ± 96 | 100 |
| 2 + Histone III S (0.5 g/L) but no casein | 52 ± 10 | 4.1 ^c |
| 3 + poly (Glu:Tyr) 4:1 (0.4 g/L) but no casein | 125 ± 24 | 9.9 ^c |
| 4 + Heparin (8 mg/L) | 507 ± 45 | 40.1 ^c |
| 5 + DRB (40 $\mu\text{mol/L}$) | 326 ± 71 | 25.7 ^c |
| 6 + Spermine (2.5 mmol/L) | 1840 ± 107 | 145.5 ^c |
| 7 + Ca^{2+} (5 mmol/L) | 1249 ± 63 | 98.7 |
| 8 + cAMP (10 $\mu\text{mol/L}$) | 1366 ± 112 | 108.0 |
| 9 + cGMP (10 $\mu\text{mol/L}$) | 1268 ± 108 | 100.2 |

Assay of CK2 holoenzyme activity was described in Materials and Methods using equimolar amounts (14 pmol) of CK2 α and β subunits. In group 1 (control) casein was used as a substrate. In group 2 and 3 histone and poly (Glu:Tyr) 4:1 were used as substrate respectively, without casein. In group 4 to 9, different agents indicated in Tab 1 was added into corresponding group on the basis of control group respectively.

Direct effect of tyrphostin AG1478 on recombinant human CK2 holoenzyme Fig 1 showed that tyrphostin AG1478 had stronger inhibitory effect on recombinant human CK2 holoenzyme in a concentration-dependent manner. Using logarithm of AG1478 concentration as horizontal coordinate, probit of inhibitory rate to corresponding AG1478 concentration as vertical coordinate, the equation of linear regression was elicited, which was $\hat{Y} = 1.1425X + 3.3861$, correlation coefficient r was 0.9808, IC_{50} was 25.9 $\mu\text{mol/L}$.

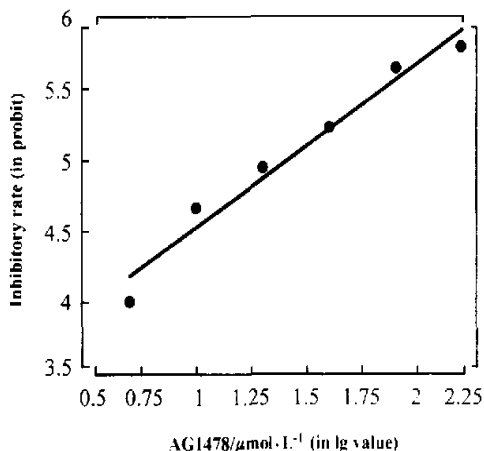


Fig 1. Effect of AG1478 on recombinant CK2 holoenzyme activity.

Kinetic analysis of tyrphostin AG1478 inhibitory effect on recombinant human CK2 holoenzyme activity in the presence of various concentrations of GTP The experimental result of enzyme kinetics of tyrphostin AG1478 showed that the inhibition was competitive with GTP (Fig 2).

Kinetic analysis of tyrphostin AG1478 inhibitory effect on recombinant human CK2 holoenzyme activity in the presence of different concentrations of casein Fig 3 indicated that the inhibitory effect of AG1478 on recombinant human CK2 holoenzyme was also competitive with casein, thus AG1478 was as bisubstrate inhibitor.

Inhibitory effects of DRB and A3 on recombinant human protein kinase CK2 holoenzyme activity 5, 6-Dichloro-1- β -D-ribo-furanosylbenzimidazole (DRB) and *N*-(2-aminoethyl)-5-chloronaphthalene-1-sulfonamide (A3) were known as potent and specific inhibitors of protein kinase CK2^{1,31}. Our data confirmed that they strongly inhibited recombinant human CK2 holoenzyme with IC_{50} of 10.4 and 25.5 $\mu\text{mol/L}$,

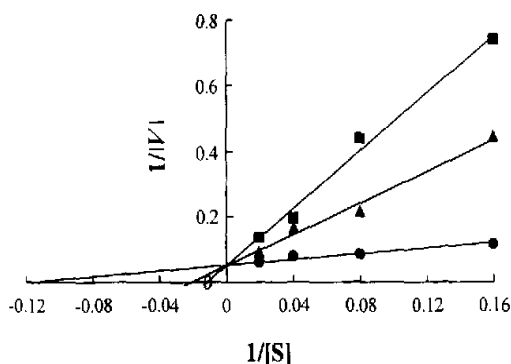


Fig 2. Lineweaver-Burk plot for kinetic analysis of AG1478 inhibitory effect on recombinant human CK2 holoenzyme in the presence of various concentrations of GTP. Casein was fixed at concentration (2 g/L), but GTP was at indicated concentration. Each value represents the mean of the three separate experiments. AG1478: 0 $\mu\text{mol/L}$ (●); 20 $\mu\text{mol/L}$ (▲); 80 $\mu\text{mol/L}$ (■). [S]: GTP concentration, $\mu\text{mol/L}$; [V]: velocity, $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$.

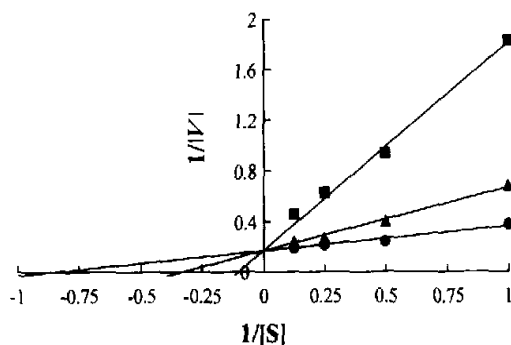


Fig 3. Lineweaver-Burk plot for kinetic analysis of AG1478 inhibitory effect on recombinant CK2 holoenzyme activity in the presence of different concentrations of casein. GTP was fixed at concentration (12.5 $\mu\text{mol/L}$), but casein was at indicated concentration. Each value represents the mean of the three separate experiments. AG1478: 0 $\mu\text{mol/L}$ (●); 20 $\mu\text{mol/L}$ (▲); 80 $\mu\text{mol/L}$ (■). [S]: casein concentration, g/L; [V]: velocity, $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$.

respectively. These results revealed that AG1478 strongly inhibited the holoenzyme activity of recombinant human CK2 with an IC_{50} of 25.9 $\mu\text{mol/L}$, the inhibition was almost the same as that of A3, but less potent than that of DRB.

DISCUSSION

It is proposed that CK2 is an attractive target

molecule for tumor and acquired immune deficiency syndrome (AIDS) therapy. The inhibitors of CK2 might have clinical therapeutic potential for neoplastic and human immunodeficiency virus (HIV-1)^[1-3,6]. Many tyrophostins had selective and distinct inhibitory activities on tyrosine protein kinase of epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), and Erb2/neu^[7,13]. Kinetic studies had shown that some tyrophostin compounds were pure competitive inhibitors *vis-à-vis* the tyrosine substrate and noncompetitive *vis-à-vis* ATP site, while many of tyrophostins showed competitive inhibitory effect against both the substrate and ATP^[7].

Tyrophostin AG1478, one of AG compounds, is a very potent and selective inhibitor of EGFR kinase with IC_{50} of 3 nmol/L versus HER2-neu (IC_{50} > 100 $\mu\text{mol/L}$) and PDGFR kinase (IC_{50} > 100 $\mu\text{mol/L}$)^[7,13]. Recently some observations indicate that AG1478 abolishes also p42/p44 mitogen-activated protein kinase (MAPK) activation induced by angiotensin II^[14], and p38 MAPK activation induced by thrombin^[15]. AG1478 inhibited also the EGF-dependent proliferation of cells and had been as novel antiproliferative agent of blocking targets of signal transduction for diseases therapy^[13,16-18]. However, there is no report on effect of AG1478 on protein kinase CK2 at present. Most reports about AG1478 focused on its inhibitory effect on EGFR kinase.

In present study we first reported the direct effect of tyrophostin AG1478 on recombinant human CK2 holoenzyme and its enzyme kinetics. The results indicated that AG1478 had significant inhibitory effect on this recombinant CK2 with IC_{50} of 25.9 $\mu\text{mol/L}$ (Fig 1). The inhibitory effect of AG1478 on recombinant human CK2 was less than that on EGFR kinase, stronger than that on HER2-neu and PDGFR kinase. These results were novel and very significant, and illuminated AG1478 was not only highly specific TPK inhibitor of EGFR, but also blocker of protein kinase CK2.

In contrast to most other kinases CK2 may utilize GTP, besides ATP, as phosphate donor, so it is more specific to assay protein kinase CK2 using GTP as phosphate donor. Enzyme kinetics clarified the action mechanism of AG1478 on CK2 and displayed that inhibitions were competitive with both GTP (Fig 2) and casein (Fig 3), thus acting as bisubstrate inhibitor. The present work extended the action or use of AG1478.

DRB, known as potent and specific inhibitor of protein kinase CK2, had been also used to inhibit RNA

polymerase II transcription which might be dependent on CK2. A3 had inhibitory effect on the activities of protein kinase CK I, protein kinase A, protein kinase G, and protein kinase C, besides CK2^[1-3]. The present work confirmed that purified recombinant human CK2 holoenzyme was inhibited by the two known inhibitors, DRB and A3, with IC₅₀ of 10.4 and 25.5 μmol/L respectively. Interestingly, AG1478 strongly inhibited the holoenzyme activity of recombinant human CK2 with an IC₅₀ of 25.9 μmol/L, the inhibition was close to that of A3, but was slightly less than that of DRB. These results indicated that AG1478 was not only a novel blocker of recombinant human protein kinase CK2, but also a potent and effective inhibitor of CK2.

CK2 is probably the most pleiotropic member of the protein kinase family, the still increasing list of CK2 substrates includes more than 200 proteins. Among them calmodulin, p34^{cdc2}, estrogen receptor 2, and insulin receptor concerned with signal transduction are also phosphorylated by PKC and TPK^[1-3]. These substrates indicate that there are close relationships between CK2 and several other protein kinases involved in signal transduction and regulation of cellular metabolism^[1-3]. However further study is required to determine these precise mechanisms.

Due to we obtained enough pure and biologically active human protein kinase CK2, the method is very simple and rapid to investigate the inhibitory effect and their action mechanisms of tested agents as drug target using this CK2 enzyme. So this paper suggests that the recombinant human protein kinase CK2 may be used as a molecular target for simpler screening and development of more effective inhibitors of CK2.

ACKNOWLEDGMENT We are grateful to Prof Aviv GAZIT for presenting generously tyrphostin AG1478.

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Tyrphostin AG1478 对重组人蛋白激酶 CK2 全酶的抑制作用及其动力学分析¹

R96 A

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关键词 酪蛋白类; 蛋白激酶类; 重组蛋白质类; 全酶类; tyrphostins; 动力学

目的: 观察 Tyrphostin AG1478 [4-(3-氯苯胺基)-6,7-二甲氧喹啉]对重组人蛋白激酶 CK2 全酶的直接作用及其酶动力学机制。 **方法:** 在体外等摩尔数混合重组蛋白激酶 CK2 α 和 β 亚基构成 CK2 全酶, 在不同条件下测定 CK2 的活性。 CK2 活性通过测定转移到 CK2 底物上的 $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ 或 $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ 的 ^{32}P

放射活度来检测。 **结果:** 重组人 CK2 是一种 Ca^{2+} 、 $[\text{KG} \times 2]\text{cAMP}$ 和 cGMP 等第二信使非依赖性蛋白激酶, 与天然 CK2 的性质一致。 AG1478 对重组人 CK2 全酶具有很强的抑制作用, IC_{50} 为 $25.9 \mu\text{mol/L}$, 抑制作用接近于已知的 CK2 抑制剂 *N*-(2-氨基)-5-氯萘-1-磺胺(A3), 稍小于 5,6-二氯-1- β -呋喃糖苯并咪唑(DRB)。 AG1478 对重组人 CK2 的动力学研究表明: 它与 GTP 和酪蛋白均呈竞争性抑制作用, 是一种双底物抑制剂。 **结论:** AG1478 不仅是高效特异的表皮生长因子受体酪氨酸蛋白激酶的抑制剂, 而且也是一种新型有效的蛋白激酶 CK2 抑制剂。 重组人蛋白激酶 CK2 可作为一种较为简便的筛选和开发有效 CK2 抑制剂的分子靶点。

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