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2-Methoxy-1,4-Naphthoquinone (MNQ) Suppresses Protein Kinase C βI, δ, and ζ Expression in Raji Cells

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ABSTRACT

Protein Kinase C (PKC) is widely documented to be involved in the regulation of cancer cell growth, proliferation, survival, inflammation and apoptosis. This study evaluates the capability of 2-Methoxy-1,4-Naphthoquinone (MNQ) in regulating PMA-induced PKC expression in human Burkitt's lymphoma cell line (Raji cells). MNQ exhibited stronger anti-tumour-promoting activity than genistein based on the inhibitory assay of Epstein-Barr virus (EBV) activation. The IC_{50} values attained were 2.92 and 7.40 μM, respectively. The suppressive effects of MNQ on PKC expression was determined by using the PepTag® non-radioactive detection of PKC assay. The IC_{50} values achieved for staurosporine and rottlerin (standard control), and MNQ were 0.01, 6.38, and 13.13 μM, respectively. These preliminary results indicate that MNQ specifically suppressed the expression of PKC βI, δ, and ζ in a concentration-dependent manner in Raji cells.

INTRODUCTION

Cancer is a highly prevalent health burden with increasing incidents worldwide that still requires much attention for medical solutions. One of the factors that contribute to the challenges in cancer research is the complexity of carcinogenesis which can be underlined by defective cellular signaling pathways. Advanced high-throughput technologies have been employed to understand the complexity and dynamic interactions of proteins that are involved in these multiple dysfunctional cancer signaling pathways, which include Protein Kinase C (PKC) pathway. Cellular signaling cascades, comprising upstream and cytoplasmic signaling networks, include many protein kinases such as MAPKs, PKC, PI3K, PKB/Akt, and GSK. The aberrant activation of these aforementioned kinases has been implicated in various cancers. Dysregulation of these kinases may also lead to activation of diverse downstream transcriptional factors such as nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) (Yap *et al*., 2011). PKC is a family of serine/threonine kinases in which each PKC isoform more or less has been implicated in carcinogenesis and displays variable expression profiles during cancer progression (Mackay and Twelves, 2003).

Notably, PKC has the strongest association with the promotion and progression of cancer and becomes the prime targets of diverse classes of chemopreventive phytochemicals (Fields and Gustafson, 2003). MNQ isolated from *Impatiens balsamina*, Linn from the family of Balsaminaceae. This plant is an ornamental plant that has been widely used in Traditional Chinese Medicine to treat rheumatism, isthmus and crural aches, fractures, superficial infections, fingernail inflammation, various skin diseases treatment of difficult labor and puerperal pain, treatment of pains in the joint and skin affliction (Farnsworth and Cordell, 1976; Ding *et al*., 2008). In some areas of China, people ingest this plant as anti-cancer herb (Ding *et al*., 2008). MNQ exhibited antifungal (John *et al*., 1948; Rodriguez *et al*., 1995), antimicrobial (Yang *et al*., 2001) activities and showed strong killing effect on K562, HL-60, Raji, MCF-7, H23, IMR-32, LA-174-T, HSC-2, and SK-MEL-28 (Teng, 2010).

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MNQ was able to trigger the intrinsic apoptotic pathway in K562 cells, as well as causing p53-dependent cell cycle arrest in the p53-wild type cell line (Tan, 2011). Besides, MNQ inhibited the TCF/β-catenin (TOP) transcriptional activity at IC_{50} 2.9 µM, while it decreased the transcriptional activity of FOP (mutated TCF-binding site)-transfected cells (Mori *et al*., 2011). Recent study has revealed that MNQ exerted an anti-metastatic effect against MDA-MB-231 cells (Liew *et al*., 2014). Therefore, this study was designed to investigate the suppressive effect of MNQ on the PKC activity and to identify the type of PKC isoform(s) expressed in Raji cells that could be regulated by MNQ.

MATERIALS AND METHODS

Materials

Genistein, phorbol 12-myristate 13-acetate (PMA), sodium *n*-butyrate (S*n*B), anti-human IgG FITC-conjugate, and staurosporine were obtained from Sigma. Epstein-Barr virus-early antigen (EBV-EA)-positive sera from nasopharyngeal carcinoma (NPC) patients were kindly provided by Dr. Paul Lim Vey Hong from Tung Shin Hospital, Kuala Lumpur, Malaysia. Rottlerin was purchased from Calbiochem.

General experimental procedures

MNQ was isolated from the pericarps of *Impatiens balsamina*, Linn. The purity of this crystallized pure compound is at 99.27 % determined by HPLC. The chemicals were prepared in DMSO and stored at 4 ºC.

Cell culture

Raji cells obtained from Riken Cell Bank, Japan were maintained in commercial Roswell Park Memorial Institute Media (RPMI-1640) supplemented with 10 % fetal bovine serum (FBS) (GIBCO, South America). The cells were incubated in a humidified atmosphere at 37 ºC with 5 % carbon dioxide (CO_2) incubator (Esco, USA).

Inhibitory assay of Epstein-Barr virus (EBV) activation

This assay was performed according to the protocol described by Lim *et al*. (2010). 5×10^5 Raji cells were incubated in 1 mL of RPMI 1640 medium (supplemented with 10 % FBS) containing 0.05 µM PMA, 3 mM S*n*B and test substance (MNQ or DMSO as the vehicle control) at 37 ºC under 5 % CO_2 for 48 hours. Genistein was used as the standard drug. The cells were then harvested, washed with PBS and fixed onto Teflon-coated slides.

The activation of EBV in Raji cells was examined through the expression of the EBV-Early antigen (EBV-EA). These early antigens were detected by using an indirect immunofluorescence approach in which the EBV-EA positive sera (1:30 dilution) were used as the primary antibody, whereas the FITC-labelled IgG (1:30 dilution) was used as the secondary antibody. Images of the fluorescent and non-fluorescent cells were captured using a laser microscope (Nikon, Japan) and their numbers were counted. The inhibitory activity on EBV activation of each sample was compared with a control (only PMA and S*n*B) in which the induction rate of the control was less than 40 %.

Protein Kinase C assay

PepTag® Assay for Non-Radioactive Detection of Protein Kinase C (Promega) was used to determine PKC activities. This assay was performed according to protocol recommended by Promega (2009). Approximately 1×10^7 cells were washed with phosphate-buffered saline (Sigma), and then suspended in 0.5 mL of cold PKC extraction buffer, and homogenized. The lysate was centrifuged for 5 minutes at 4 ºC, 14,000 × g and the supernatant (extracted PK C) was collected. The PKC sample was then incubated at 30 ºC for 30 minutes. A 0.8 % agarose solution in 50 mM Tris-HCl (pH 8.0) was prepared. Gel was run at 100 V for 15 minutes. Once electrophoresis was completed, the gel was removed from the chamber and photographed under chemiluminescence in gel imager (UVP BioSpectrum® Imaging System with VisionWorks®LS software, USA). The bands of interest were then cut and heated at 95 ºC to dissolve the gel slice. 125 µL of the hot agarose was transferred to a tube containing 75 µL of Gel Solubilization Solution and 50 µL of glacial acetic acid. A total of 250 µL sample was quickly vortex and transferred to a well in a 96-well plate. The absorbance was read at 570 nm with microplate reader (Tecan Infinite® 200 Pro NanoQuant with Magellan® software). PKC activity was calculated based on the Beer's law.

Western Blotting

Ten µL of each protein sample and 4 µL of Kaleidoscope Prestained Standards (Bio-Rad) were loaded on Acrylamide/ Bisacrylamide gels (12 % stacking gel) and the separated proteins were then transferred onto an Immobilon PVDF (poly-vinylidene-difluoride) membrane. The membrane was then probed with monoclonal or polyclonal antibodies against various isoforms of PKC (GeneTex, USA) at a dilution of 1:500 (for PKC δ), 1:1000 (for PKC α, βI, βII, η and ε) and 1:20000 (for PKC ζ). The enzyme-coupled secondary antibody (affinity purified mouse or rabbit anti-human IgG horseradish peroxidase conjugate from Cell Signaling Technology) was used at a dilution ratio of 1:10000 and detected using the Immobilon Western Chemiluminescent HRP Substrate from Millipore.

Statistical analysis

The results were obtained from three separate experiments in triplicates. The mean values ± standard deviations (SD) were calculated for all experiments.

RESULTS AND DISCUSSION

MNQ and genistein were demonstrated to inhibit the EBV-early antigen activation in Raji cells in a dose-dependent manner (Figure 1). MNQ recorded an IC_{50} value of 2.92 µM which was lower than that of Genistein (IC_{50} = 7.40 µM).

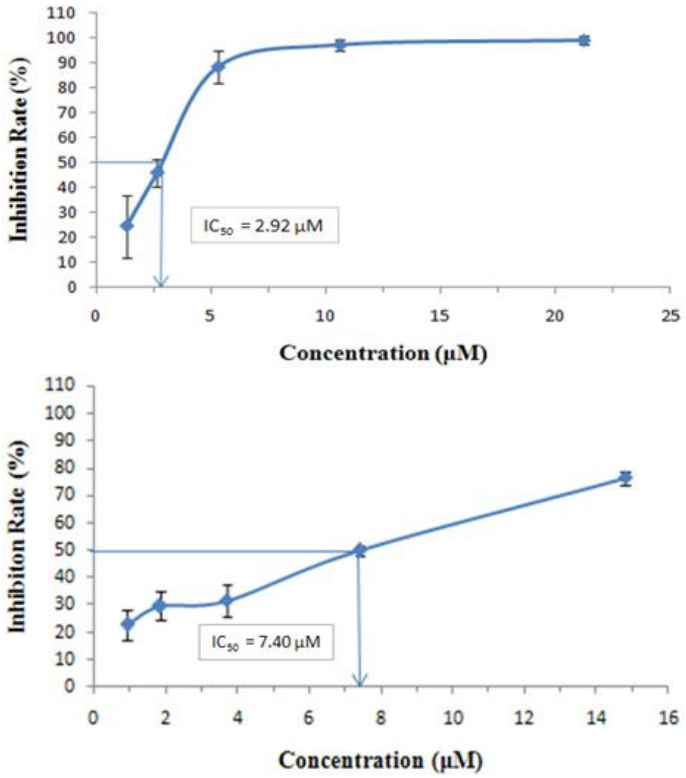

Fig. 1: The inhibition rate of (A) MNQ, (B) genistein on EBV activation in PMA-induced Raji cells.

The suppressive effect of MNQ on PKC expression was compared to two standard of PKC inhibitors, rottlerin and staurosporine. MNQ suppressed the PKC expression in a concentration-dependent manner in Raji cells with a IC_{50} values of 13.13 µM, compared to rottlerin (IC_{50} values of 6.38 µM) and staurosporine (IC_{50} values of 0.01 µM) (Figure 2). In comparison, the effect of MNQ is weaker than the standard PKC inhibitors.

Figure 3 shows that MNQ suppress PKC βI, PKC δ, and PKC ζ expression in a concentration-dependent manner. At 20 µM concentration, PKC βI expression was almost undetectable. Significant reduction of PKC βI and ζ at lane 3 band intensity (treated with 10 µM MNQ) and lane 4 band intensity (treated with 20 µM MNQ) suggested that MNQ have remarkable selectivity towards PKC βI and ζ suppression. MNQ suppressed PKC δ moderately, where MNQ suppressed half of the PKC δ expression at the concentration of 10 µM. The result shows that MNQ did not suppress PKC βII. Raji cells are EBV-positive B lymphoblast cells which harbour the EBV latently and carry multiple copies of the EBV genome (Yao *et al*., 1989; Anvret and Villalba, 2003). EBV activation is associated with oncogenesis by promoting genomic instability leading to uncontrolled cell proliferation and tumour promotion (Ooka *et al*., 1984; Young and Rickinson, 2004). In this study, PMA and S*n*B was used as inducer and enhancer respectively to cause EBV activation in Raji cells and directly transform Raji cells into promotion stage. Since PMA and S*n*B stimulate cell proliferation, alteration of cell morphology, and enhancing cell transformation in Raji cells (Ruddon, 2007), thus the suppressive effect of MNQ on PMA and S*n*B -induced EBV-early antigen activation in Raji cells is likely to contribute to its anti-tumour-promoting effects in Raji cells.

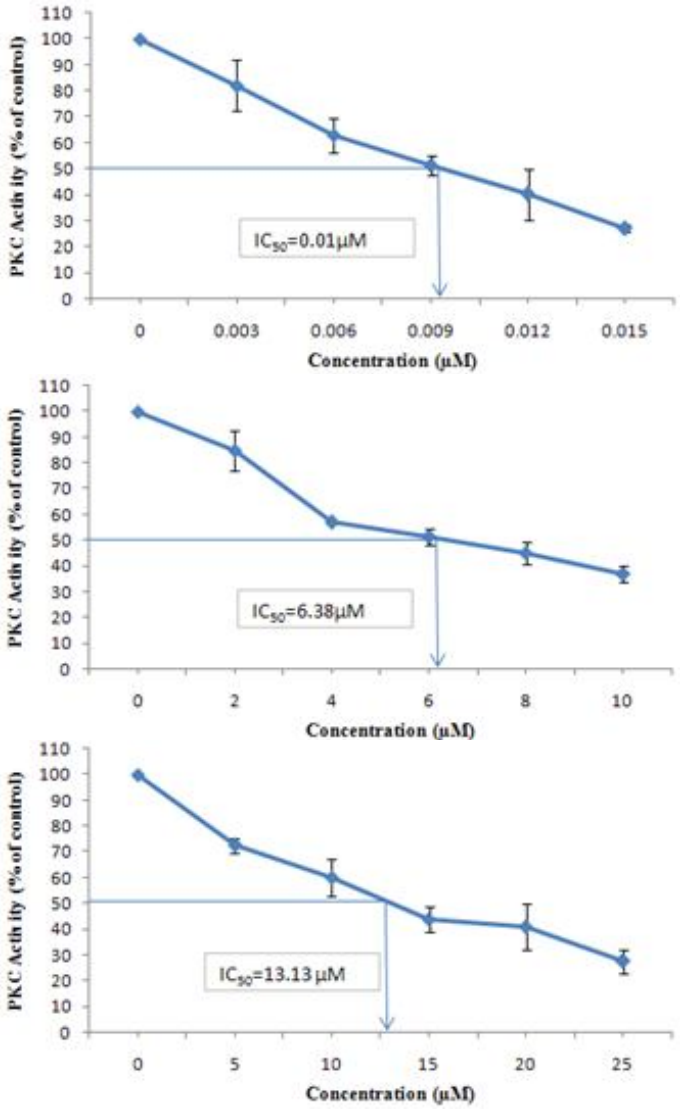

Fig. 2: The suppressive effects of (A) staurosporine, (B) rottlerin, and (C) MNQ on PKC activities in Raji cells. PKC activities were determined using the PepTag® Assay for Non-Radioactive Detection of PKC (Promega). The PKC activities obtained were plotted against the sample concentrations. Result represented the mean of three independent experiments carried out in triplicate.

The linkage between activation of PKC and phorbol ester-induced tumour promotion in Raji cells was previously demonstrated (Davies *et al*., 2001), where the induction of EBV lytic cycle of EBV-positive B-lymphoid cell lines (including Raji cells) by phorbol esters such as PMA required an phosphorylated PKC. Phosphorylation of PKC occurred via the high affinity binding of PMA onto the DAG-binding site of PKC and leading to the activation of PKC. The highest PKC induction in Raji cells was obtained with 20 nM PMA at 6 hours incubation time (Lim *et al*., 2012). In the present study, Raji cells were pre-treated with various concentrations of MNQ for 1 hour before incubating with 20 nM PMA for another 6 hours (Lim *et al*., 2012).

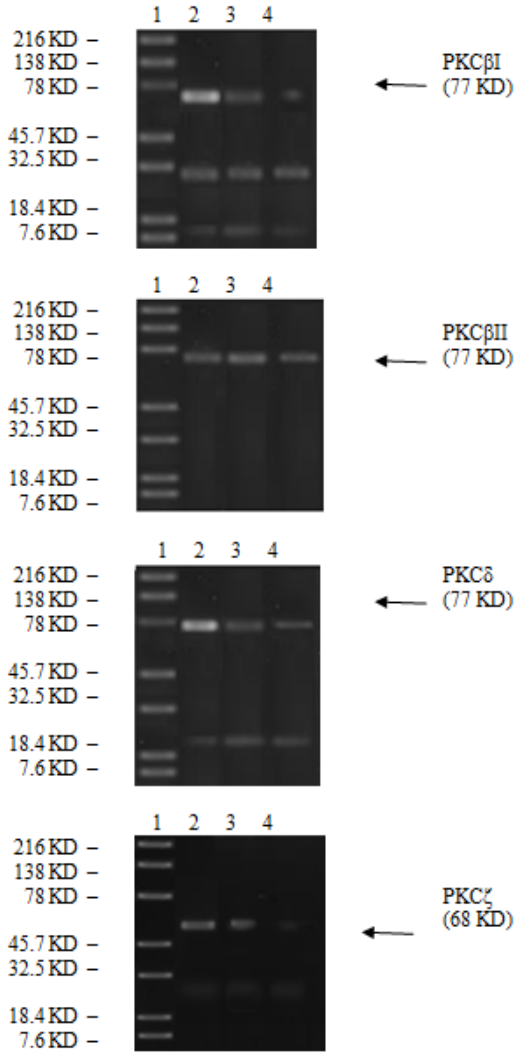

Fig. 3: Immunoblot showing the suppressive effect of 5 µM (lane 2), 10 µM (lane 3) and 20 µM (lane 4) of MNQ on the four PKC isoforms in Raji cells. Lane 1 is kaleidoscope prestained standards ladder. Essentially identical results were obtained in three independent experiments.

The study of isoform-selective PKC inhibitors can be used to elucidate the physiological and pathophysiological roles of individual PKC isoforms. Thus, this study is continued to evaluate the suppressive effects of MNQ specifically on PKC isoforms through western blotting analysis. The PKC family consists of at least 12 serine-threonine kinases, which are classified into three major groups: classical (α, β, and γ), novel (δ, ε, η, and θ) and atypical (µ, ζ, and ι). B cells expressed the cPKC isoforms of α, βI and βII, nPKC isoforms of δ, ε and η, and aPKC of isoform ζ (Brick-Ghannam *et al*., 1994; Morrow *et al*., 1999). Recent study showed that only four PKC isoforms (βI, βII, δ, and ζ) were expressed in Raji cells incubated with PMA (Lim *et al*., 2012). Thus, these four PKC isoforms were selected and incubated with three different concentrations of MNQ at 5, 10, and 20 µM, respectively.

Possible inhibition mechanisms for PKC expression may due to the interference of PKC membrane translocation by MNQ. Studies have shown that PMA binds to transmembrane domains, followed by, cholesterol (Cho) that bind to hydrogen-bonded ester carbonyl groups of PKC (Prades *et al*., 2011), with that, PKC will increase the affinity to Ca^{2+} and phosphatidylserine, thereby causing the translocation of PKC from cytosol to plasma membrane (Nishizuka, 1984; Sharkey *et al*., 1984). Thus, it is suggested that MNQ blocking PMA from intercalating into the membrane to activate PKC βI, PKC δ, and PKC ζ and thereby disturbing physiological function of PKC in Raji cells.

CONCLUSION

This study documented that MNQ specifically suppress the expression of PKC βI, PKC δ, and PKC ζ, and hence could be regarded as a potent PKC inhibitor. This preliminary study laid down the essential fundamental knowledge that warrant for further mechanism study on drug-protein interaction of MNQ and PKC, particularly target on the PKC βI and PKC ζ.

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REFERENCES

Anvret M, Karlsson A, Bjursell G. Evidence for integrated EBV genomes in Raji cellular DNA. Nucleic Acids Research, 1984; 12(2):1149-61.

Brick-Ghannam C, Ericson ML, Schelle I, Charron D. Differential regulation of mRNAs encoding protein kinase C isoenzymes in activated human B cells. Human Immunology, 1994; 41(3): 216-24.

Davies AH, Grand RJA, Evans FJ, Rickinson AB. Induction of Epstein-Barr Virus lytic cycle by tumor-promoting and non-tumor-promoting phorbol esters requires active Protein Kinase C. Journal of Virology, 1991; 65(12):6838-44.

Ding ZS, Jiang FS, Chen NP, Lv GY, Zhu CG. Isolation and Identification of an Anti-tumour Component from Leaves of Impatiens balsamina. Molecules, 2008; 13:220-29.

Farnsworth NR, Cordell GA. A review of som biologically active compounds isolated from plants as reported om 1974-1975 literature. Lloydia, 1976; 39: 420-55.

Fields AP, Gustafson WC. 2003. Protein Kinase C in disease: Cancer. In: Newton AC, ed. Methods in molecular biology, Protein Kinase C protocols. New Jersey, USA: Human Press. 56.

John EL, Thomas JS, Murray WF. Isolation and antifungal action of naturally occurring 2-methoxy-1,4-napthoquinone. Biological Chemistry, 1948; 174:335-42.

Liew K, Phelim YVC, Lim YM, Navaratnam V, Anthony HSH. 2-Methoxy-1,4-Naphthoquinone (MNQ) suppresses the invasion and migration of a human metastatic breast cancer cell line (MDA-MB-231). Toxicology in Vitro, 2014; 28:335-339.

Lim YM, Norhanom AW, Nordin HL, Abdul MA. Chemopreventive property of phytosterols and maslinic acid extracted from Coleus tuberosus in inhibiting the expression of EBV-early antigen in Raji cells. Chemistry Biodivesity, 2010; 7:1267-75.

Lim YM, Wong TY, Yap WH, Khoo KS, Lim SH, Yeo CC. Suppressive Effect of Maslinic Acid on PMA-induced Protein Kinase C in Human B-Lymphoblastoid Cells. Asian Pacific Journal of Cancer Prevention, 2012; 13:1177-82.

Mackay HJ, Twelves CJ. Protein Kinase C: a target for anticancer drugs? Endocrine-Related Cancer, 2003; 10:389-96.

Mori N, Toume K, Arai MA, Koyano T, Kowithayakorn T, Ishibashi M. 2-methoxy-1,4-naphthoquinone isolated from Impatiens

balsamina in a screening program for activity to inhibit Wnt signaling. J Nat Med, 2011; 65(1):234-36.

Morrow TA, Muljo SA, Zhang J, Hardwick JM, Schlissel MS. Pro-B-cell-specific transcription and proapoptotic function of protein kinase Cη. Molecular and Cellular Biology, 1999; 19(8):5608-18.

Nishizuka Y. The role of Protein Kinase C in cell surface signal transduction and tumour promotion. Nature, 1984; 308:693-8.

Ooka T, de Turenne M, de The G, Daillie J. Epstein-Barr virus-specific DNase activity in nonproducer Raji cells after treatment with 12-O-Tetradecanoylphorbol-13-Acetate and sodium butyrate. Journal of Virology, 1984; 49(2):626-28.

Prades J, Vogler O, Alemany R, Gomez-Florit M, Funari SS, Ruiz-Gutierrez V, Barcelo F. Plant pentacyclic triterpenic acids as modulators of lipid membrane physical properties. Biochim Biophys Acta, 2011; 1808:752-60.

Promega, 2009. PepTag Assay for Non-Radioactive detection of Protein Kinase C or cAMP-Dependent Protein Kinase. Madison, Wisconsin, USA: Promega. 4-18.

Rodriguez S, Wolfender JL, Hakizamungu E, Hostettmann K. An antifungal naphthoquinone, xanthones and secoiridoids from Swertia calycina. Planta Medica, 1995; 61(4):362-64.

Ruddon RW. 2007. Cancer Biology. New York: Oxford University Press.

Sharkey NA, Leach KL, Blumberg PM. Competitive inhibition by diacylglycerol of specific phorbol ester binding. Proc Natl Acad Sci, 1984; 81:607-10.

Tan SY. Differential Protein Expression in K562 Following Exposure to MNQ isolated from Impatiens balsamina, Linn. Master Thesis, Universiti Tunku Abdul Rahman, Malaysia, 2011.

Teng OE. Isolation and charactersation of a ctyototix napthoquinone from Impatiens balsamina. Master thesis, Universiti Tunku Abdul Rahman, Malaysia, 2010.

Yang X, Summerhurst DK, Koval SF, Ficker C, Smith ML, Bernards MA. Isolation of an antimicrobial compound from Impatiens balsamina L. using bioassay-guided fractionation. Phytotherapy Research, 2001; 15(8):676-80.

Yao QY, Ogan P, Rowe M, Wood M, Rickinson AB. Epstein-Barr virus-infected B cells persist in the circulation of acyclovir-treated virus carriers. International Journal of Cancer, 1989; 43(1):67-71.

Yap WH, Wong TY, Paul LVH, Khoo KS, Lim SH, Yeo CC, Lim YM. Cancer chemopreventive activity of Maslinic acid:P suppression of COX-2 expression and inhibition of NF-κB and AP-1 activation in Raji cells. Planta Medica, 2011; 77:152-157.

Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nature Reviews Cancer, 2004; 4:757-68.

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