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Molecular characteristics of CTA056, a novel Itk inhibitor which selectively targets malignant T cells and modulates oncomirs

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Running title: Itk inhibitors and T cell leukemia/lymphomas

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Abbreviations

Itk, Interleukin 2 inducible T-cell kinase; Btk, Bruton's tyrosine kinase; Etk, Endothelial and Epithelial tyrosine kinase; miR, MicroRNA; IFNγ, Interferon γ; IL-2, Interleukin 2; TLC, Thin layer chromatography; PI, Propidium Iodide.

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Abstract

Interleukin-2 inducible T cell kinase (Itk) is a member of the Btk (Bruton's tyrosine kinase) family of tyrosine kinases. Itk plays an important role in normal T cell functions and in the pathophysiology of both autoimmune diseases and T cell malignancies. Here, we describe the initial characterization of a selective inhibitor, CTA056, which was developed through screening a 9,600-compound combinatorial solution phase library, followed by molecular modeling, and extensive structure-activity-relationship studies. CTA056 exhibits the highest inhibitory effects toward Itk, followed by Btk and Etk. Among the 41 cancer cell lines analyzed, CTA056 selectively targets acute lymphoblastic T-cell leukemia and cutaneous T-cell lymphoma. Normal T-cells are minimally affected. Incubation of Jurkat and MOLT-4 cells with CTA056 resulted in the inhibition of the phosphorylation of Itk and its effectors including PLC-γ, Akt, and ERK, as well as the decreased secretion of targeted genes such as IL-2 and IFNγ. Jurkat cells also underwent apoptosis in a dose dependent manner when incubated with CTA056. The potent apoptosis-inducing potential of CTA056 is reflected by the significant modulation of microRNAs (miR) involved in survival pathways and oncogenesis. The in vitro cytotoxic effect on malignant T cells is further validated in a xenograft model. The selective expression and activation of Itk in malignant T cells, as well as the specificity of CTA056 for Itk, make this molecule a potential therapeutic agent for the treatment of T cell leukemia and lymphoma.

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Introduction

T cell malignancies are a heterogeneous group of cancers which encompass several entities, including acute lymphoblastic leukemia (T-ALL), adult T-cell leukemia/lymphoma (ATL), and Sezary syndrome/cutaneous T cell lymphoma (CTCL). Although there are marked differences between these cancers, there is some overlap in their management, especially at advanced stages of the disease. Also, in terms of survival, patients with relapsed T cell malignancies uniformly have a poor outcome. Results from the large UKALL XII/ECOG 2993 trial demonstrated that following therapy, 42% (95% CI, 36%-47%) of patients with T-ALL relapsed within five years (Marks et al., 2009). Similar to T-ALL, patients with Sezary/CTCL have a dismal 5-year survival rate of only 11% (Kim et al., 2003). Management of these patients is therefore an important area of research. Even if a patient responds well to therapy, there are significant short-term, and potentially long-term, side effects from the intensive chemotherapy regimens needed to attain sustained remission. Thus, the identification of new targets for drug development is critical.

Protein tyrosine kinases play a central role in the regulation of signals originating from the cell surface (August et al., 1994). Itk is one of the five members of the Btk family of non-receptor tyrosine kinases, and is known to be essential for T cell function (Siliciano et al., 1992; Berg et al. 2005). It is a critical modulator of TCR (T cell receptor) signaling, which connects receptor proximal signal molecules such as Lck, ZAP70, LAT and SLP-76 to PLC-γ (Kim et al., 2003; Grasis et al., 2010), as well as other effectors in the cytosol (Andreotti et al., 2010). Phosphorylation and activation of PLC-γ by Itk mediates calcium flux, resulting in the activation of calcineurin and NFATc with consequent upregulation of cyotkines. Recently, Itk was also shown to engage GPCR (G-protein-coupled receptor) signaling mediated by CXCL14/SDF1, and induce actin polymerization and cellular migration (Berge et al., 2010). Itk deficient animals, while viable, have profound defects in T cell differentiation and development, especially with

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respect to CD4+ T cells (Fowell et al., 1999; Schaeffer et al., 2001). At the cellular level, Itk deficient cells have impaired T cell proliferation, adhesion, migration, and F-actin reorganization (Dombroski et al., 2005; Woods et al., 2001). Although Itk inhibitors are not commercially available, selective Itk inhibitors are currently under development (Lin et al., 2004; Cook et al., 2009). Such inhibitors have been tested in models of lung inflammation, skin dermatitis, asthma (Lin et al., 2004), and HIV infection models with promising results (Readinger et al., 2008), although none have entered clinical trials (Hao et al., 2006). In addition, as the prior emphasis of these inhibitors was on inflammation, the characterizations of these inhibitors were primarily limited to cytokine release and immune modulation, with a paucity of information regarding their effects on T cell proliferation and apoptosis. We are interested in targeting Itk for T cell malignancies, due to Itk's heightened expression and aberrant activation in these cells (Kaukonen et al., 1999). Itk has also been shown to be up-regulated in skin lesions of patients with cutaneous T cell lymphoma (Shin et al., 2007), and the Itk-dependent CD25 signaling pathway is known to be important in T cell malignancies. Targeting CD25-expressing T cell leukemias/lymphomas with anti-CD25 monoclonal antibodies has shown therapeutic efficacy (Zhang et al., 2003; Dancey et al., 2009). Recently, a t(5;9)(q33;q22) chromosomal translocation was identified in a subset of unspecified peripheral T cell lymphomas. The translocation resulted in an Itk-Syk fusion gene (Streubel et al., 2006), which is oncogenic in animal models (Pechloff et al., 2010). In this case, Itk's PH (pleckstrin homology), but not kinase, domain is involved. These findings point to Itk's involvement in the pathogenesis of T cell malignancies and support Itk as a potential therapeutic target for anti-cancer drug development. Herein, we report the development of a new class of Itk inhibitors, which are structurally different from the other reported Itk inhibitors. We chose to characterize CTA056, which has selectivity toward Itk and preferentially targets T cell malignancies expressing Itk. The signals diminished by CTA056 treatment are consistent with the inhibition of Itk kinase activity. Interestingly, microRNA profiling of malignant T-cells treated with CTA056 display the upregulation of miRs which suppress survival

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factors, and the down-modulation of those which increase oncogenicity. CTA056 thus appears to be a promising new lead to target Itk in T-cell malignancy. To our knowledge, this is the first report of the application of an Itk selective inhibitor as an anti-cancer agent.

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Materials and Methods

Materials

Purified Itk, Btk, Etk, and Src kinases were obtained from Millipore Inc (Dundee, UK). Propidium Iodide (PI), *N*,*N*-diisopropylethylamine (DIEA), *N*,*N*-dimethylformimide (DMF), ethanol, acetonitrile (ACN), 1-(3-aminopropyl)piperidine, trifluoroacetic acid (TFA), palladium on carbon (Pd/C), ammonium formate and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Saint Louis, MO). L-Phenylalanine methyl ester hydrochloride and 4-(4-pyridyl)benzaldehyde were purchased from Chem-Impex International Inc (Wood Dale, IL). The annexin V-FITC apoptosis detection kit was obtained from Abcam (Cambridge, MA). Reversed-phase high-performance liquid chromatography (RP-HPLC, C18 column, 5 μm, 19 mm × 150 mm and 4.6 mm x 150 mm for preparative and analytical HPLC, respectively) from the Waters Corporation (Milford, MA) was used for analysis and purification of CTA056.

One-pot synthesis of CTA056

The synthetic approach of CTA056 is similar to that previously reported (Zhang et al., 2005), but with slight modification as shown in Figure 1A. In brief, a solution of L-Phenylalanine methyl ester hydrochloride (215.7 mg, 1.0 mmol) and DIEA (383.2μL, 2.2 mmol) in DMF (1.5 ml) was added dropwise under vigorous stirring to a solution of 1,5-difluoro-2,4-dinitrobenzene (204.0 mg, 1.0 mmol) in DMF (0.5ml) . The reaction solution was stirred at room temperature for 30 min. This was followed by the addition of a solution of 1-(3-aminopropyl) piperidine (159 μL, 1.0 mmol) and DIEA (174.2 μL, 1.0 mmol) in DMF (1 ml). The resulting mixture was agitated at room temperature overnight. Ethanol (20 ml), Pd/C (10%, 200 mg), and ammonium formate (1.50g, 23.8 mmol) were added to the solution. The solution was heated to reflux for 3 h and then cooled to room temperature. The Pd/C was filtered out and the filtrate was concentrated with

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rotary evaporator. 4-(4-Pyridyl)benzaldehyde (183.2 mg, 1.0 mmol) in DMF was added to the solution. The resulting solution was stirred at room temperature for 1 day. The DMF solution was poured into 40 mL of ice. The precipitate was collected by filtration and washed with water, followed by RP-HPLC purification. The fraction was collected and lyophilized to give a yellow powder as the final product. The homogeneity of the compound was checked by analytical RP-HPLC. The purity was determined to be >95% pure. The identity of the compounds was confirmed by Orbi-trap high resolution mass spectrometry. Found: 555.2868 dalton (calculated: 555.2866 dalton for MH^+).

Molecular modeling

Itk kinase domain in complex with staurosporine was used to create the docking pocket (Brown et al., 2004). Staurosporine was deleted and the Itk crystal structure was optimized for docking using Autodock 4.0. Docking was performed using Autodock Vina 1.0. The docking results were analyzed using Pymol 0.99 (Trott et al., 2010; Seeliger et al., 2010).

Kinase activity assay

For tyrosine kinases, kinase activity was measured using thin-layer-chromatography (TLC). Briefly, purified kinases (20 nM), the corresponding substrate (500 μM, TSFYGRH for Itk, YIYGSFK for the other kinases), and CTA056 (0-10 μM) were incubated in a kinase reaction (100 mM Hepes, pH 7.4, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM DTT) for 5 min, and the reaction was started by adding 5 μCi ^{33}P-labeled ATP. The reaction (10 μl) was incubated at room temperature for 1 h and was stopped by adding 10 μl H_3PO_4. The radioactivity of the peptide substrate was analyzed using TLC as previously described (Luo et al., 1996). For Ser/Thr kinases, the kinase activity was measured using kinase-glo assay (Promega Inc.). Briefly, purified kinases (20 nM), the corresponding substrate (Map kinase substrate peptide for Mkk1 and Erk1, PDKtide

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for PDK1, Akt/SGK peptide substrate for Akt, Millipore Inc.), and CTA056 (0-10 μM) were incubated in a kinase reaction (100 mM Hepes, pH 7.4, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM DTT) for 5 min. and the reaction was started by adding 50 μM ATP. The reaction (10 μl) was incubated at room temperature for 1 h and the kinase activity was measured using kinase-glo assay according to the manufacture's instruction.

Itk autophosphorylation assay

Itk autophosphorylation activity was measured by an *in vitro* kinase assay. Briefly, purified Itk (100 ng) was mixed with CTA056 inhibitor in the kinase assay buffer (20 mM Hepes pH 7.55, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 500 μM Na_3VO_4). The cold ATP (5 μM) and hot r-33p-ATP (5 μci) were added to the mixture and the kinase reaction was performed at 30°C for 30 min. The reactions were terminated with 4X SDS-PAGE sample buffer and then loaded onto an 8% SDS-polyacrylamide gel for electrophoresis. The gel was vaccum dried and the ITK auto kinase activity was analyzed with a phosphoimager (Biorad).

Cell culture

Jurkat, MOLT-4, LNCaP, PC3, and RWPE1 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin/glutamine. BCBL-1 cells were in RPMI 1640 medium containing 15% fetal bovine serum and 1% penicillin/streptomycin/glutamine.

Western blotting

Western blotting was performed as described previously (Grasso et al., 1997). Proteins were detected by the following antibodies: β-actin (Sigma-Aldrich, Saint Louis, MO), Itk (Santa Cruz

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Inc., Santa Cruz, CA), and phospho-Itk (Epitomics Inc., Burlingame, CA). For phospho-Itk, cells were pre-treated with 100 μM pervanadate for 15 min before harvest.

MTT assay

Cells were seeded in 96-well plates and cultured overnight, followed by treatment with 0.1% DMSO, as vehicle control, and CTA056 at the indicated concentrations for 72 h. Growth inhibition was measured using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostic, Mannheim, Germany).

Flow cytometry

Jurkat cells were treated with 0.1% DMSO (control) and CTA056 at the indicated concentrations for 24 h. Cell cycle arrest was determined by the incorporation of propidium iodide (Sigma-Aldrich) into permeabilized cells. Cells undergoing apoptosis were identified using an Annexin V-FITC kit (Abcam), following the manufacturer's instructions. The cells were analyzed using a Coulter Epics XL flow cytometer (Beckman Coulter, Miami, FL).

Stimulation of phospho-Itk assay

Jurkat cells were pre-treated with CTA056 at 37 °C for 30 min and then incubated with anti-CD3 and anti-CD28 monoclonal antibodies (Ebiosciences) at 10 μg/ml and protein G (BD Biosciences) 10 μg/ml. At 30 sec, 1 min, and 5 min the stimulation was stopped by addition of Fix Buffer I (BD Biosciences), and then the cells were permeabilized with Perm Buffer III (BD Biosciences). A phospho-Itk-specific antibody (BD Biosciences) was then used for intracellular staining. Samples were analyzed on a BD FACS Calibur flow cytometer.

Cytokine secretion assay

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Jurkat cells were pre-treated with CTA056 0.5 μM for 30 min at 37°C and then stimulated with anti-CD3 and anti-CD28 antibodies (Ebiosciences) at 1 μg/ml for 8h. Cells were harvested and RNA was isolated using trizol reagent (Ambion) and mRNA levels of IL-2 and IFN-γ were measured using real-time PCR with primers 5'GTCTTGCACTTGTCACAAACAG3' and 5'CAGTTCTGTGGCCTTCTTGG' for IL-2, 5'AATTGGAAAGAGGAGAGTGACAG3' and 5'ATTCATGTCTTCCTTGATGGTCTC3' for IFN-γ. For cytokine release, IL-2 was quantified using a sandwich ELISA kit and IFN-γ was quantified using ELISPOT assay (BD Biosciences).

Microarray assay for MiRNAs

Jurkat cells were pre-treated with CTA056 0.5 μM for 30 min at 37°C and then stimulated with anti-CD3 and anti-CD28 antibodies (Ebiosciences) at 1 μg/ml for 8h. Cells were harvested and RNA was isolated using trizol reagent (Ambion). MiRNA profile was examined using miroRNA array. miRs with the most significant changes from miR array were further confirmed using real-time PCR with the corresponding primers (Qiagen Inc.)

Inhibition of MOLT-4 xenograft tumor growth by CTA056

10^7 MOLT-4 cells were injected subcutaneously to nude mice. The tumors were grown to the indicated size and the mice were randomly divided into two groups (8 mice/group). The control group was treated with vehicle. The treatment group was treated with CTA056 at 5 mg/kg twice a week with intratumoral injection. The tumor size and body weight were measured once a week.

Statistics

A one-way ANOVA was used in combination with a Tukey test for pair wise comparison. P values less than 0.05 were considered significant.

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Results

CTA056 as a selective inhibitor against Itk tyrosine kinase

Through screening a 9,600-diversity combinatorial solution phase small molecule library, compounds with inhibitory activities against Btk were discovered. Subsequent molecular modeling and extensive structure-activity-relationship studies led to the identification of CTA056 (Figure 1A). Molecular modeling studies based on the structure elucidated by Brown et al. (Brown et al., 2004) indicates that this compound binds well to the kinase domain of Itk, where it fits into the ATP-binding pocket. The binding appears to be mediated through hydrogen bonds, as well as π-π hydrophobic interactions with residues in the ATP-binding pocket (Figure 1B).

Having isolated a small molecule that fits well into the ATP binding pocket of Itk, the next logical step was to determine the molecule's ability to inhibit the kinase function of Itk, and to determine its specificity for this enzyme. To accomplish this, purified Itk, Btk, and Etk were incubated in a kinase reaction buffer with CTA056 (0-10 μM) in the presence of ^{33}P-labeled ATP. TSFYGRH was used as substrates for Itk, and YIYGSFK for Btk and Etk. YIYGSFK was previously identified as an efficient substrate for Src through screening a one-bead-one-compound (OBOC) combinatorial peptide library (Lam et al., 1995). We subsequently found that YIYGSFK is also a very good substrate for both Btk and Etk, but not for Itk. We then screened XXXYXXX OBOC library with Itk, and identified TSFYGRH as an Itk substrate (data not shown). A kinase assay with TLC technique (Luo et al., 1996) revealed that CTA056 was a potent and selective inhibitor for Itk, with an IC_{50} of approximately 100 nM (Figure 2A). Inhibition was observed in a concentration dependent manner, and at higher concentrations, CTA056 could also inhibit Btk ($IC_{50} \approx 400$ nM). However, Etk was significantly more resistant to

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CTA056 inhibition with an IC_{50} of approximately 5 μM (Figure 2A). In summary, CTA056 showed selectivity for Itk over other Tec family members.

Btk family non-receptor tyrosine kinases are characterized by the presence of an autophosphorylation site within their non-catalytic Src homology 3 (SH3) domain. Thus, in addition to characterizing the ability of CTA056 to inhibit Itk's ability to phosphorylate cognate peptides, it was also important to determine the ability of CTA056 to inhibit Itk autophosphorylation. Therefore, an *in vitro* Itk autophosphorylation assay was established in which purified Itk was mixed with CTA056 in the presence of ^{33}P-ATP. After 30 min, the reaction was terminated, and the samples were loaded onto an SDS-polyacrylamide gel for electrophoresis. After drying, the gel was analyzed with a phopshorimager. Figure 2B reveals that CTA056 was able to inhibit Itk autophosphorylation in a concentration dependent manner.

In addition to Btk family tyrosine kinases Itk, Btk and Etk, the inhibitory activity of CTA056 to other kinases, including Src, Yes, Lyn, Axl, Mer, EGFR, Abl, was investigated using a TLC assay. As shown in Table 1, CTA056 appears to have reactivity toward Btk family kinases and Src, but not toward any of the other kinases tested.

CTA056 preferentially inhibits the growth of malignant cells harboring Itk

Since CTA056 selectively inhibits Itk, Itk expression in 41 different cancer cell lines was examined and Itk was detected only in the T cell acute lymphoblastic leukemia cell lines, Jurkat and MOLT-4, and in the cutaneous T cell lymphoma cell line, Hut78 (Figure 3A). To determine the effect of CTA056 on proliferation, a panel of cancer cell lines including Jurkat and MOLT-4 cells were incubated with CTA056 and proliferation was measured using the MTT assay. At 2 μM, CTA056 was very effective in inhibiting the growth of Itk-high cancer cells (Jurkat and

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MOLT-4) but not the Itk-null cancer cells (HCT116, HL60, HepG2, MCF7, and SKOV3). Significantly, the three normal cell lines LO2, WI38, and MCF10A, as well as normal T cells, were resistant to CTA056 (Figure 3B). Titration experiments further confirmed these findings and revealed that Jurkat cells were most sensitive to CTA056-induced growth inhibition (Figure 3C). Since CTA056 also showed moderate inhibition to Btk (Figure 2A), two B cell leukemia/lymphoma cell lines, BCBL-1 and Raji, were examined. As expected, CTA056 showed moderate growth inhibition to these two cell lines (Figure 3C). These results strongly suggest that the *in vivo* target of CTA056 is Itk. The inhibitory effect of CTA056 on BCBL-1 and Raji is likely due to its modest inhibitory activity against Btk (Figure 2A).

We then synthesized and tested a series of analogs with a fused three-ring core structure identical to CTA056, differing only in the side groups R_1, R_2 and R_3 (Figure 4). These compounds showed significant variability in their abilities to inhibit Itk (open bar), with CTA056, CTA151, and CTA164 being the most potent. Interestingly, these three compounds have the same R2 and very similar R1 and R3 groups. And when their abilities to inhibit Jurkat growth were measured (solid bar), there was a strong correlation between Itk inhibition and Jurkat growth retardation (Figure 4). This data further suggests that Itk is indeed the target responsible for the growth inhibition observed for Jurkat cells.

CTA056 induces apoptosis in target cells

To determine whether the growth inhibition induced by CTA056 on Jurkat cells was due to apoptosis, flow cytometric analysis was carried out. Following treatment with CTA056 for 24 h, a dose dependent accumulation of a “sub-G1” fraction was observed using PI staining (Figure 5A). Data based on Annexin-V reactivity also indicated a dose-dependent increase of apoptosis of Jurkat cells following treatment with CTA056 (Figure 5B).

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CTA056 inhibits the formation of phosphor-Itk in Jurkat cells and its downstream signals

The inhibitory activity of CTA056 against auto-phosphorylation of Itk in intact cells was examined by Western blot. Itk phosphorylation in Jurkat cells was significantly inhibited at 100 nM and completely inhibited at 200 nM. Interestingly, this decrease in Itk phosphorylation was accompanied by a decrease in the total Itk levels in Jurkat cells (Figure 6A). Moreover, the stimulation of Itk phosphorylation in Jurkat cells by anti-CD3 and anti-CD28 antibodies was also hampered by CTA056 (Figure 6B). Itk inhibition in Jurkat and MOLT-4 cells leads to the suppression of phosphorylation of downstream effectors such as PLC-γ1, Erk, and Akt (Figure 7). Interestingly, treatment of Jurkat and MOLT-4 cells with CTA056 resulted in the inhibition of Src phosphorylation. This could be due to its moderate inhibitory effect on Src (Table 1), and the reported cross-activation of Src and Btk family kinases (Tsai et al., 2000).

CTA056 inhibits IL-2 and IFN-γ secretion

Itk plays an important role in transmitting signals at the immunological synapse, $Itk^{-/-}$ cells had severe reduction of IL-2 and IFN-γ secretion (Schaeffer et al., 1999; Von et al., 2011). Following incubation of Jurkat cells with 0.5 μM CTA056, the mRNA levels of IL-2 and IFN-γ were measured using real-time PCR, and the IL-2 and IFN-γ secretions were measured using an ELISA assay. As expected, the mRNA levels of IL-2 and IFN-γ, as well as the IL-2 and IFN-γ release by Jurkat cells, was significantly decreased following treatment with CTA056 (Figure 8), indicating the effect of CTA056 as inhibiting T cell activation and cytokine release.

CTA056 modulates cancer related miRs

Many miRs have been reported to play a role in carcinogenesis. To explore whether CTA056 modulates miRNAs, 272 miRNA levels in Jurkat cells were examined using a microarray assay following treatment with CTA056. Among them 97 exhibited alterations more than 2 fold, of

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which most of them the functions to oncogenesis have not been fully established. A number of them however are known to possess oncogenic and tumor suppressive properties. We found that those miRNAs with tumor suppressive (green bars) properties were generally up-regulated and those with oncogenic (red bars) properties were down-regulated following treatment with CTA056 (Figure 9). For instance, Mir-135a, which is up-regulated 285.3 fold following treatment with CTA056, has been reported to target JAK2 and decrease the expression of the anti-apoptotic protein Bcl-xL (Navarro et al., 2009). Mir-365 (28.8 fold increase) and Mir-195 (27.0 fold increase) both down-regulate the expression of the anti-apoptotic protein Bcl-2 (Chen et al., 2011; Nie et al., 2012). Likewise, Mir-1224-5p (112.9 fold increase) down-regulates TNF through SP1 (Niu et al., 2011). Mir-520b targets MEKK2 and IL-8 (Hu et al., 2011; Zhang et al., 2012) (Supplemental Figure 1A). On the other hand, CTA056 down regulates Mir-421, which has been reported to down regulate ATM, a pro-apoptotic protein (Hu et al, 2010) (supplemental data Figure 1A). The effects of CTA056 on these miRs could in part account for its ability to induce apoptosis and suppress cytokine release. To validate the microarray results, we have used RT-PCR to confirm the modulation of selected miRs by CTA056 (supplemental data Figure 1B).

CTA056 inhibits MOLT-4 xenograft tumor growth *in vivo*

Given the *in vitro* activity of CTA056 against T cell leukemia, it is important to validate these results *in vivo*. Due to the low take rate and weak oncogenicity of Jurkat in our experimental model system, we resorted to a MOLT-4 xenograft model. As shown in Figure 10, CTA056 prevented MOLT-4 xenograft tumor growth at 5 mg/kg (twice a week, intra-tumoral injection) without significant toxicity.

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Discussion

Given the initial success of Imatinib, tyrosine kinases have become appealing targets for drug development (Demetri et al., 2002; O'Brien et al., 2003). Excellent combinatorial chemistry approaches and high throughput screening assays have led to successful identification of many selective enzyme inhibitors (Lam et al., 1991; McDonald et al., 1999; Su et al., 2000; Wilhelm et al., 2006). For drug development, the universal goal is to find specific kinase inhibitors with selectivity toward a particular malignant cell type. In theory, the more specific an inhibitor is, the less toxic it will be. On the other hand, a broader specificity may contribute to higher efficacy. Although effective clinically, the current kinase inhibitors have usually proven to be less specific than initially realized (Wilhelm eta l., 2006). Specificity comes from both the selectivity of an inhibitor for a particular kinase, and the expression of that kinase among different cell types. Our results indicated that among tumors, Itk expression was limited to malignant cell types of T cell origin (Figure 3A). This result is consistent with earlier reports of the Itk expression profile in non-malignant cell types. The expression profile of Itk gives Itk inhibitors an advantage in targeting T cell malignancies, while not affecting other cell types. Our inhibitor, CTA056, binds to the ATP binding pocket of Itk and blocks the kinase activity. This inhibitor showed specificity for Itk over other Tec family kinases. It has demonstrated selective cytotoxicity toward T cell malignancies, such as Jurkat and MOLT-4 cells, and it has inhibited IL-2 and IFN-γ release from Jurkat cells. In this regard, we found CTA056 to be more effective than other Itk inhibitors currently under development at inducing apoptosis (data not shown). We attribute this to the differential specificities of these inhibitors toward other kinases or toward different conformational isoforms of Itk (e.g., monomer vs. multimer, phosphorylated vs. unphosphorylated, etc.), which may engage different substrates (Dombroski et al., 2005; Qi and August, 2009; Joseph et al., 2010). As discussed before, our inhibitor also inhibits Src kinase, albeit with less potency, which may contribute to the observed apoptosis-inducing effects.

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Interestingly, in addition to inhibition of Itk phosphorylation, CTA056 treatment of Jurkat cells also decreased total Itk levels. Bunnell *et al*. reported that Itk could bind Cbl, a ubiquinitin ligase, through the SH3 domain, while phosphorylated Itk loses its ability to bind Cbl (Bunnell et al., 1996; Andreotti et al., 1997). Therefore, inhibition of Itk phosphorylation by CTA056 may lead to Itk degradation. This is important, as Itk has been shown to possess kinase-independent signal potential (Dombroski et al., 2005; Hao et al., 2006). The selective expression of Itk, as well as the specificity of CTA056 toward Itk, coupled with its ability to induce apoptosis, make CTA056 an appropriate inhibitor for T cell malignancies. Treatment of Jurkat and MOLT-4 cells with CTA056 resulted in the decrease of phoshorylation of PLC-γ, AKT, and ERK, as well as the expression and release of IL-2 and IFN-γ, as expected from Itk inhibition. Perhaps the most striking consequence of CTA056 is its ability to modulate oncomiRs (onocgenic microRNAs), which regulate survival and apoptosis. Many up-regulated miRs are involved in suppressing survival factors (Bcl-XL, Bcl-2, NF-kB, Jak2 etc.), and down-modulated miRs target oncogenes or cytokine genes involved in growth and metastasis. By contrast, CTA056 has little effect on the survival of normal T-cells, suggesting that leukemic T-cells exemplified by Jurkat and MOLT-4 are “addicted” to Itk signals for survival. Further investigation will be required to identify the Itk signals involved in the regulation of these oncomiRs.

In summary, we have identified an Itk inhibitor, CTA056, with good selectivity toward Itk and T cell malignancies. Further evaluation of its PK/PD and animal studies in new formulations are underway. Itk-inhibition holds exceptional promise as a novel anti-leukemia and anti-lymphoma treatment strategy.

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Authorship Contributions

Participated in research design: Guo, Liu, Maverakis, Lam, and Kung

Conducted experiments: Guo, Liu, Ono, Ma, Martinez, Sanchez, Wang, Huang, Mazloom, Li, and Ning

Performed data analysis: Guo, Liu, and Ono

Wrote or contributed to the writing of the manuscript: Guo, Liu, Mazloom, Maverakis, Lam, and Kung

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Footnotes

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Figure legends

Figure 1. Synthetic scheme of CTA056 (A) and molecular modeling studies of binding conformation of CTA056 to the kinase domain of human Itk (B). The docking pocket was created from the crystal structure of the kinase domain of Itk using Autodock 4.0. Docking was performed using Autodock Vina 1.0 and the docking results were analyzed using Pymol 0.99. CTA056 was shown to bind to the ATP binding pocket in the kinase domain of Itk.

Figure 2. Kinase inhibitory activity of CTA056 (A) and inhibition of Itk autophosphorylation by CTA056 (B). Kinase activity was measured using TLC assay. Purified kinase (20 nM), CTA056 (0-10 μM), and the peptide substrate were incubated with ^{33}P-ATP in a kinase reaction. The resulting product was analyzed on a TLC plate. The relative kinase activity to control was calculated using densitometry. Itk autophosphorylation activity was measured by an *in vitro* kinase assay. Purified Itk (100 ng) was mixed with CTA056 inhibitor, the cold ATP and hot r-^{33}P-ATP in the kinase assay buffer, and the Itk auto kinase activity was analyzed by phosphorimager (Biorad). Columns, mean; bars, standard deviation, n=3.

Figure 3. Expression profile of Itk in 45 cell lines (41 cancer cell lines and 4 normal cell lines) and CTA056-induced growth inhibition of a panel of cancer cells. The expression profile of Itk was measured using Western blot (A). For growth inhibition, cells were seeded at 5,000 cells/well in 96-well plate overnight and treated with CTA056 (0 and 2 μM, B; 0-10 μM, C). After 72 h, cell proliferation was measured using the MTT assay. Columns, mean; bars, standard deviation, n=3.

Figure 4. Itk inhibition and growth inhibition to Jurkat cells of a series of CTA compounds. Kinase activity was measured using TLC assay. Purified Itk (20 nM), CTA compounds (1 μM),

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and the peptide substrate TSFYGRH were incubated with ^{33}P-ATP in a kinase reaction. The resulting product was analyzed on a TLC plate. For growth inhibition, cells were seeded at 5,000 cells/well in 96-well plate overnight and treated with CTA compounds (2 μM). After 72 h, cell proliferation was measured using the MTT assay. Columns, mean; bars, standard deviation, n=3. CTA056 was shown to inhibit Itk and inhibit the growth of Jurkat cells.

Figure 5. Induction of apoptosis of Jurkat cells following treatment with CTA056. Jurkat cells were seeded at 10^6 cells/ml (2 ml) in a 6-well plate and treated with CTA056 at the indicated concentrations for 24 h. Cell cycle arrest was analyzed using PI staining (A). Apoptosis was analyzed using Annexin-V FITC apoptosis detection kit (B). Columns, mean; bars, standard deviation, n=3. 125 nM, 250 nM, 500 nM and 1000 nM are significantly different from control (*, $p<0.05$, one-way ANOVA with Tukey test for pair wise comparison).

Figure 6. Inhibition of Itk phosphorylation in Jurkat cells following treatment with CTA056. Itk phosphorylation in Jurkat cells was examined using Western blot (A). Cells were seeded at 10^6 cells/ml (5 ml) and treated with CTA056 at the indicated concentrations for 24 h. Before harvest, cells were pretreated with pervanadate for 15 min. pItk, Itk, and actin levels were measured using the corresponding antibodies through Western blot. B) Jurkat cells were pretreated with CTA056 30 min and then incubated with anti-CD3, anti-CD28 monoclonal antibodies (10 μg/ml), and protein G (10 μg/ml). At 30 sec, 1 min, and 5 min the stimulation was stopped. The cells were then permeabilized and stained with a phospho-Itk-specific antibody. One of three similar experiments depicted. Values are averages of triplicate samples.

Figure 7. Inhibition of cell signaling in Jurkat and MOLT-4 cells following treatment with CTA056. Cells were seeded at 10^6 cells/ml (5 ml) and pre-treated with 0.5 μM CTA056 for 30 min. Then cells were stimulated with anti-CD3, anti-CD28 monoclonal antibodies (10 μg/ml),

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and protein G (10 μg/ml) for 1 min. pPLC-γ1, PLC-γ1, pERK, ERK, pSrc, Src, pAkt and Akt levels were measured using the corresponding antibodies through Western blot. One of three similar experiments depicted.

Figure 8. Inhibition of IL-2 and IFN-γ mRNA level (A) and inhibition of IL-2 (B) and interferon-γ (C) release of Jurkat cells following treatment with CTA056. For mRNA levels, Jurkat cells were pretreated with CTA056 0.5 μM for 30 min then stimulated with anti-CD3 and anti-CD28 antibodies (1 μg/ml) for 6 h. RNA was isolated and the mRNA levels were measured using real-time PCR. Supernatants were then harvested and IL-2 was quantified using a sandwich ELISA. For interferon-γ release assay, Jurkat cells were pretreated with CTA056 (0 nM, 50nM, 500nM) or MTX for 30 min then stimulated with rhIL-2 and anti-CD3 antibody (1 μg/ml) for 6 h. Interferon-γ was quantified using ELISPOT assay. Values are averages of triplicate samples (columns, mean; bars, standard deviation). CTA056 treatment significantly decreased IL-2 and interferon-γ secretion from Jurkat cells compared with control. *, $p<0.05$, one-way ANOVA with Tukey test for pair wise comparison.

Figure 9. Induction of tumor suppressive MiRNAs and decrease of oncogenic MiRNAs in Jurkat cells following treatment of 0.5 μM CTA056 for 8h. RNA was isolated and miR profile was examined using miR array. The fold change between treatment and control was indicated. Green columns, tumor suppressive miRs; red columns, oncogenic miRs.

Figure 10. Inhibition of MOLT-4 xenograft tumor growth by CTA056. 10^7 MOLT-4 cells were injected subcutaneously to nude mice. The tumors were grown the indicated size and the mice were randomly divided into two groups (8 mice/group). The control group was treated with vehicle. The treatment group was treated with CTA056 at 5mg/kg twice a week with intratumoral

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injection. The tumor size (A) and body weight (B) were measured once a week. Marks, mean; bars, mean; n=8.

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Table 1. Kinase inhibition profile of CTA056

Kinase	IC_{50} (μM)
Itk	0.1
Btk	0.4
Etk	3
Src	1.2
Yes	>10
Lyn	>10
Axl	>10
Mer	>10
EGFR	>10
Abl	>10
Mkk1	>10
PDK1	>10
Akt	>10
Erk1	>10

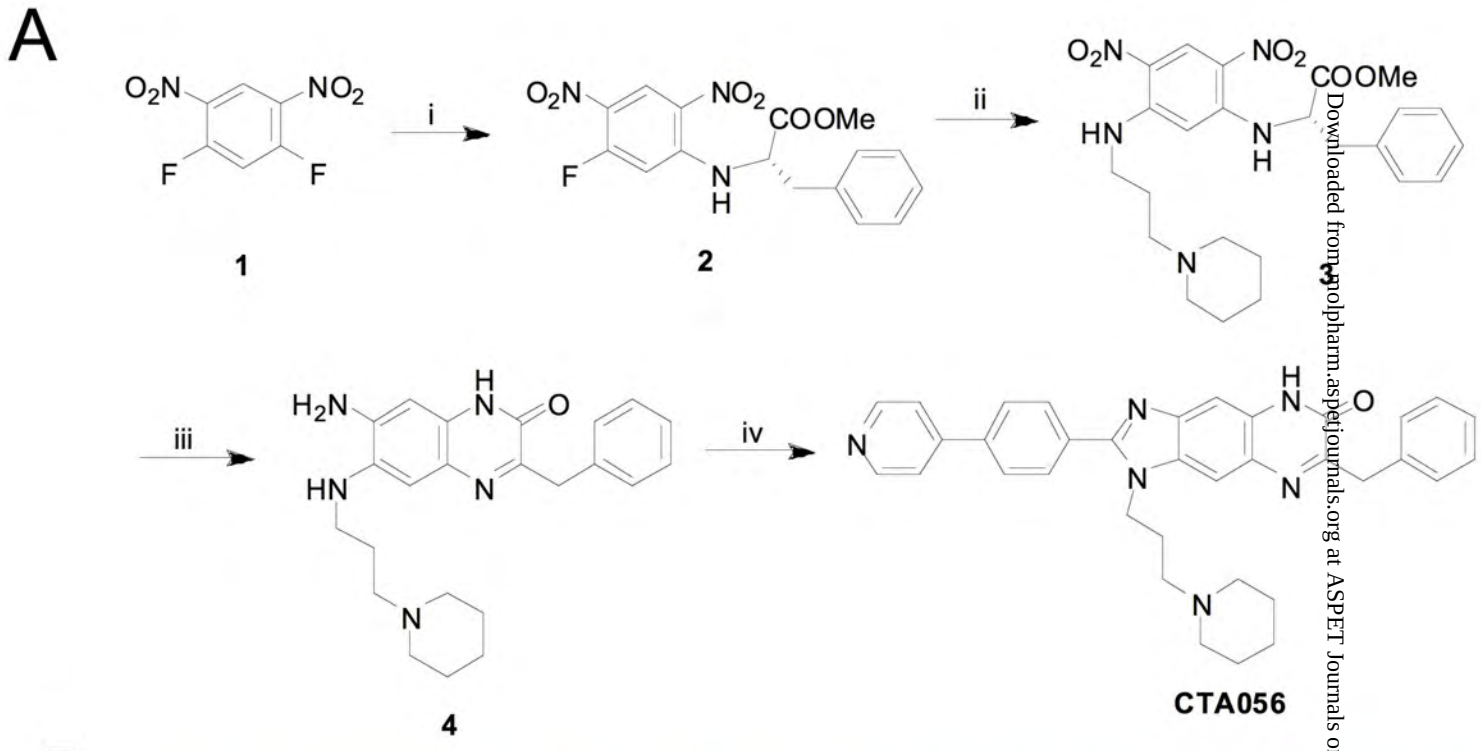


Figure 1

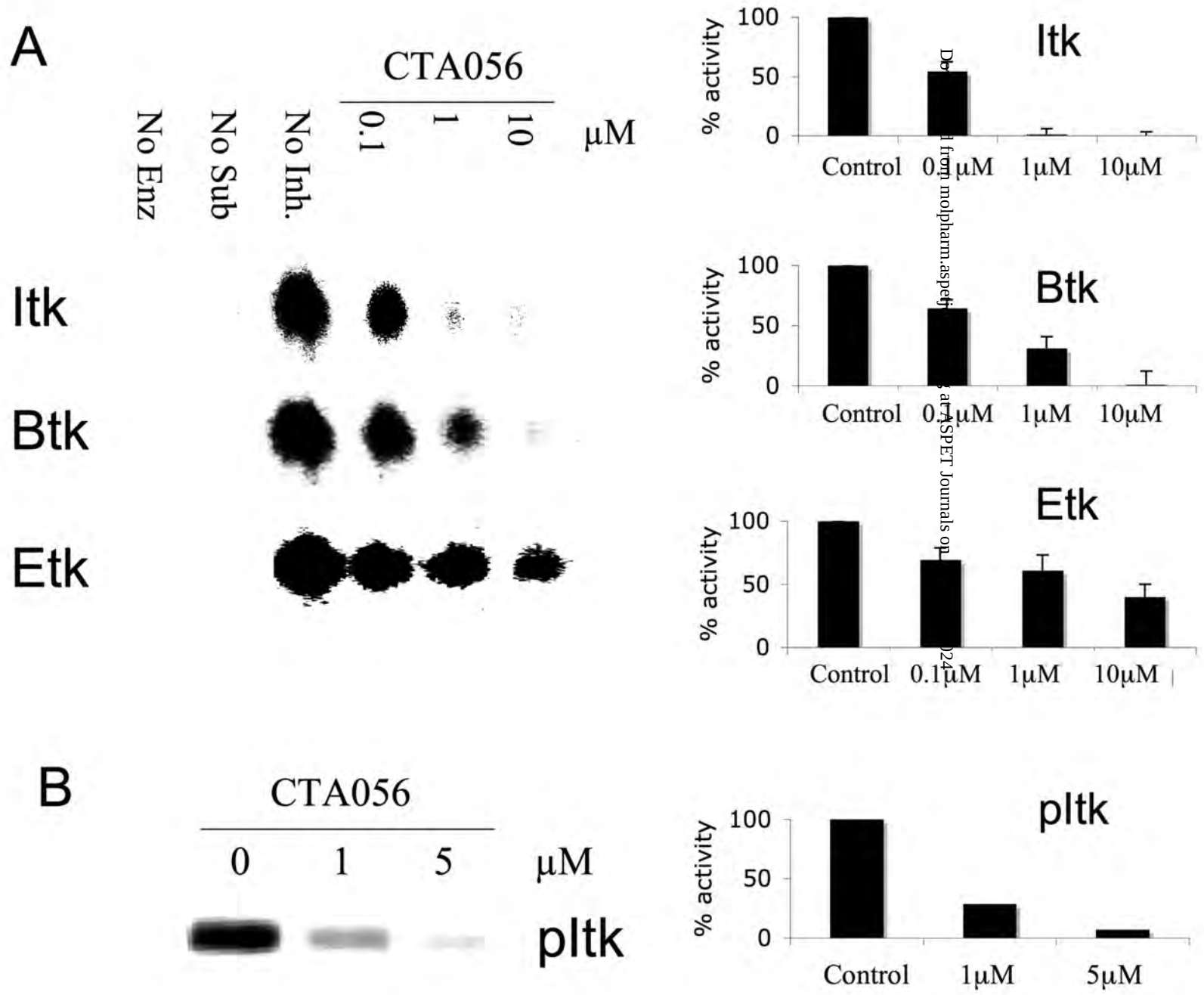


Figure 2

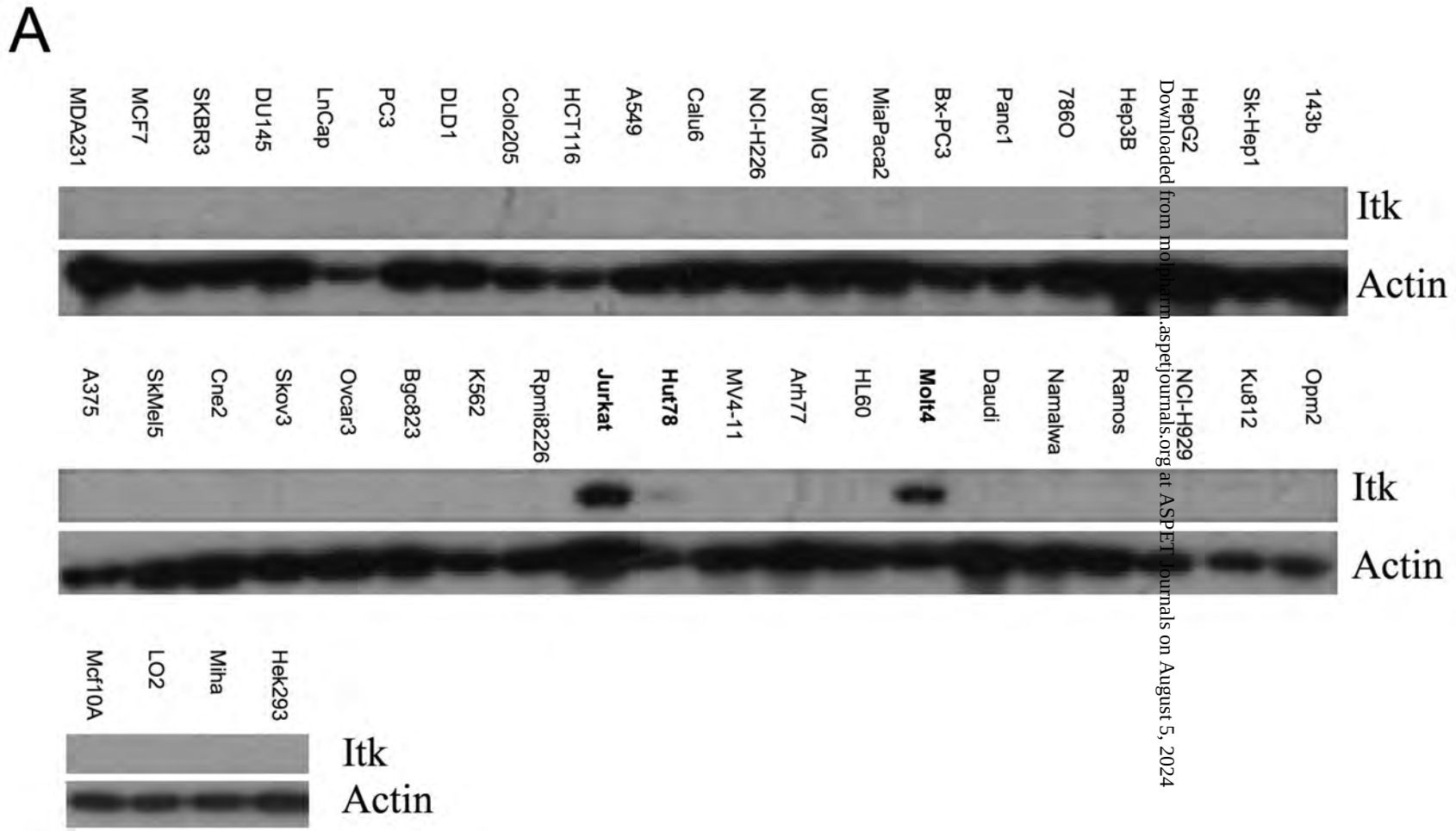


Figure 3A

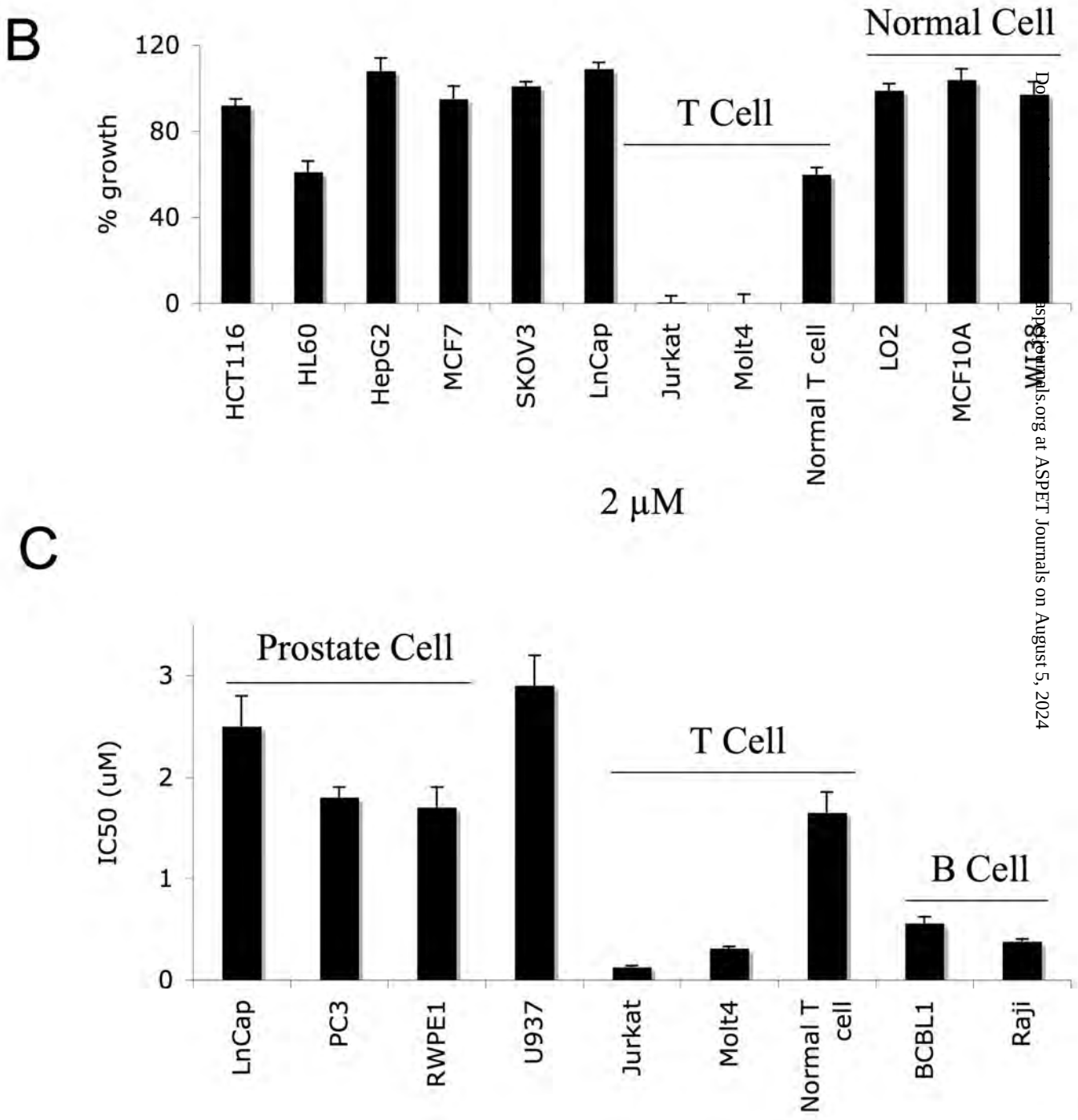


Figure 3B and 3C

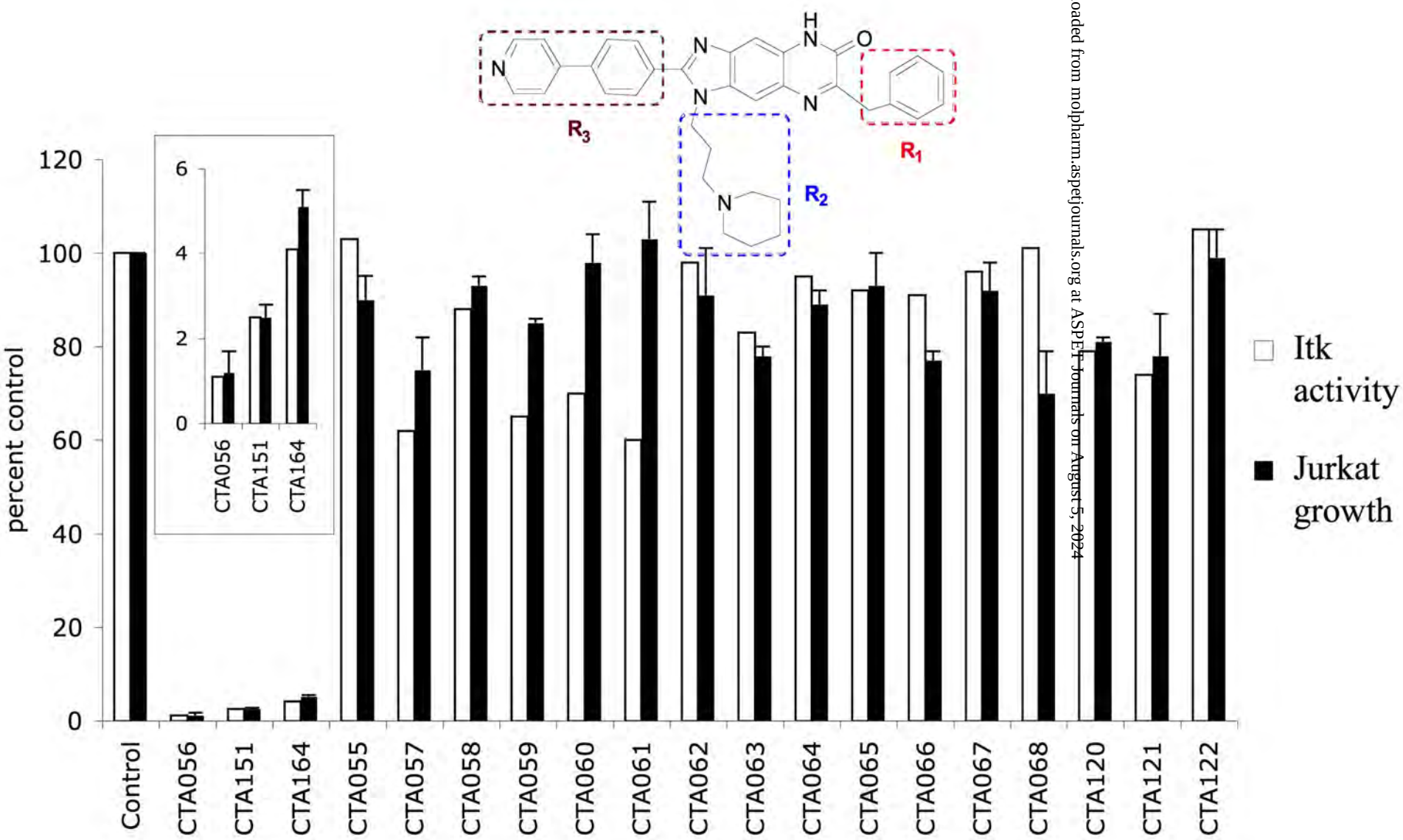
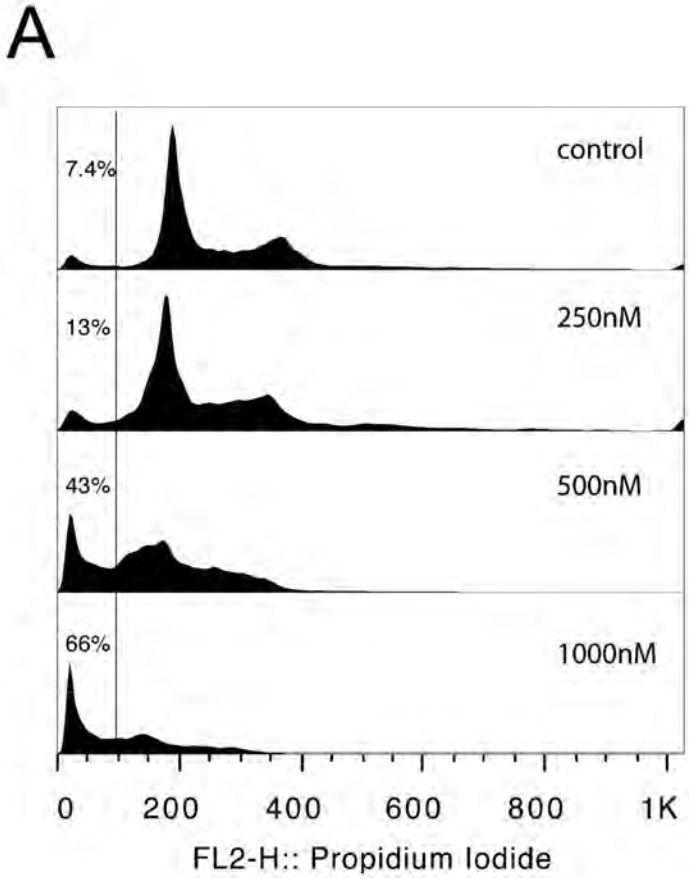


Figure 4



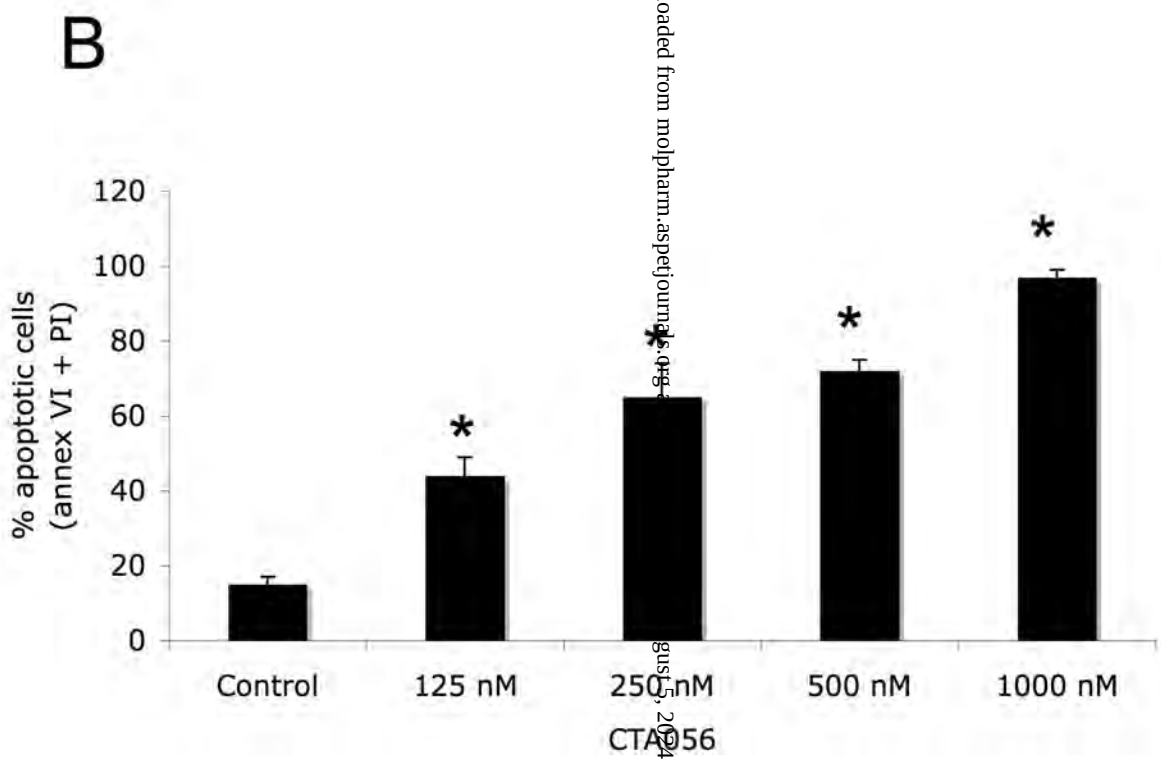


Figure 5

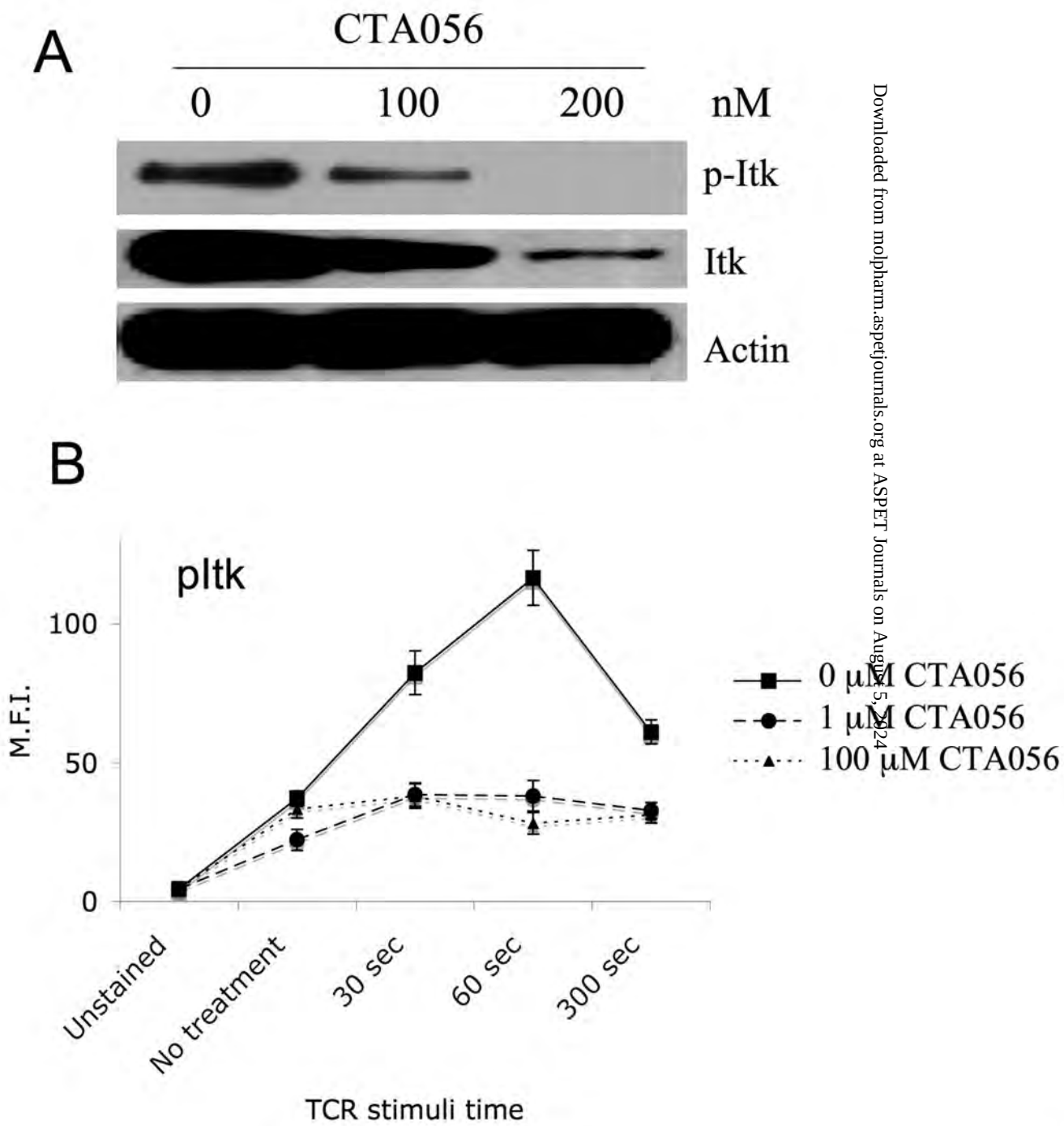
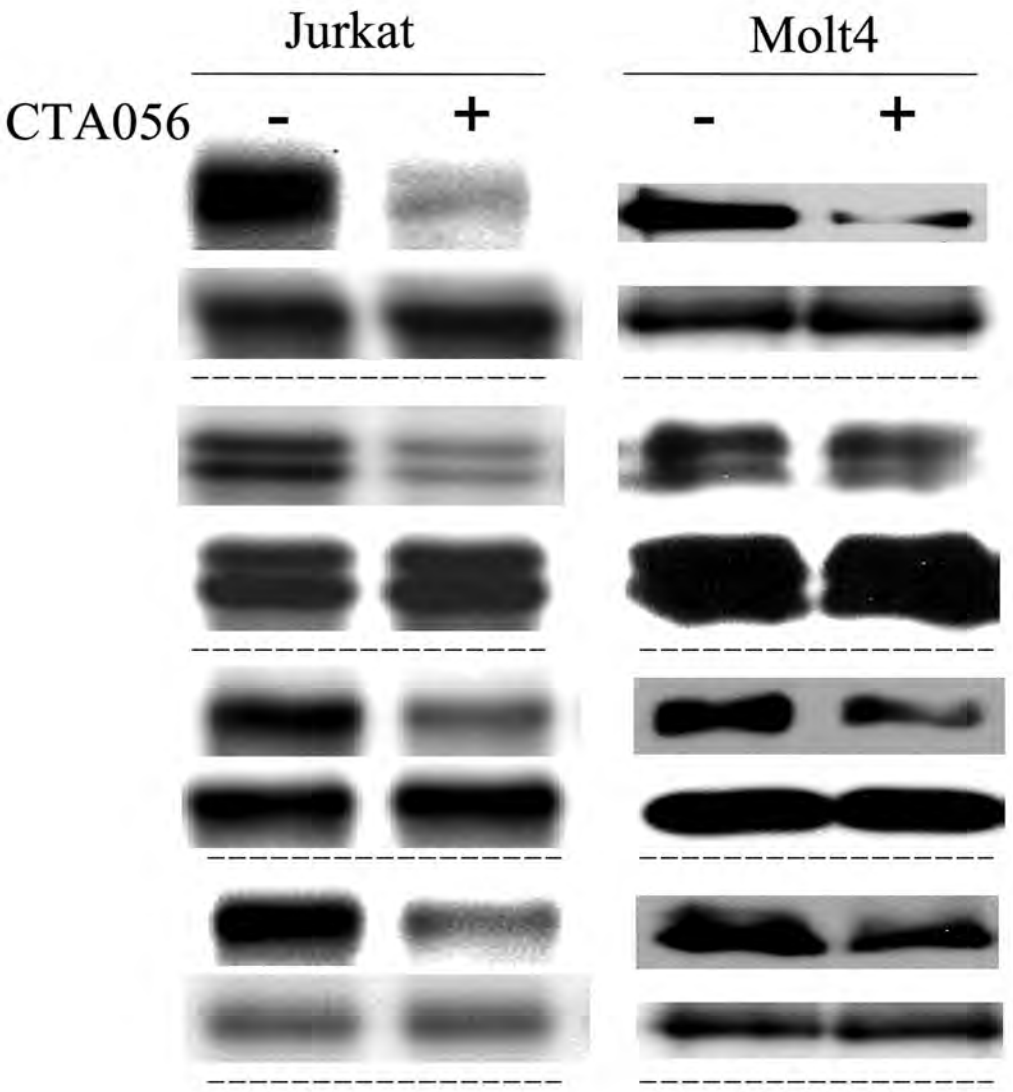


Figure 6



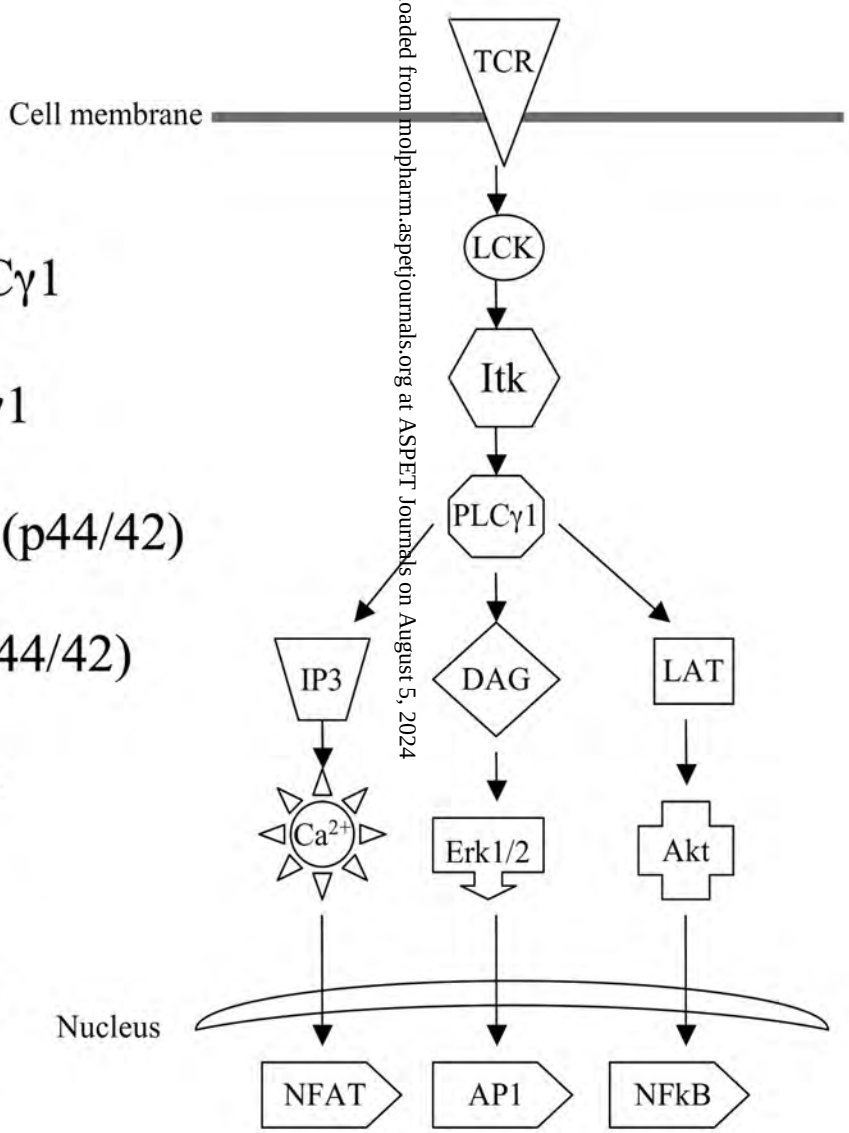


Figure 7

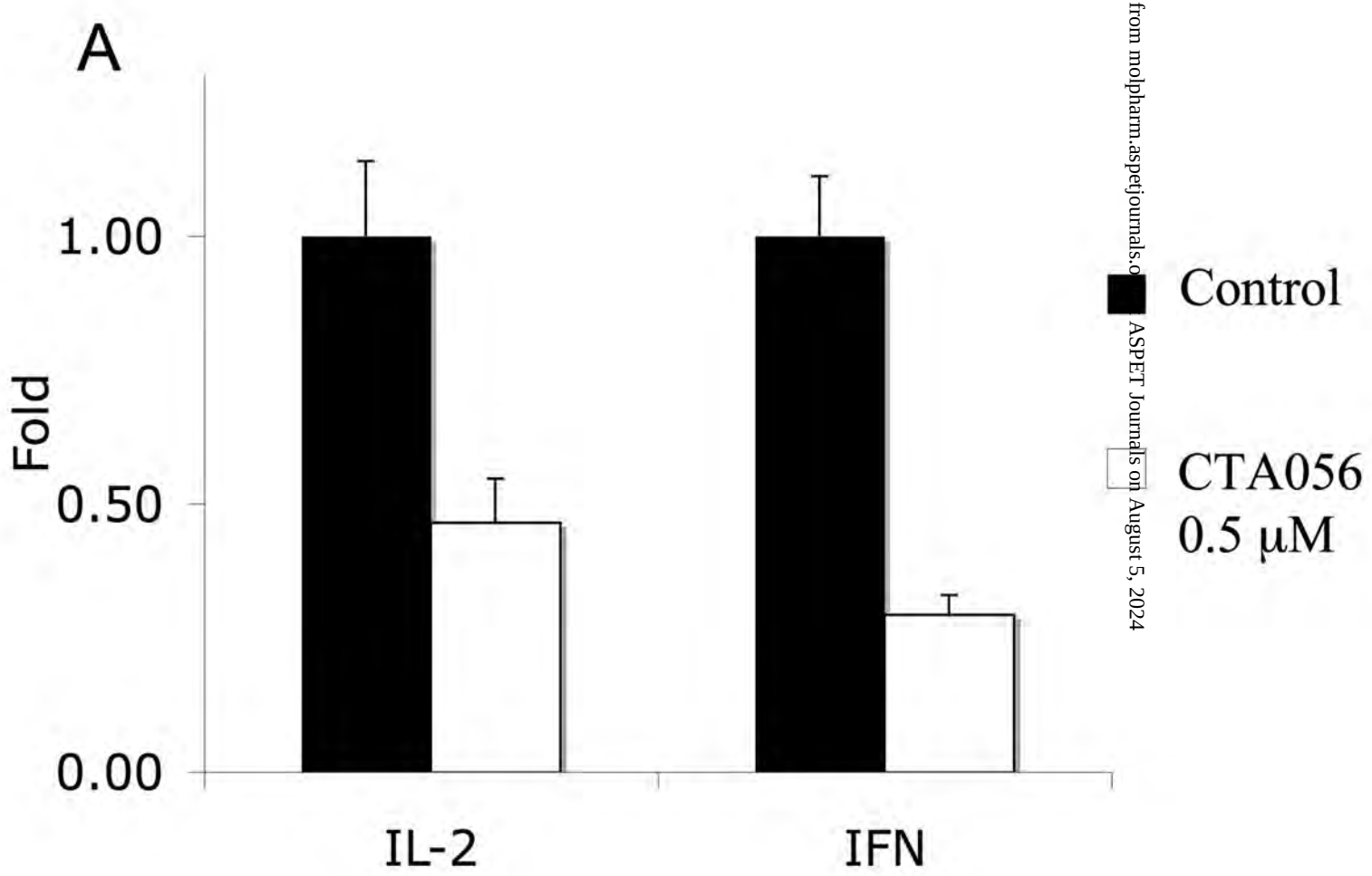


Figure 8A

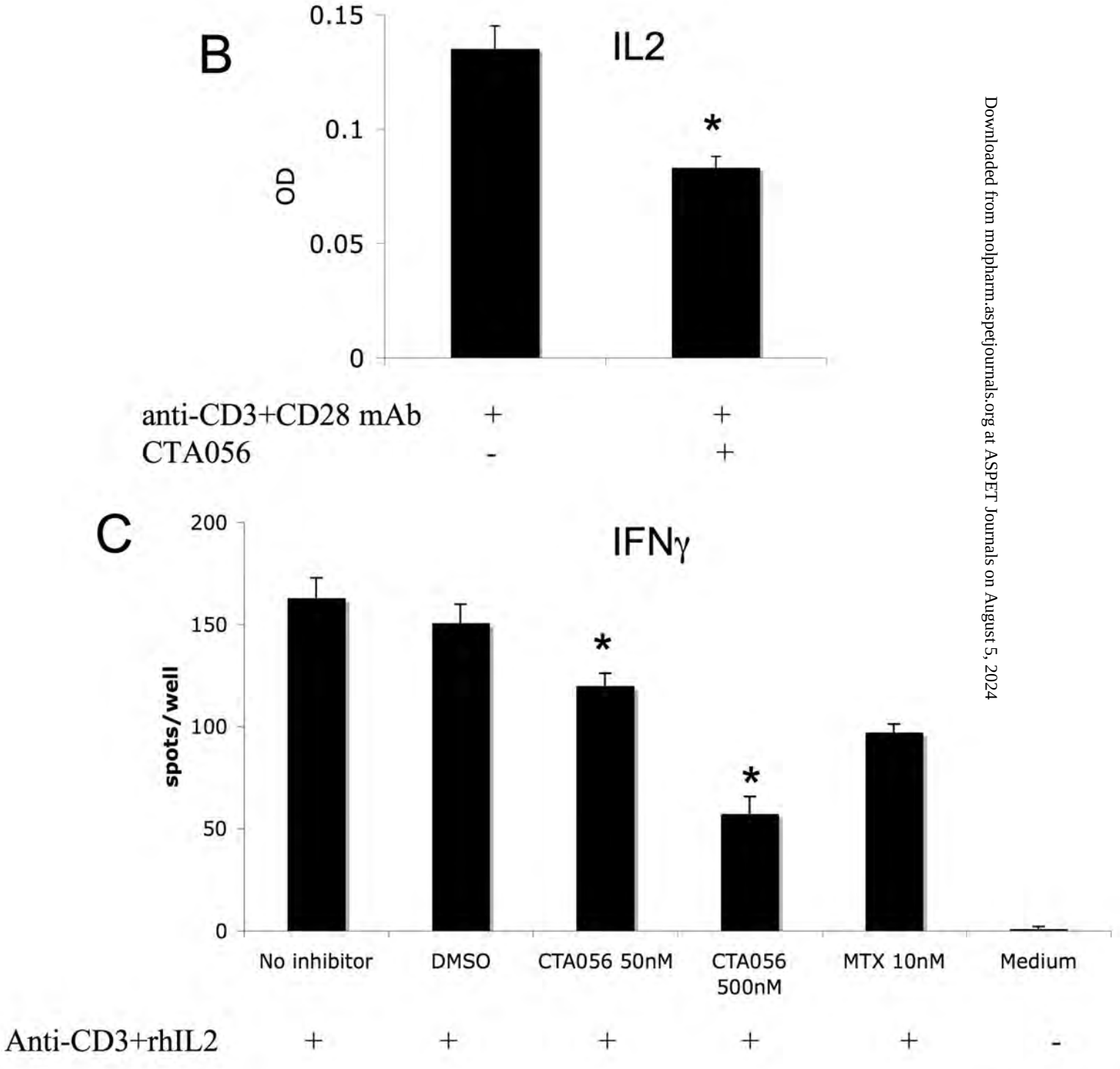


Figure 8B and 8C

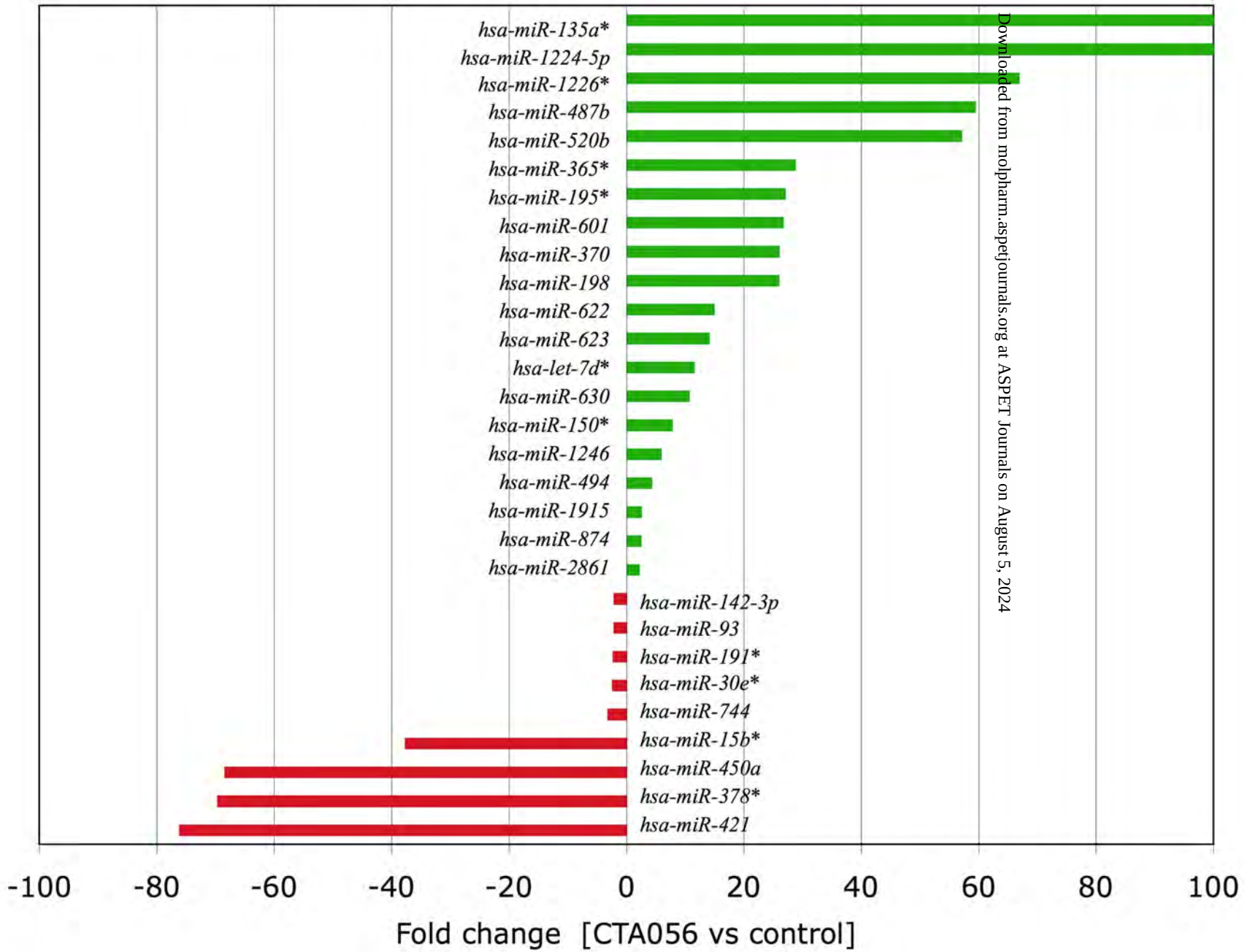


Figure 9

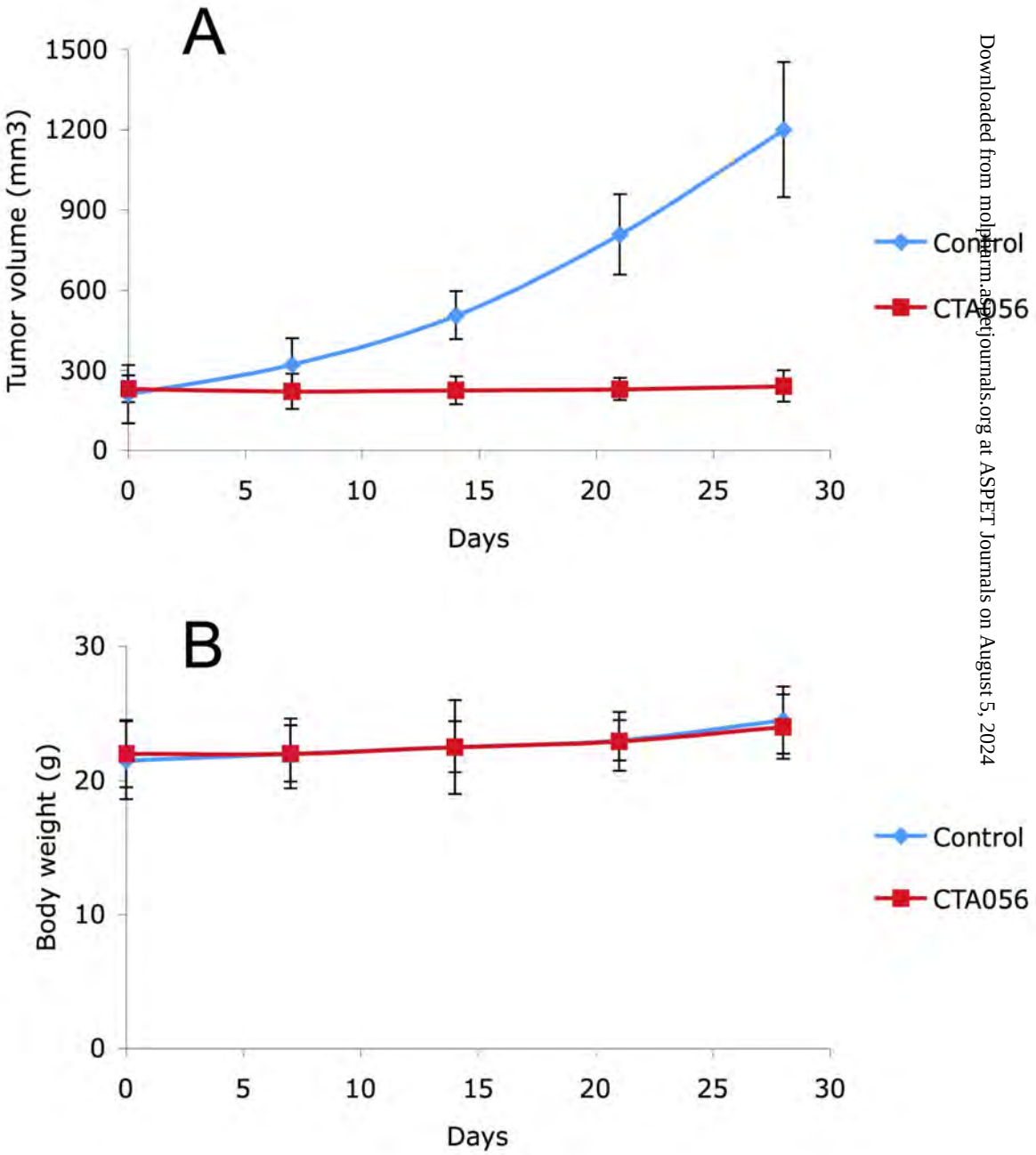


Figure 10