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# Novel Dicinnamate Analogue Inhibits Casein Kinase 2: A Potential Cancer Therapeutic

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## ABSTRACT

CK2 is a serine/threonine-protein kinase that has been implicated in a number of cellular processes, including maintenance of cell viability, protection of cells from apoptosis, and tumorigenesis. Elevated CK2 activity has been pinned to numerous cancers where it is believed to promote oncogenesis. As such the role of CK2 in the progression of tumorigenesis has become increasingly evident as the dysregulation of a number of pathways has been attributed to abnormally high levels of CK2 activity. In this study, the CK2 inhibitory properties of four novel dicinnamate analogues, namely, 3-(3,4-diemthoxy-phenyl)-acrylic acid; 2-hydroxy-3-(3-ptolyl-acryloyloxy)-propyl ester; 2-acetoxy-5-((E)-3-(3-((E)-3-(3,4-dimethoxyphenyl)acryloyloxy)-2-

hydropropoxy)-3-oxoprop-1-enyl) benzoic acid; 4-((E)-3-(3-((E)-3-(3,4-dimethoxyphenyl)acryloyloxy)-2-hydropropoxy)-3-oxoprop-1-enyl)benzoic acid were investigated. The results to date showed that 4-((E)-3-(3-((E)-3-(3,4-dimethoxyphenyl)acryloyloxy)-2-hydropropoxy)-3-oxoprop-1-enyl) benzoic acid, exhibited tremendous inhibition activity with low % Ctrl, high CK2 affinity, Kd=24 μM and high selectivity to CK2 (0.005). The results of the study suggest HL-04 as a suitable candidate for further development and possible therapeutic treatment of aggressive high grade astrocytoma (HGA) brain cancer and some neurodegenerative disorders.

Key words: casein kinase 2, CK2-inhibitor, glioblastoma, Alzheimer's, Parkinson's

Abbreviations: CK2 - casein kinase-2; HGA - high grade astrocytoma; GBM - glioblastoma multiforme; AA - anaplastic astrocytoma; CK2-Is - casein kinase 2-inhibitors

## 1. INTRODUCTION

It is a known fact that cancer affects all communities, whether, directly or indirectly, irrespective of ethnicity, social class, gender, sexuality, financials or spirituality. Results from the global cancer burden and epidemiology report published by the World Health Organization (Boyle and Levin, 2008), emphasize this growing dilemma. The reported incidence and mortality rate show in excess of 12 million new cases, 6.5 million deaths annually, and over 22 million people living with the disease (Boyle and Levin, 2008). Currently the four most prevalent types of cancers includes, prostate, breast, colon and lung (Boyle and Levin, 2008; Bray et al, 2013; Ferlay et al, 2013). In the UK, cancer remains the major killer in adults aged 50-74 accounting for 41% of deaths in males and 48% in females Bray et al, 2013; Ferlay et al, 2013). Similar mortality rates exist in other developed and emerging economies.

Clinical interventions using cocktail cytotoxic drugs (Gottesman, 2002), surgery, radiation therapies (Jaffray, 2012), and combination treatments (Suntharalingam et al, 2011), have proven successful. However, despite advancements in therapeutic management of cancer extremely aggressive high grade astrocytoma (HGA) brain cancer; glioblastoma multiforme (GBM) and anaplastic astrocytoma (AA), remain very difficult to treat (Dixit et al, 2012). These high malignancy tumors rapidly metastasize to other parts of the brain (Dixit et al, 2012; Donson et al, 2012). The ongoing challenge is that GBM and AA are resistant to TNF$\alpha$-mediated cell death and modern chemotherapeutic drugs (Dixit et al, 2012). This coupled with the location of the tumors in the brain which often renders them inoperable (Donson et al, 2012) pose major challenges in managing these types of cancers. Moreover, the recurrence rate is usually high with prognosis for short-term survival ranging between 17-36 months. High mortality rates are also observed with long-term survival being exceptionally rare.

Over-expression of gene signatures encoding casein kinase-2 (CK2) occurs in HGA tumors (Munstermann et al, 1990). CK2 down-regulates the activity of caspase (Yamane and Kinsella, 2005), promotes tumor cell survival and apoptotic resistance (Litchfield, 2003). However, several CK2-inhibitors (CK2-Is) are known to have positive effects on HGA, increasing sensitivity of tumor cells to TNFα, thus suppressing NF-$\kappa\beta$ activation, and promote apoptosis (Romieu-Mourez et al, 2001; Izeradjene et al, 2004; Rieger et al, 2007). Glioma cells treated with small interfering RNAs (siRNAs) triggered a down-regulation of CK2 $\alpha/\beta$ subunits, revealed a 25% reduction in tumor cell viability (Dixit et al, 2012). CK2-I proposed apoptotic mechanism of action causes the activation of p53, through the inhibition of mdm2 and SIRT1 (Dixit et al, 2012). Elevated CK2 is associated in the molecular pathology of neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases (Perez et al, 2011). In addition, CK2- $\alpha'$-mediated antiapoptotic effect is well established in the prevention of cardiac hypertrophy (Hauck et al, 2008; Force, 2008).

Results from our previous studies revealed that the crude extract of *T. recurvata* inhibited several kinases, CK-2 included (Lowe et al, 2012). Subsequently, 1,3-di-*O*-Cinnamoyl-glycerol, a natural CK2-I compound, was isolated from *T. recurvata* sp., which exhibited marginal anticancer activity in WST-1 cell cultures (Lowe et al, 2013). Furthermore, four novel dicinnamate analogues were synthesized from 1,3-di-*O*-Cinnamoyl-glycerol (Lowe et al, 2014). As such, this study sought to investigate efficacy of these novel derivatives in inhibiting CK2.

## 2. METHODOLOGY

### Enzymatic assay

*In vitro* kinase competition binding assay was carried out using, according to the methods of Fabian et al. (2005) and Karaman et al. (2008); with minor modifications (Figure 1). Kinases were prepared in two separate processes. For most assays, kinase-tagged bacteriophages (T7 strain) were used to infect *E. coli* (BL21 strain) during the exponential growth phase at 32°C. Cellular debris of the BL21 lysates was filtered after centrifugation. Kinases from T7/BL21 produced full-length constructs, which were used for small-, single-domain kinases assays. Alternatively, catalytic domain constructs needed for large multi-domain kinase assays, were synthesized in HEK-293 cells. These kinases were tagged with DNA sequences for quantitative real-time polymerase chain reaction (qPCR) detection. For the kinase competitive binding assays, affinity resins were developed using streptavidin-coated magnetic beads, incubated at 25°C for 30 mins with biotinylated ligands. Fixation of ligands to the strepatavidin-beads was done using biotin. Unbound ligands were removed with washing buffer, comprised of 1% Bovine serum albumin (BSA), 0.05 % Tween 20 and 1 mM

Dithiothreitol (DTT) (SeaBlock). Both kinase constructs, ligand affinity beads and cinnamoyl test compounds were combined, and assembled with binding buffer (20% SeaBlock, 0.17 × Phosphate-buffered Saline (PBS), 0.05% Tween 20 and 6mM DTT).

The cinnamoyl compounds were prepared in 100% Dimethyl sulfoxide (DMSO), however, a concentration of 2.5% DMSO was used for the assays. Cinnamoyl-kinase reactions were performed in polypropylene (96 well) plates and incubated at 25°C for 60 minutes. After which, the reaction affinity beads were washed with buffer solution (1 x PBS, 0.05% Tween 20). The beads were then re-suspended in elution buffer (1 x PBS, 0.05% Tween 20, 0.5 µM non-biotinylated affinity ligand) and incubated at room temperature for 30 min, with continuous shaking. Kinase concentration in the eluates was measured by qPCR.

## Test Compounds

The test compounds were *1,3-di-O-cinnamoyl-glycerol* (HL-01A) and its analogues, synthesized via the Wittig process shown in Figure 2 (Lowe et al., 2014). The dicinnamate analogues were: 3-(3,4-diemthoxy-phenyl)-acrylic acid (HL-01); 2-hydroxy-3-(3-ptolyl-acryloyloxy)-propyl ester (HL-02); 2-acetoxy-5-((E)-3-(3-((E)-3-(3,4-dimethoxyphenyl)acryloyloxy)-2-hydropropoxy)-3-oxoprop-1-enyl)benzoic acid (HL-03); and 4-((E)-3-(3-((E)-3-(3,4-dimethoxyphenyl)acryloyloxy)-2-hydropropoxy)-3-oxoprop-1-enyl)benzoic acid (HL-04).

### *Calculations*

Binding constants (Kds) were calculated with a standard dose-response curve using the hill equation [1].

$$\text{Response} = \text{Background} + \frac{\text{Signal Background}}{1 + (\text{Kd}^{\text{Hill Slope}} / \text{Dose}^{\text{Hill Slope}})} \quad [1]$$

Where:
Hill Slope was set to -1

### *Percentage Control (%Ctrl)*

Binding interactions of the test compounds were reported as %Ctrl, where lower number indicate stronger hits. %Ctrl was calculated using equation [2]:

$$\left( \frac{\text{test compound signal - positive control signal}}{\text{negative control signal - positive control signal}} \right) \times 100 \quad [2]$$

Where:
test compound = cinnamoyl analogues
negative control = DMSO (100%Ctrl)
positive control = control compound (0%Ctrl)

### *Selectivity Score (S-score)*

Selectivity score is a quantitative measure of a test compound selectivity to a particular kinase, excluding all mutant variants. This was calculated using %Ctrl as a potency threshold (below) using equation [3]:

$$\text{S-score} = \text{Number of hits} / \text{Number of assays} \quad [3]$$

Where:
S(35) = (number of non-mutant kinases with %Ctrl <35)/(number of non-mutant kinases tested)
S(10) = (number of non-mutant kinases with %Ctrl <10)/(number of non-mutant kinases tested)
S(1) = (number of non-mutant kinases with %Ctrl <1)/(number of non-mutant kinases tested)

## Statistical analysis

Statistical analysis was carried out using a non-linear least square fit with the Levenberg-Marquardt algorithm.

# 3. RESULTS AND DISCUSSION

The kinase panel consisted of 451 kinases, including gene signatures of 392 non-mutant human kinases. Of the 4 novel dicinnamate analogues, only HL-04 had significant inhibitory effect on CK2 (Table 1). HL-04 demonstrated a very high CK2 binding affinity, with Kd=24 µM. This corresponded to the qPCR analytical curves which measured the affinity or amount of CK2 that bind to HL-04 (Figure 3). The qPCR signal (y-axis) was plotted against the HL-04, at varying concentrations (nM), in log10 scale (x-axis).

**Table 1**

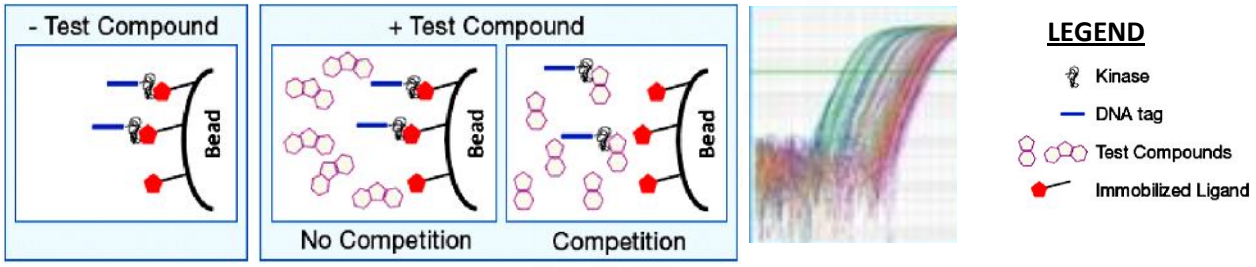
Affinity of dicinnamates to CSNK2A2 and corresponding Selectivity Score

| | **1,3-di-*O*-cinnamoyl-glycerol Derivatives** | | | | |
|---|---|---|---|---|---|
| | **HL-01A** | **HL-01** | **HL-02** | **HL-03** | **HL-04** |
| Primary screen inhibition % (10 µM) | 0 | 9 | 10 | 8 | 77 |
| Selectivity Score (S35) | N/A | N/A | N/A | N/A | 0.005 |
| Kd (µM) | N/A | NT | NT | NT | 2.4 |

N/A: Not applicable
NT: Not tested

**Figure 1**

Graphical illustration of the kinase competition binding and quantification




The data revealed yet another significant finding, where HL-04 demonstrated a selectivity score of 0.005 in relation to CK2 affinity (Table 1). The s-score is a powerful quantitative measure of kinase inhibition selectivity by a molecule (Karamanet al, 2008) and the smaller the s-score, the better the drug candidate as inhibition of multiple kinases is an indication of lack of specificity and usually would lead to toxicity concerns.

The activity observed with HL-04 against the CK-2 kinase seems to suggest structural influence on the binding affinity with the kinase. While the five compounds screened do share the same basic dicinnamate moiety, with compound HL-01A being structurally present in all the other 4 compounds, this compound showed zero inhibition in the primary screen against CK-2. Compounds HL-01, HL-02 and HL-03 exhibited negligible inhibition. An examination of the structures indicates that the presence of the dimethoxy group in positions C3 and C4 renders some activity (HL-01, HL-02 and HL-03). The presence of a methyl group in position C4 of the second phenyl ring had no effect on activity. Most interestingly is the activity of HL-03 versus HL-04. Compound HL-03 differs from HL-04 in that it has an acetate group on C4 of the second phenyl ring in addition to the presence of benzoic acid which is also present in HL-04. The presence of the acetate group in this molecule seems to antagonize activity against CK-2 despite the presence of benzoic acid. From the data obtained in this study and based on structure activity relationship (SAR) analysis, we can conclude that the benzoic acid group is crucial for the CK-2 inhibition activity of cinnamoyl derivatives. Future studies will determine whether the two methoxy groups contribute to activity or not.

## 4. CONCLUSION

The study is the first to reveal the kinase inhibition effect of 4-((E)-3-(3-((E)-3-(3,4-dimethoxyphenyl)acryloyloxy)-2-hydropropoxy)-3-oxoprop-1-enyl) benzoic acid. More importantly, we have highlighted the unique potential of this novel compound, to specifically target and inhibit CK-2. This strong evidence could possibly lead to the development of a novel anticancer therapeutic for aggressive malignances, including glioblastoma multiforme brain cancer. In addition, this new dicinnamate compound could also prove beneficial to persons suffering from neurodegenerative diseases and cardiovascular disorders, where CK2 levels are significantly elevated.

**Figure 2**
Structures of dicinnamate derivatives studied

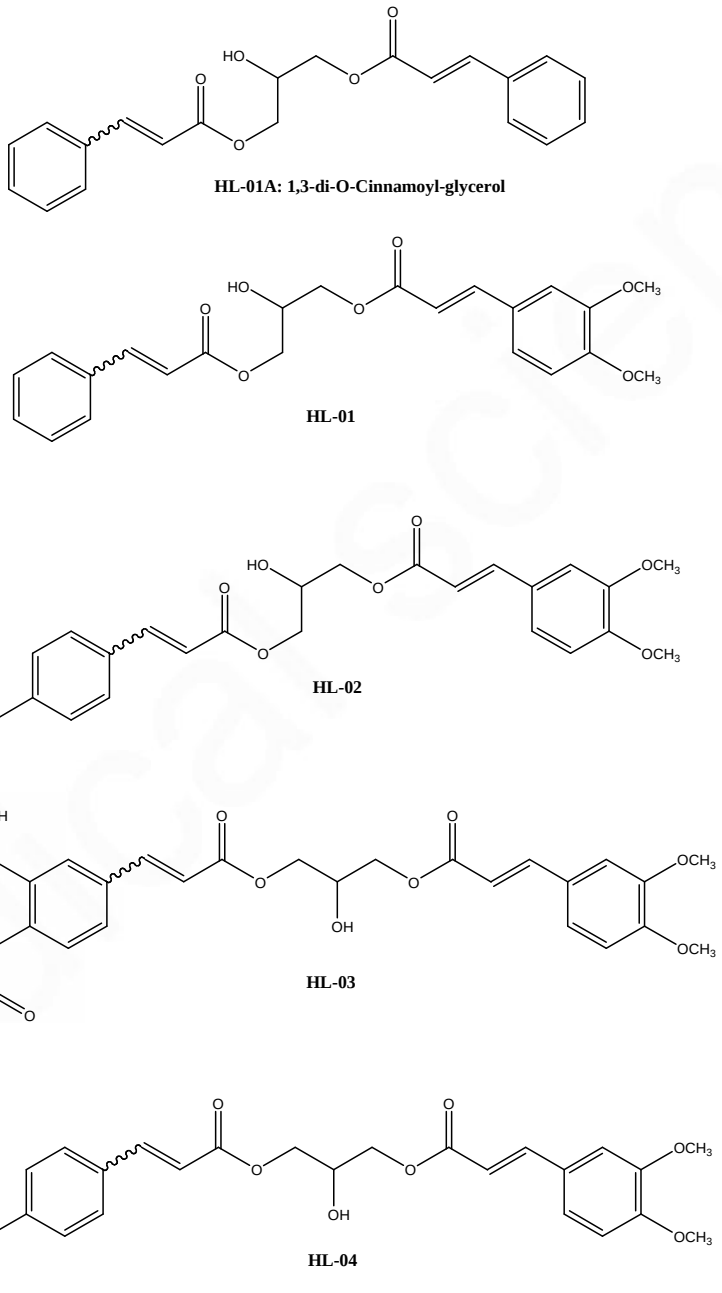





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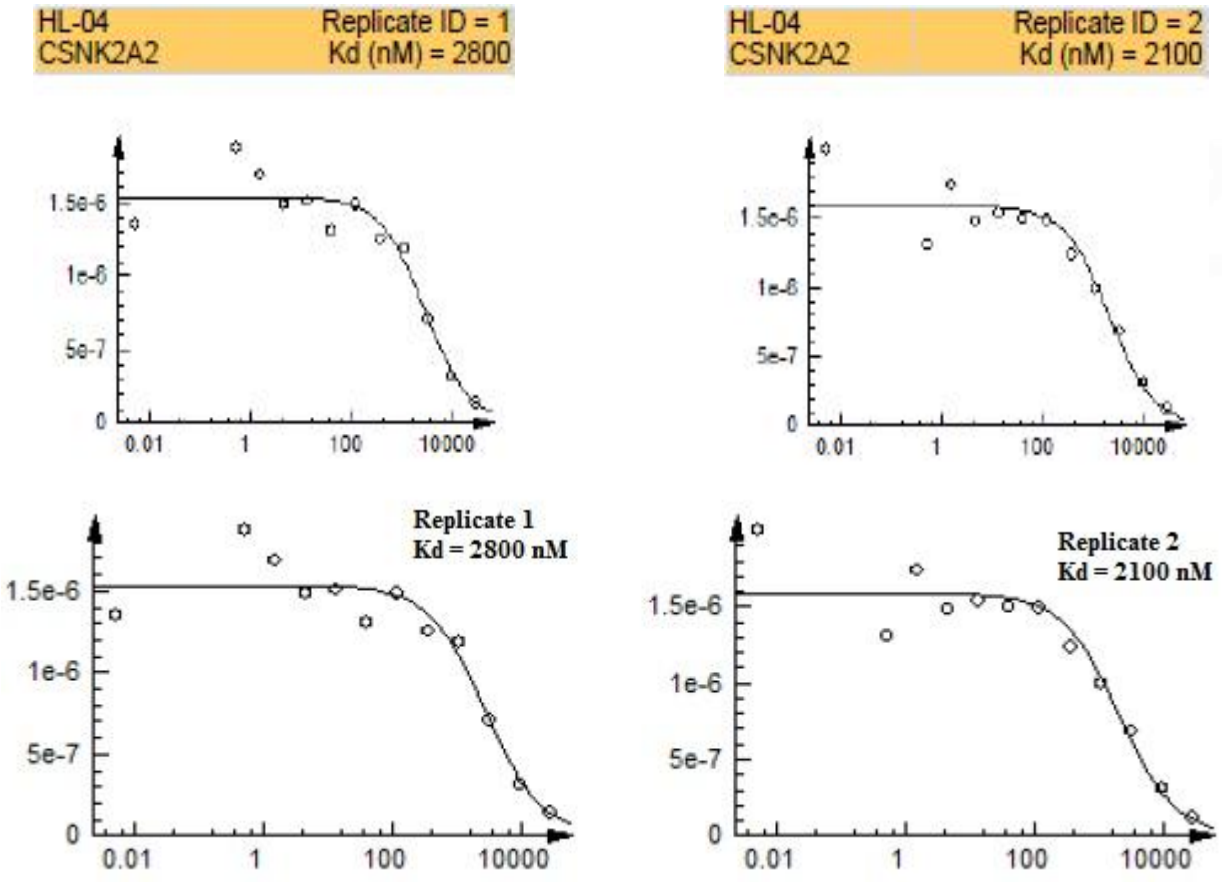
**Figure 3**
PCR Quantification Curves showing CK2 binding to HL-04




## DISCLOSURE STATEMENT

Authors have no conflicting interest.

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