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Effect of kanglemycin C on lymphocyte proliferation induced by tetradecanoylphorbol acetate and ionomycin[1]

LI Jian-Ming, LIN Zhi-Bin[2]
(*Department of Pharmacology, Beijing Medical University, Beijing 100083, China*)

KEY WORDS kanglemycin C; cyclosporine; tetradecanoylphorbol acetate; ionomycin; protein kinase C; calcineurin; lymphocytes; cell division; concanavalin A; interleukin-2

ABSTRACT

AIM: To compare the suppressions of kanglemycin C (Kan) with that of cyclosporine (Cyc) on lymphocyte proliferations induced by tetradecanoylphorbol acetate (TPA) with ionomycin (IM), and concanavalin A (Con A). METHODS: Cell proliferation was quantified with 3-(4, 5-dimetyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) or [^{3}H]thymidine ([^{3}H]TdR) incorporating method. Calcineurin (CN) activity was measured with molybdate dye colorimetry. RESULTS: Kan (8, 40, 80, and 400 nmol·L^{-1}) strongly suppressed splenocyte proliferation induced by TPA and IM, and the suppressive effect of Kan gradually decreased along with the increasing concentrations of TPA (1 – 100 μg·L^{-1}) and IM (125 – 500 μg·L^{-1}). But, the suppression of Cyc on the splenocyte proliferation induced by TPA was mild. Cyc suppressed CN activity of mouse splenocytes stimulated by Con A stronger than Kan. Moreover, Kan and Cyc strongly suppressed spleen enriched T-cell proliferation induced by Con A and TPA + IM, and the suppression of Kan on proliferation was partly attenuated by exogenous IL-2. CONCLUSION: Kan competitively suppressed the proliferation induced by TPA and IM, and Cyc mainly suppressed IM part of the proliferation induced by TPA and IM.

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[2] Correspondence to Prof LIN Zhi-Bin. Phn/Fax 86-10-6209-1686. E-mail linzb@public3.bta.net.cn

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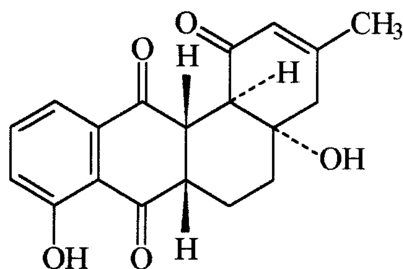
INTRODUCTION

Protein kinase C (PKC) is still a expanding family of related isozymes[1], which induce phosphorylation of serine, threonine, and tyrosine of transcription factors and signal molecule inducing gene expression and cell proliferation[2]. Tetradecanoylphorbol acetate (TPA) alone can activate PKC by direct binding and induce IL-2 receptor expression[3], however, TPA alone or calcium ionophore-ionomycin (IM) alone is not sufficient to activate T-cells[4]. The combination of TPA and IM can induce the synthesis of IL-2 and expression of IL-2 receptors in T-cells and activate T-cells.

Calcineurin (CN) is a calcium/calmodulin-regulated serine/threonine phosphatase that is ubiquitously expressed[5] and a major calmodulin-binding protein in lymphocyte[6]. CN regulates the function of nuclear factor of activated T cells (NF-AT) by controlling the nuclear translocation of NF-AT cytoplasmic component in response to T cell activation[5] and plays a critical role in signal transduction pathways necessary for T cell activation[7]. Immunosuppression of Cyclosporine (Cyc) takes effect through the inhibition of CN[8,9].

Kanglemycin C (Kan, $C_{19}H_{18}O_5$, yellow pin-shaped crystal, mp 170 ℃) *in vivo* and *in vitro*, like Cyc, prolonged allografts survival time, and inhibited DH and hemolysin production[10], strongly inhibited the mouse splenocyte proliferation stimulated by mitogens [concana-valin A



Kanglemycin C

(Con A), phytohemagglutinin (PHA), and tetradecanoylphorbol acetate (TPA) with ionomycin (IM)] and by alloantigen. Splenocyte proliferation induced by lipopolysaccharides (LPS) was suppressed by Kan, but not by Cyc[11]. In order to explore the immunosuppressive mechanisms of Kan and Cyc, the research is involved to differentiate the effects of the agents on lymphocyte activation pathways induced by TPA with IM, and Con A.

MATERIALS AND METHODS

Reagents and drugs RPMI-1640 medium was purchased from Gibco, complete medium was supplemented with *L*-glutamine 2 mmol·L^{1} (Kaibiomed Scientific Co, Beijing), 10 % fetal calf serum (FCS, Gibco), 2-mercaptoethanol 0.5 nmol·L^{-1} (Shanghai № 4 Chemical Reagent H V Co Ltd), benzylpenicillin 100 kU·L^{-1}, streptomycin 100 mg·L^{-1}. Con A (Sigma Chemical Co) was dissolved at 2 g·L^{-1} in RPMI-1640. TPA (Sigma), and IM (Sigma) were prepared as the solutions 5 g·L^{-1} in Me_2SO. Kan supplied by Prof WANG Nan-Jin (Institute of Medical Biotechnology, Chinese Academy of Medical Sciences), a solution of 10 g·L^{-1} was prepared in ethanol, stored protected from light until use. Cyc (Sandoz Pharmaceuticals, E Hanover, NJ, USA) 10 mg was dissolved in 1 mL ethanol. The reagents stated above diluted freshly to the desired concentration in complete RPMI1640.

Mice Inbred strain BALB/c (Grade Ⅱ, Certificate №: 01 – 3046) mice, ♀, 8 – 12 wk, 20 – 22 g, were purchased from the Department of Experimental Animal, Beijing Medical University, Beijing, PRC.

Cell separation Splenocyte was prepared in a general way[12], and the concentration and viability of the cells were determined by trypan blue exclusion.

Cell proliferation As the optimum concentrations (TPA 100 μg·L^{-1} + IM 500 μg·L^{-1}) described[13], the combinations of TPA 1, 10, 100 μg·L^{-1} and IM 125, 250, 500 μg·L^{-1} were chosen from the preexperiment with the combinations of TPA 1, 10, 100, 1000 μg·L^{-1} and IM 125, 250, 500, 1000 μg·L^{-1}. Splenocytes and enriched T lymphocytes (2×10^5) were incubated with mitogen (Con A 5 mg·L^{-1}, or TPA and IM) alone, or with Kan or Cyc 8, 40, 80, and 400 nmol·L^{-1}, or with recombinant hIL-2 20 ku·L^{-1} (purchased from Biotinge Biomedicine Co) in flat-bottom microtiter plates in 0.2 mL of complete media. The cultures were triplicate and incubated at 37 ℃ in a humidified atmosphere of 5 % CO_2 in air for 48 h. The proliferation was measured by tritiated thymidine ([^{3}H] TdR, 920 GBq·mol^{-1}, 37 GBq·L^{-1}, Shanghai Institute of Nuclear Research, Chinese Academy of Sciences) or MTT method[12].

Calcineurin activity Calcineurin activity was measured as the protocol with the kits bought from the Promega. Briefly, the splenocytes were cultured for 12 h as above procedure, and then homogenized in storage buffer (Tris-HCl 0.05 mol·L^{-1}, pH 7.0; egtazic acid 0.1 mmol·L^{-1}; 0.1 % 2-mercaptoethanol) and centrifuged at 100 000 × *g* at 4 ℃ for 60 min. The supernatants 250 μL was added to the sephadex G-25 resin spin column, drained by gravity and then centrifuged at 600 × *g* at 4 ℃ for 5 min. The protein concentration of the supernatants was measured with Commasie brilliant blue colorimetry[14]. Ser/Thr phosphopeptide (1 mmol·L^{-1}) 5 μL or phosphate standards (50 μmol·L^{-1}) 0 – 2000 pmol and 5 × reaction buffer (imidazole 250 mmol·L^{-1}, pH 7.2; egtazic acid 1 mmol·L^{-1}; $MgCl_2$ 50 mmol·L^{-1}; $CaCl_2$ 2 mmol·L^{-1}; Calmodulin 250 mg·L^{-1}; 0.1 % 2-mercaptoethanol) 10 μL were added to the 96 well plate and phosphate free water added to 45 μL, and placed at 30 ℃ for 3 min. Then, the supernatant (1 g protein·L^{-1}) 5 μL and phosphate free water 5 μL were respectively added to the sample wells and phosphate standard wells, and incubated at 30 ℃ for 15 min, then, molybdate dye/additive mixture 50 μL added and the plate placed at room temperature for 15 min. The absorbance was read at 630 nm in plate reader (Bio-Rad). Enzyme activity was expressed as quantity (μmol) of phosphate produced by per g protein per minute.

RESULTS

Effect of Kan on splenocyte proliferation induced by different concentrations of TPA and IM

The splenocyte proliferation induced by the combinations of TPA (1 – 100 μg·L^{-1}) with IM (250 μg·L^{-1}), and TPA (10 μg·L^{-1}) with IM (125 – 500 μg·L^{-1}) were inhibited by Kan (8 – 400 nmol·L^{-1}) (Fig 1 A, B, C, D, E).

And Kan markedly suppressed the splenocyte proliferation induced by TPA (100 μg·L^{-1}) and IM (125 μg·L^{-1}) (Fig 1F). Kan, with increasing its concentrations, was able to strongly suppress the proliferation induced by TPA and IM of the higher concentrations. Cyc

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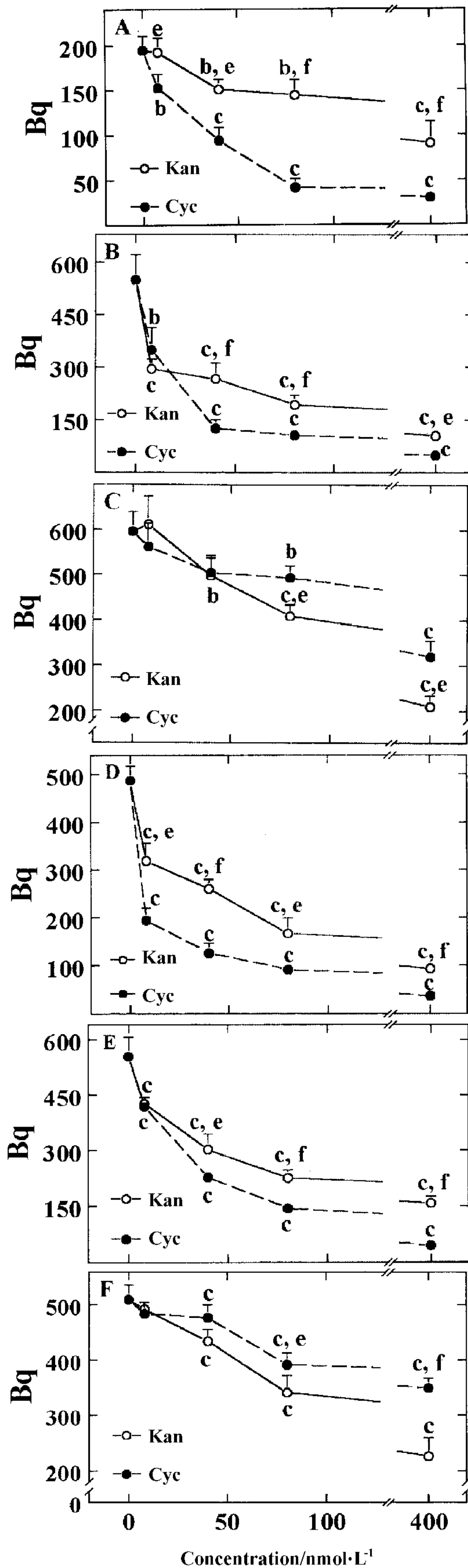

Fig 1. Effect of Kan and Cyc on the mouse splenocyte proliferation induced by the combinations of TPA and IM at different concentrations. A) TPA 1 μg·L^{-1} + IM 250 μg·L^{-1} ; B) TPA 10 μg·L^{-1} + IM 250 μg·L^{-1} ; C) TPA 100 μg·L^{-1} + IM 250 μg·L^{-1} ; D) TPA 10 μg·L^{-1} + IM 125 μg·L^{-1} ; E) TPA 10 μg·L^{-1} + IM 500 μg·L^{-1} ; F) TPA 100 μg·L^{-1} + IM 125 μg·L^{-1}. $^bP<0.05$, $^cP<0.01$ *vs* TPA + IM ; $^eP<0.05$, $^fP<0.01$ *vs* Cyc.

(8 – 400 nmol·L^{-1}) strongly suppressed splenocyte proliferations induced by the combinations of TPA and IM (Fig 1 A, B, C, D, E). However, the splenocyte proliferation induced by TPA (100 μg·L^{-1}) and IM (125 μg·L^{-1}) was mildly inhibited by Cyc (Fig 1F). The suppression of Cyc, with the enhancing of TPA concentrations and though Cyc concentrations were elevated, tended to decline. Suppression of Cyc on splenocyte proliferation induced by TPA (100 μg·L^{-1}) and IM (125 μg·L^{-1}, 250 μg·L^{-1}) was less than that of Kan (Fig 1C, F).

Effect of Kan on CN activity Both Kan and Cyc (8 – 400 nmol·L^{-1}) markedly inhibited CN activity (Fig 2). Furthermore, it was shown that Kan is a milder inhibitor of CN activity than Cyc.

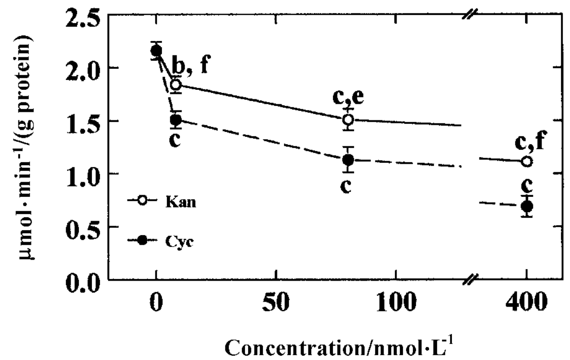

Fig 2. Effect of Kan and Cyc on mouse splenocyte calcineurin activity induced by Con A. $^bP<0.05$, $^cP<0.01$ *vs* Con A ; $^eP<0.05$, $^fP<0.01$ *vs* Cyc.

Influence of Kan on IL-2 action events Both Kan and Cyc suppressed enriched T-lymphocyte proliferation induced by Con A (5 mg·L^{-1}) and by the combination of TPA (10 μg·L^{-1}) and IM (250 μg·L^{-1}). The suppressions of Kan were milder than that of Cyc ($P<0.01$). The suppression of Cyc (8 – 400 nmol·L^{-1}) was mostly attenuated by addition of exogenous IL-2 (20 ku·L^{-1}), while, the suppression of Kan (8 – 400 nmol·L^{-1}) was partly attenuated (Fig 3 A, B).

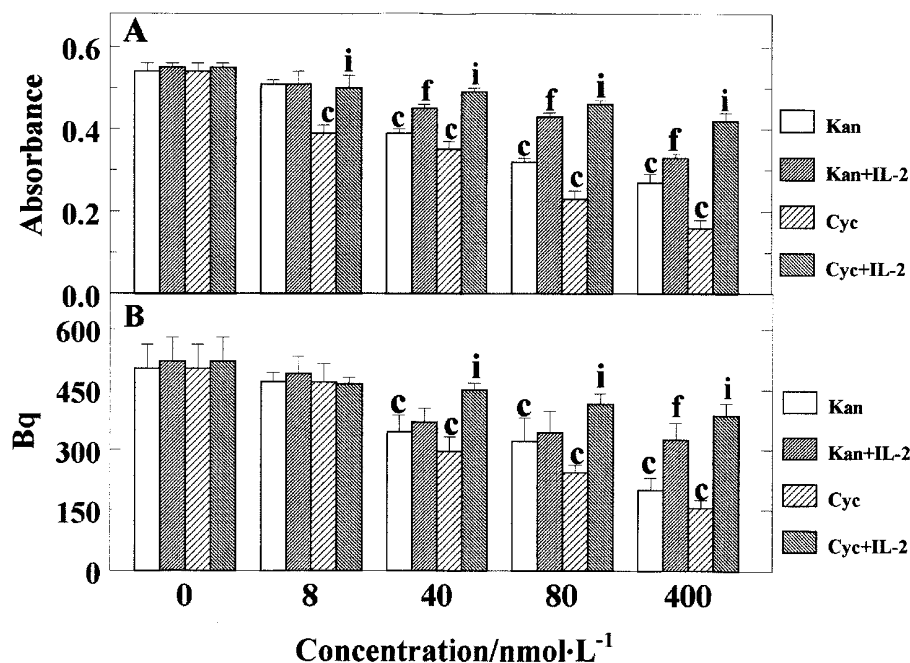

Fig 3. Effect of Kan and Cyc on enriched T-cell proliferation induced by Con A and TPA + IM by addition of exogenous IL-2. $^{c}P<0.01$ *vs* control (Con A or TPA + IM without IL-2, or with IL-2); $^{f}P<0.01$ *vs* the same dose's Cyc without IL-2; $^{i}P<0.01$ *vs* the same dose's Cyc with IL-2. (A. Inducing agent was Con A; B. Inducing agents were TPA + IM).

DISCUSSION

By means of respectively enhancing the concentrations of TPA ($1-100\ \mu g \cdot L^{-1}$) and IM ($125-500\ \mu g \cdot L^{-1}$) in culture, it was found that the influence of Kan and Cyc on cell activation and proliferation induced by the combination of TPA and IM was different. The suppression of Cyc on proliferation induced by different concentrations of TPA was milder than that on proliferation induced by different concentrations of IM. Furthermore, Cyc strongly inhibited the CN activity of splenocyte stimulated by Con A, and the Cyc-mediated suppression of enriched T-lymphocyte proliferation was powerfully attenuated by IL-2[15]. It corresponded with the reports[8,9] that Cyc mainly inhibited the calcium pathway of cell activation and action pathways before IL-2 receptor. Kan, with increasing its concentrations, was able to strongly suppress the proliferation induced by TPA and IM of the higher concentrations though the suppression of Kan was declined with the increasing of TPA and IM. It suggested that Kan and the combination of TPA and IM competitively inhibited each other in actions. Moreover, Kan also inhibited both the CN activity and the enriched T-lymphocyte proliferation induced by Con A. The suppression of Kan on the enriched T-lymphocyte proliferations induced by Con A and by the combination of TPA and IM was partly attenuated by IL-2. It suggested that Kan, compared with Cyc, suppressed the cell activation pathways at different sites.

In summary, Kan competitively suppressed the proliferation induced by TPA and IM, and Cyc mainly suppressed IM part of the proliferation induced by TPA and IM.

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康乐霉素对豆蔻酰佛波醇乙酯和伊屋诺霉素诱导淋巴细胞增殖的作用[1]

李建明，林志彬[2]

（北京医科大学药理系，北京 100083，中国）

关键词 康乐霉素 C；环孢素；十四酰佛波醇乙酯；伊屋诺霉素；蛋白激酶 C；钙调磷酸酶；淋巴细胞；细胞分裂；刀豆球蛋白 A；白细胞介素-2

目的：比较康乐霉素 C（Kan）和环孢素（Cyc）对十四酰佛波醇乙酯（TPA）和伊屋诺霉素（IM），及刀豆球蛋白 A（Con A）诱导淋巴细胞增殖的作用. **方法**：氚掺入或 MTT 法测细胞增殖；钼酸盐染料比色法测钙调磷酸酶（CN）活性. **结果**：Kan（8，40，80 和 400 nmol·L^{-1}）竞争性抑制 TPA 和 IM 刺激的脾细胞增殖. Cyc 浓度增加，对 TPA 和 IM 刺激的脾细胞增殖中 IM 变化的增殖作用抑制较强. Kan 和 Cyc 抑制 Con A 刺激的脾富集 T-细胞增殖，IL-2 拮抗 Cyc 作用较强；Kan 抑制 CN 活性的作用较 Cyc 弱. **结论**：Kan 竞争性抑制 TPA 和 IM 活化的细胞增殖，而 Cyc 抑制 IM 活化作用较强.

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