BJP British Journal of Pharmacology

DOI:10.1111/bph.13051
www.brjpharmacol.org

RESEARCH PAPER

Novel immunosuppressive agent caerulomycin A exerts its effect by depleting cellular iron content

Suneet Kaur, Gautam Srivastava*, Amar Nath Sharma and Ravinder S Jolly

Department of Chemistry, CSIR-Institute of Microbial Technology, Chandigarh, India

Correspondence
Ravinder S Jolly, CSIR-Institute of Microbial Technology, Sector 39, Chandigarh 160 036, India.
E-mail: jolly@imtech.res.in

*Present address: Department of Structural Biology, Weizmann Institute of Science, Rehovot 76100, Israel.

Received
23 July 2014
Revised
3 December 2014
Accepted
14 December 2014

BACKGROUND AND PURPOSE

Recently, we have described the use of caerulomycin A (CaeA) as a potent novel immunosuppressive agent. Immunosuppressive drugs are crucial for long-term graft survival following organ transplantation and treatment of autoimmune diseases, inflammatory disorders, hypersensitivity to allergens, etc. The objective of this study was to identify cellular targets of CaeA and decipher its mechanism of action.

EXPERIMENTAL APPROACH

Jurkat cells were treated with CaeA and cellular iron content, iron uptake/release, DNA content and deoxyribonucleoside triphosphate pool determined. Activation of MAPKs; expression level of transferrin receptor 1, ferritin and cell cycle control molecules; reactive oxygen species (ROS) and cell viability were measured using Western blotting, qRT-PCR or flow cytometry.

KEY RESULTS

CaeA caused intracellular iron depletion by reducing its uptake and increasing its release by cells. CaeA caused cell cycle arrest by (i) inhibiting ribonucleotide reductase (RNR) enzyme, which catalyses the rate-limiting step in the synthesis of DNA; (ii) stimulating MAPKs signalling transduction pathways that play an important role in cell growth, proliferation and differentiation; and (iii) by targeting cell cycle control molecules such as cyclin D1, cyclin-dependent kinase 4 and $p21^{CIP1/WAF1}$. The effect of CaeA on cell proliferation was reversible.

CONCLUSIONS AND IMPLICATIONS

CaeA exerts its immunosuppressive effect by targeting iron. The effect is reversible, which makes CaeA an attractive candidate for development as a potent immunosuppressive drug, but also indicates that iron chelation can be used as a rationale approach to selectively suppress the immune system, because compared with normal cells, rapidly proliferating cells require a higher utilization of iron.

Abbreviations

CaeA, caerulomycin A; DCFDA, 2′,7′-dichlorofluorescin diacetate; DFO, desferoxamine; dNTP, deoxyribonucleoside triphosphate; ISD, immunosuppressive drug; PI, propidium iodide; RNR, ribonucleotide reductase

Tables of Links

TARGETS	
ASK1	JNK1
Cdk4	JNK2 (SAPK)
ERK	p38

LIGANDS
Cytidine
Hydroxyurea

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guideto PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Immunosuppressive drugs (ISDs) are crucial for long-term graft survival following organ transplantation. In addition, ISDs are prescribed for the treatment of autoimmune diseases, inflammatory disorders, hypersensitivity to allergens, etc. (Kovarik and Burtin, 2003; Pillans, 2006). Although currently used drugs in clinics have provided significant relief to patients (Halloran, 2004), these suffer from one or another drawback, such as poor oral bioavailability, side effects like nephrotoxicity and malignancy, non-specific mode of action, incomplete suppression of belligerent immune cells, unaffordable cost, etc. (Danesi and Del Tacca, 2004; Rosenberger *et al.*, 2005; Utecht *et al.*, 2006; Wu, 2007; Leyral *et al.*, 2008; Hsu and Katelaris, 2009; Naesens *et al.*, 2009; Thaci and Salgo, 2010; Niioka *et al.*, 2013). Thus, the need remains for discovering efficacious, selective, and safer new ISDs for improved sustenance of organ transplants.

Recently, we have described the use of caerulomycin A (CaeA) as a potent immunosuppressive agent (Singla *et al.*, 2012; 2014; Gurram *et al.*, 2014). CaeA induces generation of regulatory T-cells, significantly suppresses T-cell response and prolongs the survival of allogeneic skin graft. The objective of this study was to identify cellular targets of CaeA and decipher its mechanism of action. CaeA is known to form a complex with iron *in vitro* in stoichiometry of 2:1 (Dholakia and Gillard, 1984). Iron being redox active plays a crucial role in various metabolic processes including DNA synthesis. Iron is not only a vital component for all proliferating cells, it is also a central regulator for the proliferation and function of immune cells (Brock and Mulero, 2000; Le and Richardson, 2003). Compared with normal cells, rapidly proliferating cells require higher utilization of iron, which potentially provides a rationale for selective immunosuppressive activity of iron chelators. In the past, depriving cells of essential nutrient iron by chelators has been used as an approach for cancer treatment (Le and Richardson, 2002; Kalinowski and Richardson, 2005; Whitnall *et al.*, 2006).

Herein we report the results of our study, which established that CaeA exerts its effect at the cellular level by targeting iron. We show that CaeA causes cell cycle arrest not only by targeting ribonucleotide reductase (RNR), but also cell cycle control molecules, viz., cyclin D1, cyclin-dependent kinase (cdk) 4 and $p21^{CIP1/WAF1}$, which are important for normal cell cycle progression. While CaeA causes effective cell cycle arrest in the S phase, the effect is reversible once CaeA is withdrawn from the system. Taken together, the unique properties of CaeA not only makes it an attractive candidate for development as drug, but also indicate that iron chelation can be used as a rationale approach to suppress the immune system for successful organ transplantation and treatment of autoimmune diseases.

Methods

Cell culture

Jurkat T-lymphocyte cell line was used in this study. The cells were grown in RPMI 1640 media supplemented with 10% FBS, 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin. Cells were incubated at 37°C in humidified atmosphere of 5% CO_2.

Western blotting

Western blot analysis was performed as described previously (Gahlot *et al.*, 2010). Briefly, cell lysate was prepared in lysis buffer (20 mM Tris–HCl pH 7.4, 0.25 M NaCl, 2 mM EDTA pH 8.0, 0.1% Triton-X-100, 1 mM DTT) containing protease and phosphatase inhibitor cocktail. Protein estimation was done using the Bradford assay. Proteins were resolved on 10–12% SDS-PAGE, transferred to a PVDF membrane, incubated with specific antibody and visualized using an enhanced chemiluminescence method (ECL plus, GE Healthcare, Buckinghamshire, UK). The densitometry of each band was obtained using scion image software (Scion Corporation, Frederick, MD, USA).

Cellular iron content

The cellular iron content was determined by atomic absorption spectrometry using modified procedure (Petrak *et al.*, 2006). Briefly, 10^7 Jurkat cells were treated with 0–2.5 μM of CaeA or 100 μM DFO for 24 h. Cells were washed with PBS and digested with 5% HNO_3 at 60°C for 2 h and supernatant collected for iron estimation using a flame atomic absorption spectrometer (Spectra AA-6800 Spectrometer, Shimadzu Corporation, Kyoto, Japan).

Iron and transferrin uptake and iron efflux

The effect of CaeA on iron uptake from ^{55}Fe-transferrin and iron efflux from ^{55}Fe pre-labelled cells was performed using procedures described previously (Richardson *et al.*, 1995). Briefly, 10^6 mL^{-1} Jurkat cells were incubated with 0.75 μM ^{55}Fe-transferrin in the presence of 0–2.5 μM CaeA or 100 μM DFO for 3 h. The cells were washed with ice-cold PBS and

treated with 1 mg·mL^{-1} pronase at 4°C for 30 min to remove surface-bound ^{55}Fe-transferrin. The amount of ^{55}Fe internalized in the cells was measured by scintillation counting using a β-counter (Wallec, Microbeta Trilux, Perkin Elmer, Billerica, MA, USA).

The effect of CaeA on Tf uptake by Jurkat cells was examined using Alexa Fluor 633-labelled diferric human transferrin as described previously (Ba *et al.*, 2013). After removal of surface-bound transferrin label with pronase, the samples were analysed by flow cytometry at the FL-4 channel (BD Accuri C6, BD Biosciences, San Jose, CA, USA). The values were corrected for non-specific signal.

For the iron efflux study, 10^6 mL^{-1} Jurkat cells were prelabelled with ^{55}Fe by incubating cells with 0.75 μM ^{55}Fe-transferrin for 3 h. Cells were washed with ice-cold PBS and re-incubated at 37°C in RPMI containing 0–2.5 μM CaeA or 100 μM DFO for 3 h. The supernantant was separated and subjected to scintillation counting.

Expression of transferrin receptor (TFR1)

Expression of TFR1 in Jurkat cells was analysed by immunostaining and FACS analysis as described previously (Ludwiczek *et al.*, 2003; Rishi *et al.*, 2009). Briefly, 2×10^5 Jurkat cells were incubated with 0–2.5 μM CaeA or 100 μM DFO at 37°C for 24 h. Cells were surface stained with FITC-conjugated mouse anti-human CD71 antibody or IgG2aκ isotype control antibody (BD Biosciences) and analysed by a flow cytometer at the FL-1 channel (FACS Calibur, BD Biosciences).

[^{3}H]-cytidine incorporation assay

The ^{3}H-labelled cytidine incorporation assay was done as described previously (Heffeter *et al.*, 2009). Briefly, 5×10^6 cells were treated with 0–2.5 μM CaeA or 1 mM hydroxyurea for 24 h. After their incubation, cells were pulsed with 1 μCi [^{3}H]-cytidine for 30 min at 37°C. The cells were collected, washed and total DNA extracted. Radioactivity was determined using scintillation counting.

Deoxyribonucleoside triphosphate (dNTP) pool determination

Jurkat cells, 10^6 mL^{-1}, were treated with 0 or 2.5 μM CaeA for 24 h. On completion of treatment, cells were washed twice with HBSS buffer and suspended in ice-cold 80% acetonitrile (ACN) for the nucleotide extraction. The extracts were centrifuged to remove cellular debris and loaded on an SAX column (100 mg, Supelco, Sigma Aldrich, Bellefonte, PA, USA) preconditioned with methanol, water and ACN. Once the sample was effused completely, the cartridge was washed with 3 mL 80% ACN and 3 mL water and eluted with 1 M KCl. The nucleotides were analysed by an ion-pairing HPLC method using a Zorbax Extend-C18 column (Agilent, Santa Clara, CA, USA) as reported previously (Pierro *et al.*, 1995).

Cell cycle distribution

Jurkat cells 1×10^6 were treated with 2.5 μM CaeA or 100 μM DFO for 24 h at 37°C. Cells were fixed with cold 70% ethanol and washed twice with 1× PBS and stained with 500 μl of PI/RNAse (BD Biosciences) solution for 15 min at 25°C. The cells were analysed by FACS Aria III using FACS Diva software (BD Biosciences) (Lin *et al.*, 2007).

Real-time PCR

Total RNA was isolated from untreated cells or 2.5 μM CaeA or 100 μM DFO-treated cells for 24 h using an RNA isolation kit (Promega, Madison, WI, USA). qRT-PCR was carried out in a volume of 50 μL using the Superscript III RT kit (Invitrogen) according to the manufacturer's instructions. The primer sequences used were: human CD71 sense: 5′-ATGATGGA TCAAGCTAGATCAGCAT-3′; antisense: 5′-TTGGTTTTGTGA CATTGGCCT-3′; human p21 sense: 5′-ATGTCAGAACCGG CTGGGG-3′; antisense: 5′-GGAAGGTAGAGCTTGGGCAGG-3′; human GAPH sense: 5′-ATGGGGAAGGTGAAGGTCGG-3′; antisense: 5′-CTTGATTTTGGAGGGATCTCGC-3′. qRT-PCR was performed for p21, CD71 and GAPDH in the Mastercycler ep realplex real-time PCR system (Eppendorf) using following conditions: 55°C for 30 min, 94°C for 2 min, 40 cycles of (i) 94°C for 15 s; (ii) 50°C for 30 s; and (iii) 68°C for 20 s, and 68°C for 5 min. Samples were quantified through C_T values using the formula, fold change = $2^{-\Delta(\Delta C_T)}$ where, $\Delta C_T = C_T$, target – C_T, GAPDH, and $\Delta(\Delta C_T) = \Delta C_T$, stimulated – ΔC_T, control (Livak and Schmittgen, 2001).

Intracellular reactive oxygen species (ROS) level

Intracellular ROS generation was quantified by flow cytometric measurement of the cellular metabolism of 2′,7′-dichlorofluorescin diacetate (DCFDA) as described previously (Yuan *et al.*, 2004).

Cell viability

Cell viability was determined by the ability of cells to exclude PI as described previously (Gahlot *et al.*, 2010).

Statistical analysis

All experiments were done at least three times to ensure the reproducibility of data. Data presented are expressed as means ± SEM. Statistical analysis was performed by Student's paired *t*-test. Differences were considered significant at a level of $P \leq 0.05$.

Materials

RPMI 1640 and FBS were purchased from GIBCO (Grand Island, NY, USA), [^{3}H]-cytidine from Moravek Biochemicals (Brea, CA, USA), $^{55}FeCl_3$ from American radiolabelled chemicals (St. Louis, MO, USA), apo-transferrin and pronase from Calbiochem (San Diego, CA, USA), propidium iodide (PI)/RNase staining buffer from BD Pharmingen (San Jose, CA, USA) and Alexa Fluor® 633-labelled diferric human transferrin from Life Technologies (Carlsbad, CA, USA). Antibodies (catalogue number in parenthesis) JNK/SAPK (pT183/pY185) (612540), JNK1/JNK2 (554285), anti-cyclin D1 (556470), FITC mouse anti-human CD71 (555536) and FITC mouse IgG2aκ isotype control (555573) were purchased from BD Pharmingen, Human anti-p-ERK (sc-7383), anti-ERK (sc-94), anti-p-p38 (sc-7973), anti-p38 (sc-7972), anti-R2 (sc-10848), anti-ferritin-H (sc-135667) and anti-ferritin-L (sc-390558) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-cdk4 (2906) from Cell Signaling (Danver, MA, USA).

Results

CaeA decreases intracellular iron content

The intracellular iron content was quantified using atomic absorption spectroscopy after incubation of Jurkat cells with 0–2.5 μM CaeA or 100 μM desferoxamine (DFO) for 24 h at 37°C. Compared with untreated cells, concentration-dependent depletion of the iron pool was observed on treatment with CaeA (Figure 1A). At 2.5 μM, CaeA caused more than 90% reduction in the intracellular iron pool. In comparison, 100 μM DFO caused only 20% reduction in the intracellular iron pool.

CaeA causes reduced uptake and increased release of iron by cells

The effect of CaeA on the transferrin mediated iron uptake was determined by incubating Jurkat cells with 0.75 μM ^{55}Fe-labelled transferrin (^{55}Fe-Tf) in the presence of CaeA 0–2.5 μM or 100 μM DFO for 3 h. A concentration-dependent inhibition of iron uptake was observed on treatment of cells with CaeA (Figure 1B); 0.3 μM CaeA caused about 14% inhibition of iron uptake by the cells, which reached 35% when the concentration of CaeA was increased to 2.5 μM. In comparison, 100 μM DFO caused only 33% inhibition.

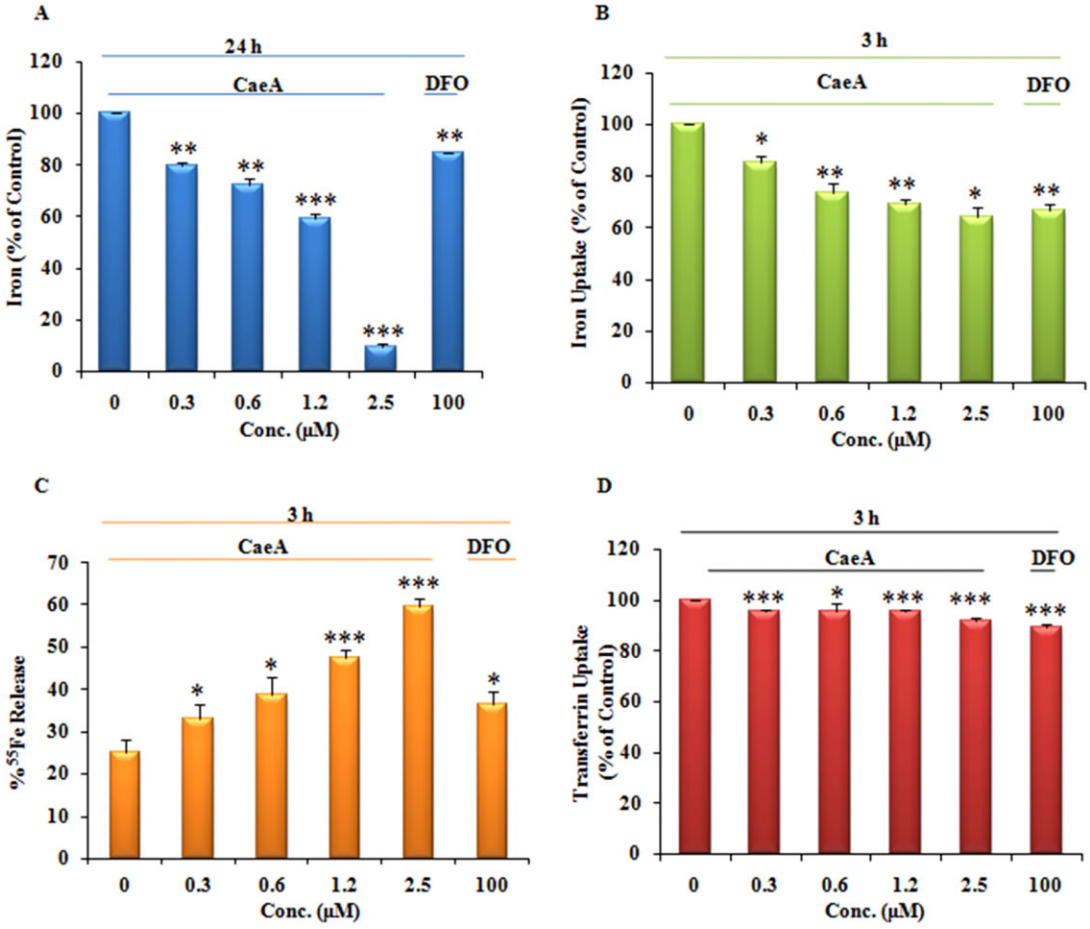

Figure 1

Effect of CaeA on cellular iron content (A), iron uptake (B), iron release (C) and transferrin uptake (D). (A) Jurkat cells were treated with CaeA (0–2.5 μM) or DFO 100 μM for 24 h at 37°C. Intracellular content of iron was determined by atomic absorption spectroscopy. Data are means ± SEM of three experiments. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. (B) Cells were treated with 0.75 μM of ^{55}Fe-Tf in the presence of 0–2.5 μM CaeA or 100 μM DFO for 3 h. Subsequently, the cells were treated with pronase (1 mg·mL^{-1}) for 30 min at 4°C to remove membrane-bound ^{55}Fe. The amount of ^{55}Fe uptake by the cells was determined by liquid scintillation counting. Iron level in untreated cells was assigned a value of 100%; all other values are relative to it $^{*}P \leq 0.05$, $^{***}P \leq 0.01$. (C) Cells were labelled with ^{55}Fe-Tf (0.75 μM) for 3 h at 37°C and then re-incubated with 0–2.5 μM CaeA or 100 μM DFO for 3 h. At the end of the re-incubation period, the supernatant and cells were separated and the amount of ^{55}Fe released was determined by scintillation counting. Data are means ± SEM of three experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. (D) Jurkat cells were incubated with 25 μg·mL^{-1} of Alexa Fluor 633-labelled diferric human transferrin in the presence of 0–2.5 μM CaeA or 100 μM DFO for 3 h. Subsequently, the cells were washed and treated with pronase (1 mg·mL^{-1}) for 30 min at 4°C to remove membrane-bound transferrin. The amount of transferrin uptake was determined by flow cytometry at the FL-4 channel. Transferrin uptake in untreated cells was considered as 100%; all other values are relative to it; $^{*}P \leq 0.05$, $^{***}P \leq 0.001$.

^{55}Fe release from Jurkat cells was examined by pre-labelling cells with ^{55}Fe-Tf (0.75 μM) for 3 h at 37°C followed by washing and re-incubation of cells with CaeA or DFO. In comparison with control, ^{55}Fe release from cells increased from 33% at 0.3 μM CaeA to 62% at 2.5 μM CaeA. Under similar conditions, 100 μM DFO exhibited only 36% ^{55}Fe release (Figure 1C).

CaeA causes small effect on transferrin uptake by cells

Tf uptake was examined by incubating cells with 25 μg·mL^{-1} Alexa Fluor 633-labelled diferric human transferrin in the presence of CaeA 0–2.5 μM or 100 μM DFO for 3 h at 37°C. After removal of surface-bound transferrin with pronase, the samples were analysed by flow cytometry; 2.5 μM CaeA induced only about an 8% decrease in Tf uptake compared with a 11% decrease caused by 100 μM DFO (Figure 1D).

CaeA increases the level of TFR1 and decreases the level of ferritin

Whereas TFR1 binds to iron-loaded transferrin and plays a pivotal role in cellular uptake of iron, ferritin sequesters and stores the cellular iron. Both the proteins are regulated by intracellular iron concentration (MacKenzie *et al.*, 2008). We have demonstrated the effect of CaeA on the surface expression of TFR1 by flow cytometry (Figure 2A) and at the transcriptional level by quantitative real-time PCR (Figure 2B). Surface expression of TFR1 was assayed by staining cells with FITC-conjugated anti-human CD71 antibody or mouse isotype antibody (IgG2aκ) after treatment with CaeA (0–2.5 μM) or 100 μM DFO for 12 or 24 h. Compared with untreated samples, CaeA induced a concentration-dependent increase in TFR1 of up to 1.36- and 1.72-fold at 12 and 24 h respectively. The corresponding increase with 100 μM DFO was 1.4- and 1.57-fold respectively (Figure 2A).

The effect on the mRNA level of TFR1 was determined after CaeA or DFO treatment for 24 h. A 2.7-fold increased expression of TFR1 mRNA was estimated by qRT-PCR on treatment with 2.5 μM CaeA; the corresponding value for 100 μM DFO was 2.2-fold (Figure 2B).

We determined the effect of CaeA on the expression level of ferritin-H and ferritin-L by treating Jurkat cells with 0–2.5 μM CaeA or 100 μM DFO for 24 h. The change in the expression level was determined through immunoblotting with anti-ferritin-H or anti-ferritin-L antibody. The fold change in expression of ferritin-H and ferritin-L was found to decrease in concentration-dependent manner from 1.0- to 0.33- and 0.36-fold, respectively, with 0–2.5 μM CaeA. The respective change with 100 μM DFO was 0.27- and 0.35-fold (Figure 2C).

CaeA inhibits activity of ribonucleotide reductase (RNR) enzyme

RNR catalyzes the conversion of ribonucleotides to deoxyribonucleotides, a rate-limiting step in the synthesis of DNA. RNR is dependent on iron for the stabilization of the tyrosyl radical essential for its activity (Shao *et al.*, 2006; Sanvisens *et al.*, 2013). The effect of CaeA on the activity of RNR was examined by (i) quantification of DNA synthesis by the [^{3}H]-cytidine incorporation assay and (ii) quantification of intracellular dNTPs.

The [^{3}H]-cytidine incorporation assay is an indirect method for estimating RNR activity in cells (Heffeter *et al.*, 2009). Following the treatment of Jurkat cells with 0–2.5 μM CaeA or 1 mM hydroxy urea (HU) for 24 h, the cells were incubated with [^{3}H]-cytidine for 30 min before the incubation was completed. HU is a known inhibitor of RNR and has been used extensively in the literature as a positive control (Koc *et al.*, 2004; Shao *et al.*, 2006; Heffeter *et al.*, 2009). Total DNA was isolated and analysed for incorporation of [^{3}H]-cytidine into DNA through radioactive counting. We observed a concentration-dependent decrease in DNA synthesis on treatment with CaeA (Figure 3A). The decrease was in the range of 21–55% with CaeA concentrations of 0.3–2.5 μM. In comparison, 1 mM HU caused 51% decrease in DNA synthesis. Significantly, no effect on the synthesis of DNA was observed when Jurkat cells were treated with CaeA in presence of excess of ferrous ions (Supporting Information Fig. S1).

Deoxyribonucleotides are precursors for DNA synthesis, which are synthesized within cells by the action of RNR on the ribonucleotides (Tholander and Sjoberg, 2012). Thus, inhibition of RNR activity should result in a decreased dNTP pool within the cells. A significant decrease in levels of dNTPs was observed on treatment of cells with 2.5 μM CaeA for 24 h. Compared with untreated cells, the respective decreased levels of dUTP, dGTP and dATP were approximately 71, 41 and 30% (Figure 3B).

CaeA increases the expression of the R2 subunit of RNR

RNR is composed of two subunits, R1 and R2, and their complexation is essential for its activity. The R1 subunit is constitutive and is expressed throughout the cell cycle. However, the R2 subunit is cell cycle-dependent and is expressed at the time of cell proliferation, which requires increased DNA synthesis (Shao *et al.*, 2006). The presence of excess of the R2 subunit is found to coordinate with the S phase checkpoint to compete with DNA damage and reduce the replication stress in the absence of p53 (Lin *et al.*, 2007; 2011). Therefore, we studied the effect of CaeA on expression of the R2 subunit of RNR (Figure 4). Jurkat cells were treated with 0–2.5 μM CaeA or 100 μM DFO for 24 h at 37°C. The expression level of R2 was analysed by Western blotting using anti-R2 subunit antibody. The expression of the R2 subunit was found to increase from 1.50- to 2.24-fold on treatment with 0.3–2.5 μM CaeA with respect to untreated samples. The corresponding increase with 100 μM DFO was 1.86-fold. Significantly, CaeA in presence of excess iron did not show any appreciable effect on expression of R2 subunit (Supporting Information Fig. S2).

CaeA induces S-phase arrest

We determined the effect of CaeA on the cell cycle. Through PI staining, the DNA content in cells was measured by flow cytometry after treating Jurkat cells with 2.5 μM CaeA or 100 μM DFO for 24 h (Figure 5). The fluorescent intensity correlates with the amount of DNA content of the cell, which further determines the distribution of the cells in different phases of the cell cycle. Compared with untreated cells, the CaeA treatment resulted in a significant decrease in the % of

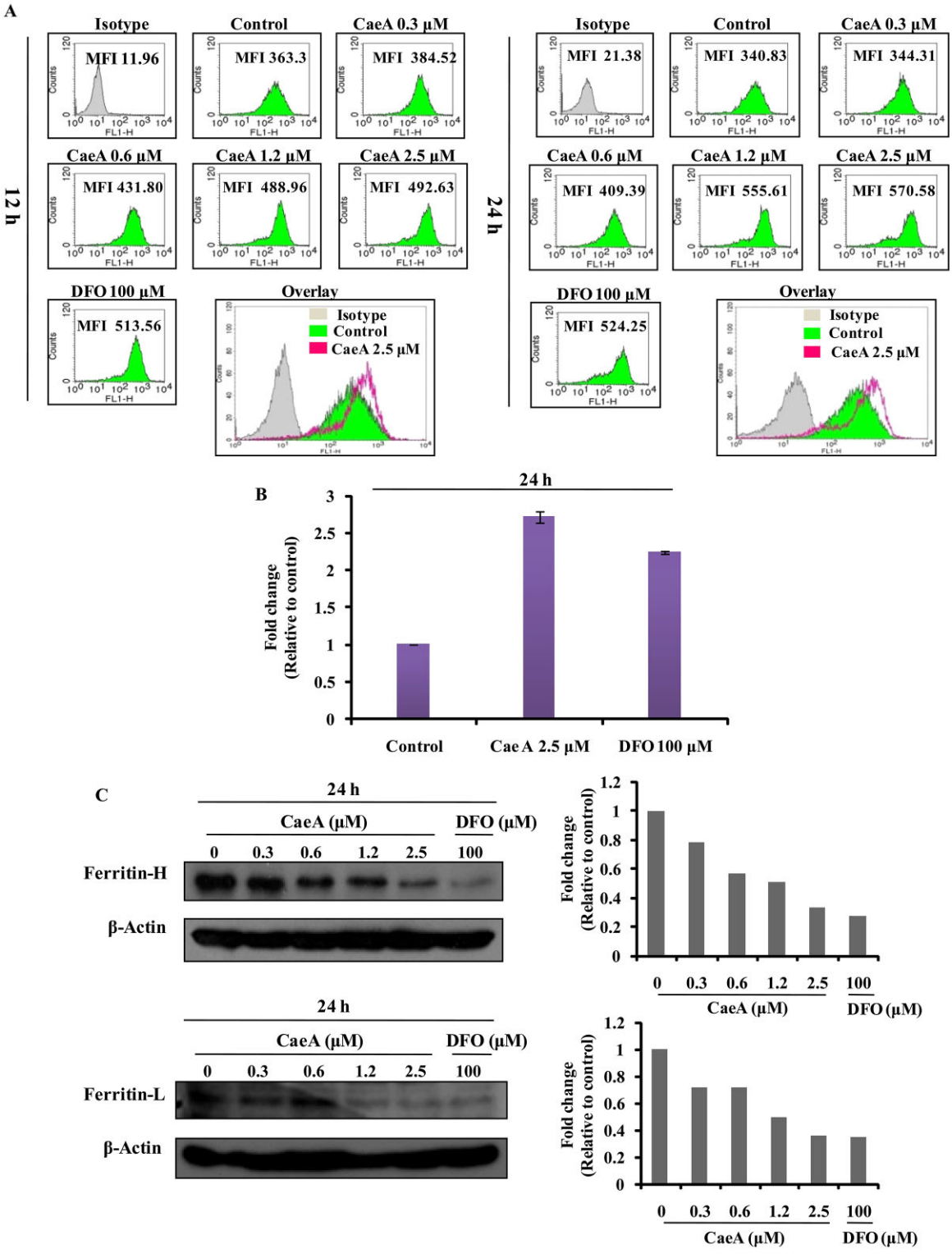

Figure 2

The effect of CaeA on CD71 expression (A and B), ferritin-H and ferritin-L expression (C) in Jurkat cells. (A) Cells were treated with various concentrations of CaeA (0–2.5 µM) or DFO (100 µM) for 12 h or 24 h at 37°C. Surface expression of CD71 was determined by flow cytometery at the FL-1 channel. Overlay plots represent changes in surface expression of CD71 at indicated time points. (B) Jurkat cells were treated with 2.5 µM of CaeA or 100 µM of DFO for 24 h at 37°C. Total RNA was isolated and increase in expression of TFR1 was confirmed by qRT-PCR. (C) Jurkat cells were treated with different concentration of CaeA (0–2.5 µM) or DFO (100 µM) for 24 h, whole-cell lysates were prepared and immunoblotted using anti-ferritin-H or anti-ferritin-L antibody. Fold change was calculated after normalization with β-actin. Right panels show fold change relative to control.

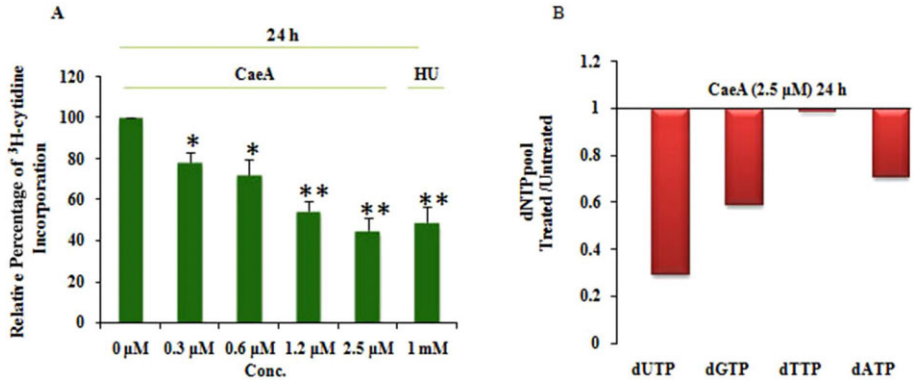

Figure 3

Effect of CaeA on RNR activity. (A) Jurkat cells were treated with different concentrations of CaeA or 1 mM hydroxyurea (HU) for 24 h and then pulsed with [^{3}H]-cytidine for 30 min. DNA was isolated and [^{3}H]-cytidine incorporation was determined. The incorporation of [^{3}H]-cytidine into the DNA of untreated cells was taken as 100%; all other values are relative to it. *$P \leq 0.05$, **$P \leq 0.01$. (B) Jurkat cells were treated with 2.5 µM CaeA for 24 h. dNTPs were isolated and measured by HPLC.

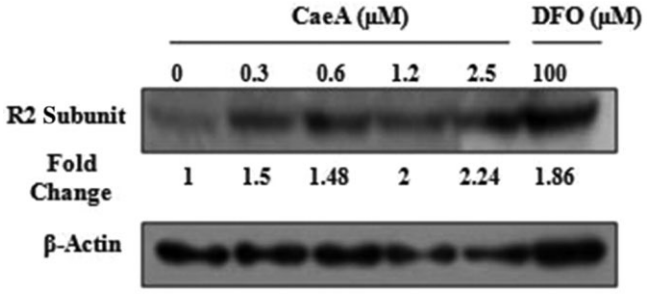

Figure 4

Effect of CaeA on R2 subunit expression. Jurkat cells were treated with different concentrations of CaeA (0–2.5 µM) or DFO (100 µM) for 24 h. Expression levels of the R2 subunit were determined by western blotting. Fold change was calculated after normalization with β-actin.

cells in G1 phase and significant increase in the % of cells in the S phase (Figure 5). In untreated cells, 51.2% were in the G1 phase and 32% in the S phase. In 2.5 µM CaeA-treated cells, 7.2% cells were in G1 phase and 79.7% in S phase. In 100 µM DFO-treated cells, G1 phase contained 3.9% and S phase 80.5%. The population of cells in G2/M phase decreased from 14.6% in control to 8.85 and 12.35% in CaeA and DFO-treated cells respectively.

CaeA stimulates MAPK signalling transduction pathways

To understand the effect of CaeA on the MAPK pathway, phosphorylation status of JNK, p38 and ERK MAPK was estimated on treatment with CaeA for various time points or with DFO for 24 h through immunoblotting with respective antibody p-ERK, ERK, p-p38, p38, JNK/SAPK (pT183/pY185) and JNK1/JNK2. We observed an increase in phosphorylation initiated at an early time point for ERK1/2 that is at 30 min of incubation with 2.5 µM CaeA. Maximum phosphorylation was observed at 6 h after which it started to decrease reaching normal levels at 24 h. In contrast, the increased phosphorylation of ERK1/2 on treatment with 100 µM DFO at 24 h incubation was noted (Figure 6A).

The phosphorylation of JNK was initiated at 3 h and reached maximum at 12 h of treatment with CaeA. However, no significant increase in phosphorylation of JNK was observed with DFO at 24 h (Figure 6B). A significant increase in phosphorylation of p38 was observed from 6 to 24 h of incubation in a time-dependent manner with 2.5 µM CaeA and 100 µM DFO. However, the increase in phosphorylation of p38 with 100 µM DFO was less as compared with 2.5 µM CaeA (Figure 6C).

Effect of CaeA on cell cycle molecules cyclin D1, cdk4 and p21$^{CIPI/WAF1}$

Iron depletion affects the expression of cell cycle control molecules in addition to the inhibition of RNR activity (Le and Richardson, 2003). Therefore, we studied the effect of CaeA on the expressions of p21$^{CIP1/WAF1}$, cyclin D1 and cdk4, some of the molecules involved in cell cycle progression. The expression of p21$^{CIP1/WAF1}$ was examined at the transcriptional level by qRT-PCR. Compared with control, 2.5 µM CaeA resulted in 3.4-fold increased level of p21$^{CIP1/WAF1}$ mRNA. In comparison, 100 µM DFO resulted in twofold increase in p21$^{CIP1/WAF1}$ mRNA level (Figure 7C).

For determining the effect of CaeA on the expressions of cyclin D1 and cdk4, whole-cell lysates were prepared after incubation of cells with various concentrations of CaeA (0–2.5 µM) or 100 µM DFO for 24 h. The change in the expression levels of cyclin D1 and cdk4 was determined through Western blotting using anti-cyclin D1 and anti-cdk4 antibodies. We observed a relative decrease in expression of cyclin D1 from 1.0- to 0.57-fold on treatment with 0–2.5 µM CaeA. In comparison, 100 µM DFO resulted in a 0.62-fold decrease in expression of cyclin D1 (Figure 7A).

Similarly, CaeA caused a concentration-dependent decrease in expression level of cdk4. Compared with control (untreated cells), CaeA caused decrease in expression of cdk4 from 1.0- to 0.1-fold, at 0–2.5 µM. The corresponding value

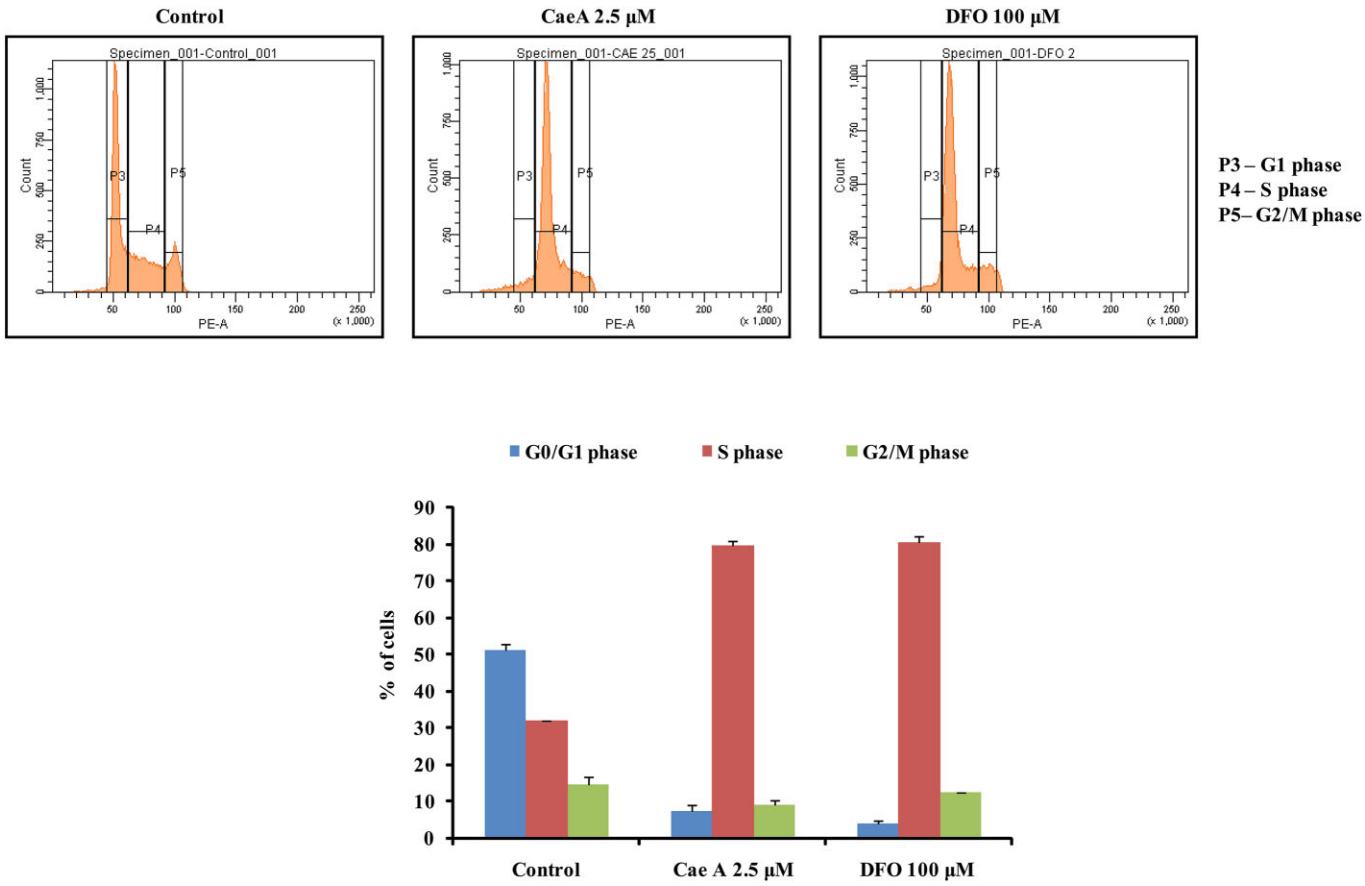

Figure 5

Effect of CaeA on cell cycle progression. Jurkat cells were incubated with 2.5 µM CaeA or 100 µM DFO for 24 h at 37°C. Cells were fixed with 70% ethanol and then washed with PBS and stained with PI/RNAse. Cellular DNA was analysed by flow cytometry. Data are means ± SEM of three experiments.

for 100 µM DFO was about 0.4-fold (Figure 7B). Significantly, the preformed CaeA–iron complex has an insignificant effect on the expression levels of cyclin D1 or cdk4 (Supporting Information Fig. S3).

CaeA caused decrease in ROS level

Jurkat cells were treated with 0–2.5 µM CaeA or 100 µM DFO for 24 h. After which the cells were washed and stained with DCFDA. Subsequently, the intracellular level of ROS was quantified by measuring fluorescent intensity by flow cytometry, which was then correlated to the ROS level in the cells. Compared with untreated cells, we observed an increase in the fall of mean fluorescent intensity in concentration-dependent manner on treatment of cells with CaeA (Figure 8). A 1.2- to 2.1-fold decrease in mean fluorescent intensity was observed on treatment of Jurkat cells with 0.3–2.5 µM CaeA. In comparison, DFO at 100 µM induced a decrease of approximately 1.7-fold.

Effect of CaeA on cell viability

Jurkat cells were treated with 0–2.5 µM CaeA for 24 h at 37°C, followed by staining with PI. Cytotoxicity was determined by flow cytometry using the Live PI dye exclusion method (Gahlot *et al.*, 2010). Compared with untreated cells, the increase in dead cells was insignificant for CaeA concentrations up to 1.2 µM (Figure 9). At 2.5 µM, the increase in dead cells was 14.2%.

Effect of CaeA on cell proliferation is reversible

Jurkat cells were incubated at 37°C in the presence or absence of 1.2 µM CaeA for 18 h and then washed by incubating in RPMI 1640 for 2 h. The wash cycle was repeated 5 times. Washed cells, 1.27×10^6 were then incubated in a fresh complete medium at 37°C for 60 h. The cells were counted by the trypan blue method. Cell count: untreated sample (control), 4.65×10^6 mL^{-1} and CaeA-treated cells, 4.38×10^6 mL^{-1} (Figure 10). Thus, the effect of CaeA on the proliferation of cells is reversible once CaeA is withdrawn from the system.

Discussion

Iron is central regulator of immune cell proliferation and function (Weiss, 2002). Lymphocytes are key molecules for

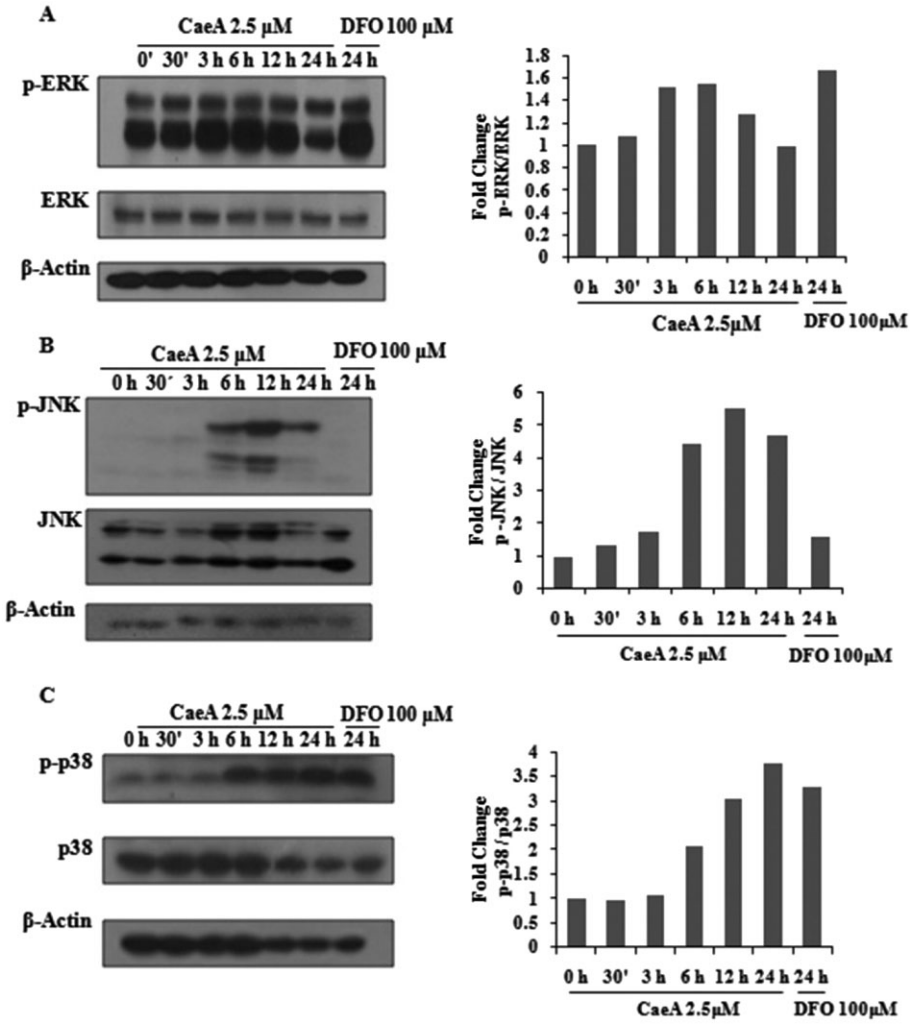

Figure 6

Effect of CaeA on phosphorylation of MAPKs. Jurkat cells were treated with CaeA (2.5 μM) for 0, 0.5, 3, 6, 12 and 24 h or DFO 100 μM for 24 h and whole-cell lysates prepared and immunoblotted against p-ERK, ERK, p-p38, p38, p-JNK/SAPK and JNK1/JNK2. Fold change was calculated after normalization with β-actin.

specific immunity. Iron is a limiting factor for the proliferation and differentiation of lymphocytes (Kuvibidila *et al.*, 2001; Weiss, 2005). In addition, iron is a crucial trace metal and cofactor of various metaloproteins required for primary biochemical activities, such as oxygen transport, energy metabolism and DNA synthesis (Le and Richardson, 2003; Yu *et al.*, 2009). RNR, which catalyse the rate-limiting step in the synthesis of DNA is one such iron containing enzyme whose activity is essential for the proliferation of cells (Shao *et al.*, 2006). Depriving lymphocytes of essential nutrient iron can therefore be used as rationale approach to suppress the immune system.

Recently, we have described the use of CaeA as a potent immunosuppressive agent. CaeA is known to form a complex with Fe (II) *in vitro* (Dholakia and Gillard, 1984). The property of CaeA to chelate iron appears noteworthy and relevant to its immunosuppression property. Accordingly, as a first step, we studied the effect of CaeA on intracellular iron pool. Jurkat cell line was chosen for this study based on the well-established role of T-cells in the graft rejection (Zelenika *et al.*, 2001; Abbas *et al.*, 2004; Issa *et al.*, 2010). CaeA caused intracellular iron depletion through decreased uptake and increased release of iron by cells. 2.5 μM CaeA caused 90% reduction in intracellular iron pool after 24 h treatment. The transferrin-mediated iron uptake and mobilization from cells are important indices for determining the ability of chelator to induce iron depletion in the cells (Le and Richardson, 2003). Compared with DFO, CaeA at 40-fold lesser concentration caused similar reduction in iron uptake and 1.78-fold increase in iron release from cells. The uptake of ^{55}Fe was about 65% of the control with 2.5 μM CaeA, whereas under similar conditions uptake of transferrin was about 92% of the control. Thus, the inhibition of transferrin uptake by CaeA was very small compared with the observed inhibition of ^{55}Fe uptake, which rules out transferrin as a primary site of action.

TFRs are transmembrane proteins that allow the controlled access of iron to cells (Aisen *et al.*, 2001). The Tf-TFR-mediated pathway for uptake of iron is central to all

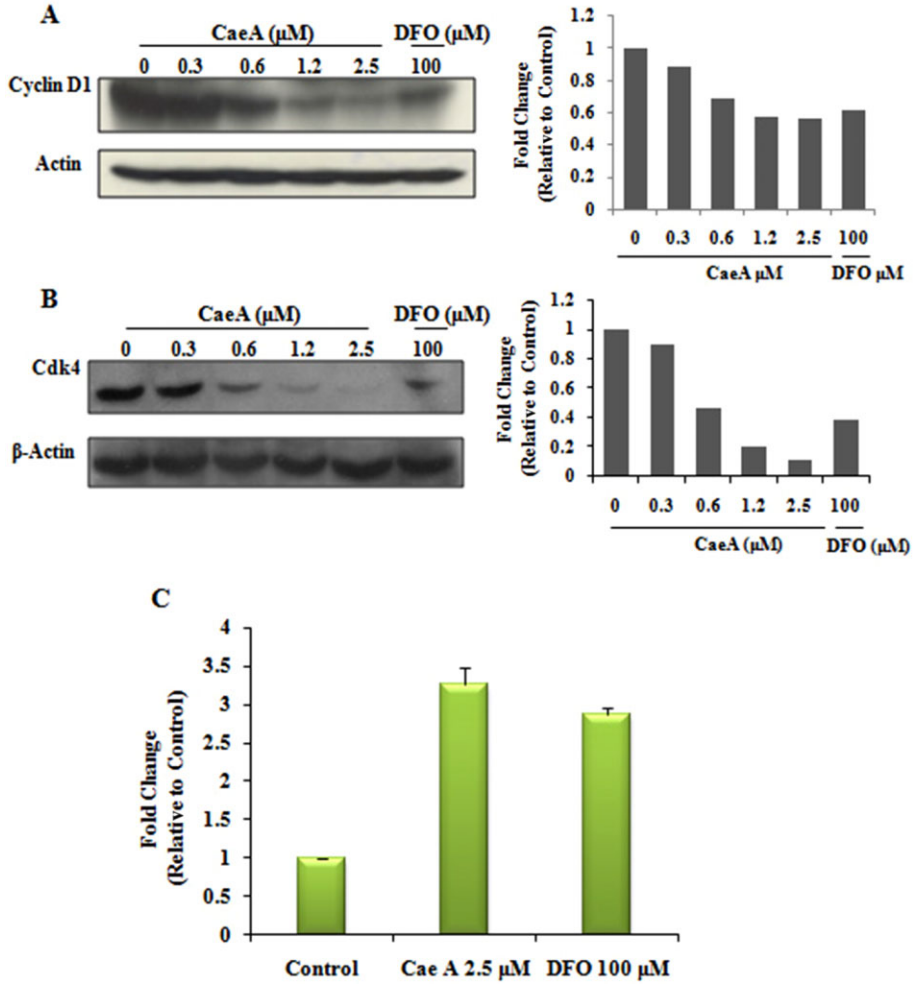

Figure 7

Effect of CaeA on the expression of molecules involved in the cell cycle and proliferation. Jurkat cells were treated with CaeA (0–2.5 μM) or DFO (100 μM) for 24 h. At the end of the incubation, the whole-cell lysates were prepared. Whole-cell lysates prepared from treated cells were subjected to immunoblotting against cyclin D1 (A) and cdk4 (B) using specific antibodies. Equal loading was confirmed using actin or β-actin. (C) Jurkat cells were treated with 2.5 μM CaeA or 100 μM DFO for 24 h. Total RNA was isolated and increase in expression of $p21^{CIP1/WAF1}$ was determined by qRT-PCR. The results are means ± SD from three determinations in a typical experiment.

iron-requiring cells (Chua *et al.*, 2007). Ferritins are ubiquitously expressed proteins, which are involved in the sequestration and storage of iron (Han *et al.*, 2011). Intracellular iron level regulates the abundance and availability of TFR and the intensity of ferritin protein post transcriptionally through iron regulatory proteins. Iron deplete conditions causes increased TFR translation, decreased TFR degradation and translation block of ferritin (Ponka, 1999; Yu *et al.*, 2007). As we have shown that CaeA causes iron-deplete condition in cells, we studied the effect of CaeA on the expression levels of TFR1 and ferritin. CaeA 2.5 μM caused a 1.72-fold increase in the level of TFR1 and approximately threefold decrease in the level of ferritin-H as well as ferritin-L after 24 h.

Under similar conditions, 100 μM DFO and 2.5 μM CaeA caused a similar decrease in ferritin levels. However, depletion in total iron content caused by DFO was only about 15% compared with about 92% caused by CaeA. This apparent discrepancy may be explained as follows. Total iron content, which was determined by atomic absorption spectrometry, included the iron in chelator-iron complex that remained within the cells. DFO being much less lipophilic than CaeA is less efficient at mobilizing iron from the cells, which would result in greater retention of DFO-iron complex within cells compared with the CaeA-iron complex. Similar observations have been previously reported by Richardson et al while studying the effect of DFO on iron uptake by human malignant melanoma cell (Richardson *et al.*, 1994). However, the fraction of iron chelated to DFO or CaeA that is retained within the cells may not enter the labile pool of iron, thereby causing iron-deplete conditions within the cells.

A major consequence of intracellular depletion of iron would be on the function of enzymes that require iron as a cofactor. RNR is one such enzyme, whose activity is essential for the proliferation of cells. Inactivation of RNR stops DNA synthesis, which in turn inhibits cell proliferation (Cerqueira *et al.*, 2007). Using the [^{3}H]-cytidine incorporation assay, we

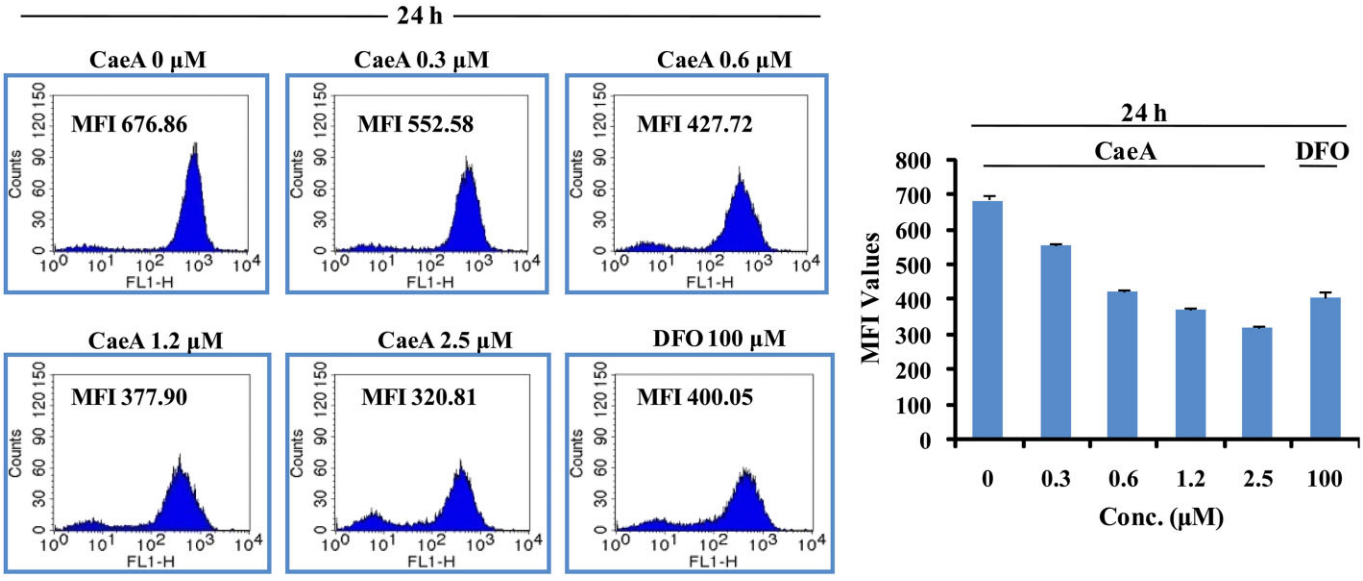

Figure 8

Effect of CaeA on ROS production. Jurkat cells were treated with CaeA (0, 0.3, 0.6, 1.2 and 2.5 µM) or DFO (100 µM) for 24 h and exposed to 2′,7′-dichlorofluorescein diacetate. Fluorescence intensity resulting from 2′,7′-dichlorofluorescein diacetate oxidation by ROS was measured by flow cytometry at the FL1-H channel. Values shown are the fluorescent intensity scores measured (MFI). Data represent the results from one of the three similar experiments. The values plotted in the graph are means ± SEM of three experiments.

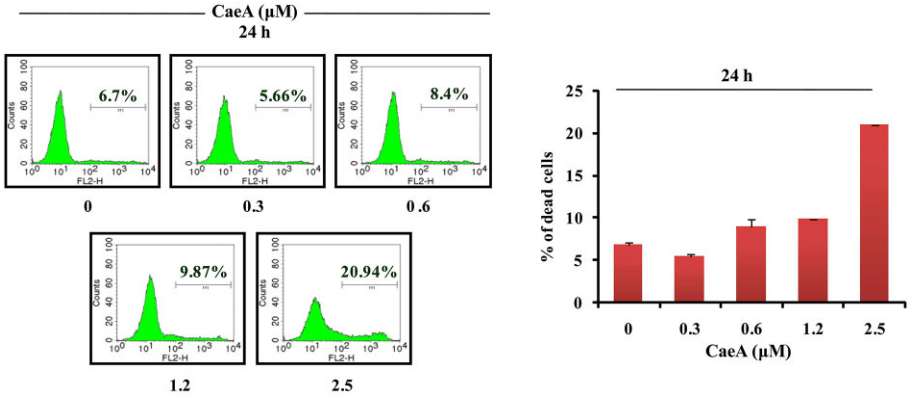

Figure 9

Cytotoxicity determination in Jurkat cells. Jurkat cells were treated with different concentrations (0, 0.3, 0.6, 1.2 and 2.5 µM) of CaeA for 24 h and cell viability was determined by the live PI dye exclusion method through flow cytometry at the FL2-H channel, where % of cells shown are dead cells. The Y axis values plotted in the graph are % of dead cells obtained from means ± SEM of three experiments.

demonstrated that CaeA caused a concentration-dependent decreased synthesis of DNA. The decrease was in the range of 21–55% at CaeA concentrations of 0.3–2.5 µM. Significantly, CaeA under the iron replete conditions showed no effect on the synthesis of DNA.

We estimated the intracellular pool of deoxyribonucletide phosphates (dNTPs). Treatment of Jurkat cells with CaeA resulted in a significant decrease in the intracellular levels of dUTP, dGTP and dATP. The effect on dTTP levels was insignificant, possibly due to a compensatory deoxyribonucleotide salvage pathway (Bianchi *et al.*, 1986; Koc *et al.*, 2004). These results confirmed that decreased synthesis of DNA is due to the impaired function of RNR.

RNR is composed of two subunits R1 and R2. R1 subunit is constitutively expressed throughout the cell cycle, whereas the R2 subunit is expressed in the S phase of the cell cycle where large amounts of deoxyribonucleotides are required by proliferating cells (Lin *et al.*, 2007). We found that treatment of Jurkat cells with 2.5 µM CaeA caused a 2.24-fold increased expression of the R2 subunit. We speculate that the increased level of the R2 subunit may be due to its decreased degradation. It has been proposed previously that the increased level of R2 may help cells to overcome the replication stress due to CaeA-induced depletion of dNTPs (Shao *et al.*, 2006; Lin *et al.*, 2007).

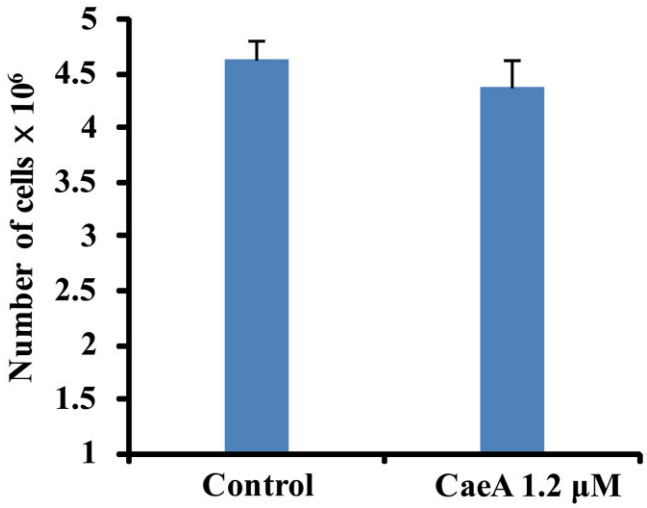

Figure 10

Effect of CaeA on cell proliferation is reversible. Jurkat cells were incubated at 37°C in the presence or absence of 1.2 µM CaeA for 18 h and then washed by incubating in RPMI 1640 for 2 h. The wash cycle was repeated five times. Washed cells, 1.27×10^6 were then incubated in a fresh complete medium at 37°C for 60 h. The numbers of cells were then estimated by the trypan blue method.

Next, we studied the effect of CaeA on MAPK signalling transduction pathways. The MAPK pathway occupies a central position in the signal transduction machinery, which links external signals to the various cellular processes (Kim *et al.*, 2002; Le and Richardson, 2002). The c-Jun NH_2-terminal protein kinase (JNK) and p38 MAPK are known to have anti-proliferative and pro-apoptotic function, while ERK1/2 MAPK is primarily known for its proliferative role (Dhillon *et al.*, 2007; Raman *et al.*, 2007). The p38 and JNK are known for cell cycle arrest by phosphorylating p53 and increasing the stabilization of the cdk inhibitor $p21^{WAF1/CIP1}$ (Yu and Richardson, 2011). Iron chelation is known to significantly reduce ASK1-thioredoxin complex formation. Reduced iron levels promote disulfide formation and consequently the dissociation of thioredoxin from the complex that leads to increased phosphorylation of ASK1, which activates the JNK and p38 MAPKs and their downstream target molecules p53 and ATF2 (Yu and Richardson, 2011).

We studied the effect of CaeA on the phosphorylation of JNK and p38 MAPKs at various time points. The phosphorylation for JNK initiated at 3 h of treatment and maximum phosphorylation with respect to total protein was observed at 12 h of treatment with 2.5 µM CaeA. However, we did not find a significant increase in phosphorylation of JNK with 100 µM DFO. A significant increase in phosphorylation of p38 was observed with both 2.5 µM CaeA and 100 µM DFO. Another member of MAPKs is ERK1/2, which is primarily known for its proliferative role. At early stages 2.5 µM CaeA caused increased phosphorylation of ERK1/2. The level of phospho-ERK1/2 increased up to 1.63-fold in 6 h; after which the level started decreasing and reached close to the normal level after 24 h. This initial increase in ERK1/2 phosphorylation may be related to an early response against stress (Yu and Richardson, 2011). However, CaeA caused a significant increase in phospho-JNK and phospho-p38 levels at 24 h, where anti-proliferative effects of these pathways may be important in the immunosuppressive activity of CaeA.

We also studied the effect of CaeA on cell cycle molecules, cyclin D1, cdk4 and $p21^{CIP1/WAF1}$. Cell cycle progression depends upon the sequential activation and subsequent inactivation of cdks. Cdks rely on cyclins to modulate their phosphorylation activity. The activity of cyclin–cdk complexes are modulated by the cyclin-dependent kinase inhibitors (CKIs). One of the inhibitors, known as $p21^{CIPI/WAF1}$ is a universal CKI (Le and Richardson, 2003). It has been demonstrated that p38 MAPK activates $p21^{CIPI/WAF1}$ on Fe chelation and results in decreased expression of cyclin D1, which leads to cell cycle arrest, JNK is also known to cause cell cycle arrest by stabilizing $p21^{CIPI/WAF1}$ expression (Yu and Richardson, 2011).

CaeA caused concentration-dependent decrease in expression levels of cyclin D1, cdk4 and increase in transcriptional level of $p21^{CIP1/WAF1}$. We observed relative decrease in expression of cyclin D1 from 1.0- to 0.57-fold on treatment with 0–2.5 µM CaeA. In comparison, 100 µM DFO resulted in 0.62-fold decrease in expression of cyclin D1. Compared with control (untreated cells), 2.5 µM CaeA caused 10-fold higher decrease in expression of cdk4, whereas 100 µM DFO caused about 2.5-fold higher decrease in expression of cdk4. 2.5 µM CaeA caused 3.4-fold higher expression of $p21^{CIPI/WAF1}$ mRNA compared with control, whereas 100 µM DFO increased the expression of $p21^{CIPI/WAF1}$ mRNA by twofold. Significantly, preformed CaeA–iron complex has insignificant effect on the expression levels of cyclin D1 or cdk4.

In conclusion, this study demonstrates that CaeA exerts its immunosuppressive effect by depleting intracellular iron concentration. It has multiple cellular targets, viz., iron containing ribonucleotide reductase enzyme, which is crucial for DNA synthesis and cell cycle control molecules cyclin D1, $p21^{CIPI/WAF1}$ and cdk4, which are important for normal cell cycle progression. Significantly, CaeA in presence of excess Fe^{2+} has insignificant effect on DNA synthesis or the expression levels of R2 subunit of RNR, cyclin D1 and cdk4. CaeA decreases ROS level in the cells. Whereas, 0.3 µM CaeA caused complete inhibition of T-cell proliferation (Singla *et al.*, 2014), 0.6 µM CaeA showed insignificant effect on the viability of cells. Moreover, this effect on the proliferation of cells is reversible once CaeA is withdrawn from the system. These findings suggest that that iron chelation is a viable rationale approach to selectively suppress the immune system; because compared with normal cells, rapidly proliferating cells require a higher utilization of iron, which is a central regulator for the proliferation and function of immune cells.

Acknowledgements

This work was supported by New Millennium Indian Technology Initiative grant TLP0009 and Supra Institutional Project Grant BSC0211 from CSIR, New Delhi, India. S. K., G. S. and A. N. S. acknowledge CSIR for award of Senior Research Fellowship. We acknowledge the technical help provided by Chander Prakash and Pradeep Patel.

Author contributions

S. K. and R. S. J. designed the study, analysed and interpreted the data, drafted the paper; S. K. performed most of the experiments; G. S. and A. N. S. performed some critical experiments. All authors approved the paper.

Conflict of interest

The authors declare no competing financial interests.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13051

Figure S1 Effect of CaeA on RNR activity in presence of excess iron. Jurkat cells were treated with 2.5 μM of CaeA in the presence of excess $FeSO_4$ and then pulsed with ^{3}H-cytidine for 30 min. DNA was isolated and ^{3}H-cytidine incorporation was determined. The incorporation of ^{3}H-cytidine into the DNA was estimated relative to control (untreated cells).

Figure S2 Effect of CaeA on R2 expression in presence of excess iron. Jurkat cells were treated with CaeA 2.5 μM alone or in presence of excess of $FeSO_4$ for 24 h. Expression levels of R2 subunit were determined by Western blotting. Fold change was calculated after normalization with β-actin.

Figure S3 Effect of CaeA on the expression of molecules involved in the cell cycle progression in presence of excess iron. Jurkat cells were treated with 2.5 μM CaeA or 2.5 μM CaeA–Fe complex or 100 μM DFO for 24 h. At the end of incubation, the whole-cell lysate was prepared. Influence on the expression levels of cyclin D1 and cdk4 by 2.5 μM CaeA-Fe complex was compared with 2.5 μM CaeA or 100 μM DFO; determined by immunoblotting of whole-cell lysates using specific antibodies. Equal loading was confirmed using actin.