Elucidation of the Roles of Cyclooxygenase-2 and Prostaglandin E_2 in Human Esophageal Squamous Cell Carcinoma

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ABSTRACT

Abstract of the thesis entitled

Elucidation of the roles of cyclooxygenase-2 and prostaglandin E_2 in human esophageal squamous cell carcinoma

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Overexpression of cyclooxygenase-2 (COX-2) and elevation of its product prostaglandin E_2 (PGE_2) are implicated in the pathogenesis of human esophageal squamous cell carcinoma. COX-inhibitors also have been demonstrated to overcome multidrug resistance (MDR) in some cancer cells. Therefore, our studies are designed to investigate the role of COX-2 and PGE_2 in cell proliferation and drug resistance on human esophageal squamous cell carcinoma cells.

PGE_2 stimulated cell proliferation of HKESC-1, a human esophageal squamous cell carcinoma cell line. PGE_2 exerts its effects through four subtypes of G-protein-coupled receptors, namely EP1 to EP4. In this regard, we showed that all four EP receptor subtypes were expressed in a panel of human esophageal squamous cell carcinoma cell lines (HKESC-1, HKESC-2, HKESC-3, KYSE150, and EC109). Further characterization by pharmacological and RNA interference approaches revealed that EP2 receptor mediated the mitogenic effect of PGE_2 in HKESC-1 cells. EP2 receptor agonist butaprost mimicked the mitogenic effect of PGE_2, whereas knockdown of the EP2 receptor attenuated the PGE_2-induced proliferation. In relation to the signaling mechanism, PGE_2 and butaprost induced phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), whose down-regulation by RNA interference significantly attenuated PGE_2-induced cell proliferation. Moreover, ERK1/2 activation by PGE_2 was completely abolished by protein kinase C (PKC) inhibitor, Ro-31-8425. In addition, PGE_2 and butaprost increased c-Fos expression and activator protein-1 (AP-1) transcriptional activity, which were abolished by the

ERK1/2 kinase inhibitor, U0126. AP-1-binding inhibitor, curcumin, also partially reversed the mitogenic effect of PGE_2. Apart from c-Fos, PGE_2 also increased c-Myc expression and its association with the binding partner Max. Knockdown of c-Myc by RNA interference attenuated PGE_2-induced cell proliferation. Further mechanistic study revealed that PGE_2 increased the protein stability and nuclear accumulation of c-Myc via phosphorylation on serine 62 in an ERK1/2-dependent manner. Moreover, the effect of PGE_2 on c-Myc expression was mimicked by butaprost. These findings suggest that PGE_2 promotes cell proliferation via EP2/PKC/ERK-dependent induction of c-Fos and c-Myc expression in human esophageal squamous cell carcinoma cells.

In the event to assess the involvement of COX in cancer cell drug resistance, different COX-inhibitors (indomethacin, SC236, SC560, nimesulide and NS398) were employed. They all substantially suppressed PGE_2 production to a similar extent. However, only the non-selective COX inhibitor indomethacin and the COX-2 selective inhibitor SC236 enhanced cytotoxic effects of doxorubicin on HKESC-1 and HKESC-2 cells, and these effects could not be reversed by the addition of PGE_2. Knockdown of COX-2 also failed to mimic the enhancing effect of indomethacin or SC236 on cytotoxicity, implicating that their action is COX- and PGE_2-independent. To this end, we observed that indomethacin and SC236 directly functioned as non-competitive inhibitors of P-glycoprotein (P-gp), which were manifested as reduction of P-gp ATPase activity. Collectively, these findings suggest that the direct inhibitory effect of indomethacin and SC236 may contribute to their ability to increase the intracellular retention of doxorubicin and thus enhance its cytotoxicity.

In summary, this study demonstrates for the first time that PGE_2 promotes human esophageal squamous cell carcinoma cell proliferation mainly through the EP2 receptor. Moreover, the activation of AP-1 and c-Myc through the PKC/ERK pathway is required for the mitogenic action of PGE_2. On the other hand, non-selective COX inhibitor indomethacin and COX-2 selective inhibitor SC236 enhance the cytotoxicity of doxorubicin via direct inhibition of P-gp ATPase activity. Our findings support the growth-promoting action of PGE_2 in esophageal squamous

cell carcinoma and the potential application of EP2 receptor antagonists in the treatment of this disease. In addition, the study also suggests that combination of indomethacin or SC236 with doxorubicin may have significant clinical application, especially in the circumvention of P-gp-mediated MDR in cancer cells.

论文摘要

环氧酶 2 (COX-2)过表达和其产物前列腺素(PGE_2)水平的提高在人食管磷癌的发病机理中起一定的作用. 环氧酶抑制剂也被证明可以克服一些肿瘤细胞的多药抗药性(MDR). 因此,我们设计试验研究 COX-2 和 PGE_2 在人食管鳞癌细胞增殖和抗药性中的作用.

PGE_2 刺激 HKESC-1 细胞的增殖. HKESC-1 是一人食管磷癌细胞株. PGE_2 通过四个亚型的 G 蛋白耦联受体(EP1, EP2, EP3, 和 EP4)发挥起功能. 我们检测了五株人食管鳞癌细胞株(HKESC-1, HKESC-2, HKESC-3, KYSE150, 和 EC109), 发现被检测的细胞株都表达所有的受体亚型. 通过药理学和 RNA 干扰的方法进一步研究发现 EP2 受体亚型介导 PGE_2 的促增殖作用. EP2 受体的激动剂 butaprost 摹拟了 PGE_2 的促增殖作用, 并且调低 EP2 受体的表达可削弱 PGE_2 的促增殖作用. 在信号转导机理方面, PGE_2 和 butaprost 引起了细胞外信号调控激酶 1/2(ERK1/2)磷酸化. 调低 ERK1/2 可显著的削弱 PGE_2 引起的细胞增殖. 并且, 蛋白激酶 C(PKC)的抑制剂 Ro-31-8425 可完全阻断 PGE_2 引起的 ERK1/2 活化. 此外, PGE_2 和 butaprost 可增加 c-Fos 的表达和激活蛋白 1(AP-1)的转录活性. 这些作用可被 ERK1/2 激酶的抑制剂 U0126 所阻断. 同时, AP-1 抑制剂姜黄素也可部分阻断 PGE_2 的促增殖作用. 除了增加 c-Fos 的表达, PGE_2 也增加了 c-Myc 的表达和它与其起结合蛋白 Max 的结合. 调低 c-Myc 的表达可削弱 PGE_2 引起的细胞增殖作用. 进一步的机理研究发现, PGE_2 可增加 c-Myc 蛋白的稳定性和增加其在细胞核中的聚集. 这些作用通过 ERK1/2 依赖的途径磷酸化 c-Myc 的 62 位丝氨酸而得以实现. 此外, butaprost 可摹拟 PGE_2 的促进 c-Myc 表达的作用. 这些发现提示 PGE_2 促进细胞增殖的作用是通过 EP2/PKC/ERK 依赖的途径促进 c-Fos 和 c-Myc 的表达而实现的.

为了研究 COX 在人食管鳞癌抗药性中的作用,不同的 COX 抑制剂(indomethacin, SC236, SC560, nimesulide, 和 NS398)用于本研究中. 虽然这些抑制剂都相同程度的抑制了 PGE_2 的产生, 只有非选择性的 COX 抑制剂 indomethacin 和选择性的 COX-2 抑制剂 SC236 可以增强阿霉素(doxorubicin)在 HKESC-1 和 HKESC-2 细胞中的细胞毒性作用. 并且, 这一增强作用并不能通过

补充 PGE_2 来逆转. 调低 COX-2 的表达也不能摹拟 indomethacin 和 SC236 的作用. 这些结果说明, indomethacin 和 SC236 对此作用不是通过抑制 COX 和 PGE_2 来实现的. 我们观察到 indomethacin 和 SC236 可直接作为 P-糖蛋白(P-gp)的非竞争性抑制剂, 减少 P-gp ATP 酶的活性. 这些发现提示 indomethacin 和 SC236 对 P-pg 的直接抑制作用可导致阿霉素在细胞内蓄积的增加从而增强其细胞毒性.

综上所述, 本研究首次证明在人食管磷癌细胞中 PGE_2 通过 EP2 受体亚型促进细胞增殖. 并且, 这一促增殖的作用需要激活 PKC/ERK 依赖的 AP-1 和 c-Myc 通路. 另一方面, 非选择性的 COX 抑制剂 indomethacin 和选择性的 COX-2 抑制剂 SC236 通过直接抑制 P-gp ATP 酶活性增强阿霉素的细胞毒性作用. 我们的发现支持 PGE_2 在人食管磷癌中的促进细胞增殖的作用, 并且提示 EP2 受体的抑制剂有潜力应用于该病的治疗. 此外, 本研究也提示 indomethacin 或者 SC236 与阿霉素联合应用可能有重要的临床意义, 特别是对于 P-gp 介导的肿瘤细胞多药抗药性.

Declaration

I declare that this thesis represents my own work, except where due acknowledgement is made, and that it has not been previously included in a thesis, dissertation or report submitted to this University or any other institution for a degree, diploma or other qualification.

Signed YU Le

YU Le

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Publications

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3. **Yu L**, Wu WK, Li ZJ, Li HT, Wu YC, Cho CH (2009) Prostaglandin E_2 promotes cell proliferation via activation of PKC/extracellular signal regulated kinase pathway dependent induction of c-Myc expression in human esophageal squamous cell carcinoma cells. Int J Cancer (Revised)

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Abbreviations

Akt	Protein kinase B
AP-1	Activator protein-1
BSA	Bovine serum albumin
cAMP	Adenosine-,3-5-cyclic monophosphate
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal gowth factor receptor
ELISA	Enzyme linked immunosorbance assay
EP	E series of prostaglandin
ERK	Extracellular-signal regulated kinase
FBS	Fetal bovine serum
IBMX	1-methyl-3-isobutylxanthine
IC50	Half maximal inhibitory concentration
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase

MDR	Multidrug resistance
MEK	Mitogen-activated protein kinase kinase
MRP	Multidrug resistance associated protein
MTT	3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
$NaVO_4$	Sodium orthovanadate
NF-κB	Nuclear factor κB
NSAIDs	Nonsteroidal anti-inflammatory drugs
p38 MAPK	p38 MAP kinase
PBS	Phosphate-buffered saline
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGHS	Prostaglandin H synthase
PGI_2	Prostacyclin
P-gp	P-glycoprotein
PKC	Protein Kinse C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonyl fluoride
RIPA	Radioimmunoprecipitation assay buffer

RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
Tris	Tis-(hydrooxymethyl)aminoethane
TXA_2	Thromboxane A_2

Chapter 1

Introduction

1.1 Esophageal squamous cell carcinoma

1.1.1 Epidemiology and etiology

Esophageal cancer, an aggressive malignant disease with dismal prognosis, is the sixth leading cause of cancer-related death in the world (Tew et al., 2005). There are two major histological types of esophageal cancer, squamous cell carcinoma and adenocarcinoma, each of which has distinct etiological and pathological characteristics. Both types of esophageal cancer remain equally virulent. Although the incidence of adenocarcinoma is continuously increasing both in the United States and in Northern and Western Europe, more than 90% of esophageal cancers worldwide are squamous cell carcinoma (Blot and McLaughlin, 1999; Stoner and Gupta, 2001). In China, esophageal squamous cell carcinoma represents more than 99% of esophageal cancer cases (Wang et al., 2006). Much rarer malignant tumors of the esophagus include small cell carcinoma, mucoepidermoid carcinoma, adenoid cystic carcinoma, and carcinosarcoma, as well as other sarcomas, carcinoid tumors, lymphomas, and metastases from other primary sites (Lieberman et al., 1994; Elton, 2005). In the present study, we focused on squamous cell carcinoma.

Esophageal squamous cell carcinoma incidence rates overall vary greatly worldwide. The highest rates in the world have been reported in central China (Henan and Shanxi Provinces) and in so-called 'central Asian esophageal cancer belt' (Sepehr et al., 2001). The incidence of esophageal squamous cell carcinoma is higher in males than females worldwide. The male-to-female ratio varies, in different

studies, from 2:1 to as high as 20:1 (Liu and Crowford, 2004). Although occasional cases are found in patients in their thirties, the incidence of this disease increases with age, with the highest rates in patients over 70. The mortality rate for esophageal squamous cell carcinoma remains high because most patients are diagnosed at a late stage of the disease. With advances in endoscopic detection and in surgical and medical treatments, the prognosis of this malignancy has slowly improved. However, the five-year overall survival rate (12-15%) remains dismal (Tew et al., 2005; Holmes and Vaughan, 2007).

The pathogenesis of esophageal squamous cell carcinoma is a multifactorial and multistep process. The occurrence and development of this malignant disease is a result of interactions between environmental and genetic factors. Table 1.1 lists the known environmental risk factors for esophageal squamous cell carcinoma. Excessive use of tobacco has been suggested as a principal factor in the etiology of esophageal squamous cell cacinoma. Several tobacco constitutes, including nitrosamines, polycyclic aromatic hydrocarbons, aromatic amines, various aldehydes and phenols, may be causally associated with this disease (Wynder and Bross, 1961; Tuyns, 1982; Hecht and Stoner, 1996). Alcohol consumption can further increase the risk of esophageal squamous cell carcinoma in the esophagus of cigarette smokers. In addition, the presence of carcinogens, such as fungus-contaminated and nitrosamine-containing foodstuffs, also contributes to the incidence of this disease (Stoner and Gupta, 2001; Liu and Crawford, 2004).

Apart from environmental factors, molecular studies of esophageal squamous

cell carcinoma have revealed frequent genetic abnormalities. Genetic alterations that are commonly related to this malignancy include p53 point mutations (Hollstein et al., 1991; Gao et al., 1994), loss of p16MST1 (Xing et al., 1999) and reduced Rb expression (Jiang et al., 1993), amplification of cyclin D1, HST-1, EGFR and INT-2 (Jiang et al., 1993; Hollstein et al., 1988; Lu et al., 1988; Guo et al., 1993), elevations in iNOS, hTERT, BMP-6, COX-2 and c-Myc expression and cytoplasmic beta-catenin levels (Lu et al., 1988; Guo et al., 1993; Tanaka et al., 1999; Hiyama et al., 1999; Raida et al., 1999; Zimmermann et al., 1999; Kimura et al., 1999). These alterations often lead to altered DNA repair, cell proliferation, apoptosis, loss of cell cycle control, and deregulation of cell signaling cascades, which finally contribute to the growth and metastatic potential of the tumors (Lam, 2000; Mandard et al., 2000). In addition, loss of heterozygosity on chromosomes 1p, 3p, 4, 5q, 9, 11q, 13q, 17q, 18q have frequently found in this malignancy (Mandard et al, 2000; Lu, 2000; Stoner and Gupta, 2001). The role of COX-2 and its product prostaglandin E_2 (PGE_2) will be discussed in detail in the following sections.

Table 1.1 Environmental factors associated with the development of squamous cell carcinoma of the esophagus (Liu and Crawford, 2004)

Dietary
Deficiency of vitamins (A, C, riboflavin, thiamine, pyridoxine)
Deficiency of trace elements (zinc, molybdenum)
Fungal contamination of foodstuffs
High content of nitrites/nitrosamines
Betel chewing
Lifestyle
Burning-hot beverages or food
Alcohol consumption
Tobacco use
Urban environment

1.2 Prostaglandin E_2 and esophageal squamous cell carcinoma

1.2.1 COX-2: A target for prevention and treatment

Compelling epidemiological evidence shows that regular or occasional use of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) is associated with a lower risk for esophageal squamous cell carcinoma (Corley et al., 2003; Altorki, 2003). In addition, experimental and preclinical evidence also suggests a possible preventive or therapeutic benefit of aspirin or other NSAIDs in this malignant disease. For instance, aspirin treatment leaded to significant growth inhibition of ten esophageal cancer cell lines in a time and dose dependent manner (Li et al., 2000). Also, another NSAID indomethacin not only prevented the occurrence of esophageal tumors induced by carcinogen, but also abrogated the development of already committed esophageal tumors in animal models (Rubio, 1984; Rubio, 1986; Altorki, 2003). Taken together, these evidences suggest a potential role for NSAIDs in the prevention, and possibly treatment, of esophageal cancer.

The anti-cancer properties of NSAIDs are thought to be mediated mainly through the inhibition of the cyclooxygenase (COX), which exists in two isoforms commonly referred to as COX-1 and COX-2. COX-1 is constitutively expressed in nearly all tissues and is thought to play a "housekeeping" role. COX-2, in contrast, is an immediate-early response gene product normally absent from most cells but highly inducible in response to inflammatory cytokines, growth factors, and tumor promoters (Dubios et al., 1998). In studies of human esophageal squamous cell carcinoma, aberrant up-regulation of COX-2 expression has been reported to occur

as early as at the stage of dysplasia and in over two thirds of cases of carcinoma *in situ* and invasive carcinoma, whereas COX-2 is weakly expressed, if at all, in normal squamous esophageal epithelium (Zhi et al, 2006). Moreover, COX-2 expression is increasingly upregulated from low grade to high grade dysplasia compared with normal esophageal epithelium in the esophagus (Shamma et al., 2000). However, normal and cancerous esophageal tissues express similar amounts of COX-1 (Zimmermann et al., 1999). Collectively, these data suggest that COX-2 but not COX-1 is involved in esophageal carcinogenesis.

The crucial role of COX-2 in the development of esophageal squamous cell carcinoma was further supported by experimental and preclinical evidences. For example, COX-2 selective inhibitors exerted inhibitory effect on cell proliferation, induced cell cycle arrest, or induced apoptosis on human esophageal squamous cell carcinoma cells *in vitro* (Zimmermann et al., 1999; Yu et al, 2004; Kase et al., 2004; Zhi et al., 2006). Moreover, COX-2 selective inhibitors reduced tumor multiplicity or inhibited tumor development in carcinogen-induced esophageal tumors in rats (Li et al., 2001; Li et al., 2002; Stoner et al., 2005). In addition to pharmacological methods, knockdown of COX-2 expression in a human esophageal squamous cell carcinoma cell line using a specific COX-2 siRNA dramatically inhibited cell growth and, more importantly, colony formation and tumorigenesis in nude mice (Zhi et al., 2006).

COX is the key enzyme for the conversion of arachidonic acid to prostaglandin (PG) G_2 and PGH_2. PGH_2 is substantially converted to a variety of prostanoids,

which include PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2 by each respective prostaglandin synthase (Figure 1.1). One of the prostanoids produced at high levels in the tumor microenvironment is PGE_2, which is thought to play a major role in cancer progression (Cha and DuBois, 2007). Its role in the development of esophageal squamous cell carcinoma will be discussed in detail in the following section.

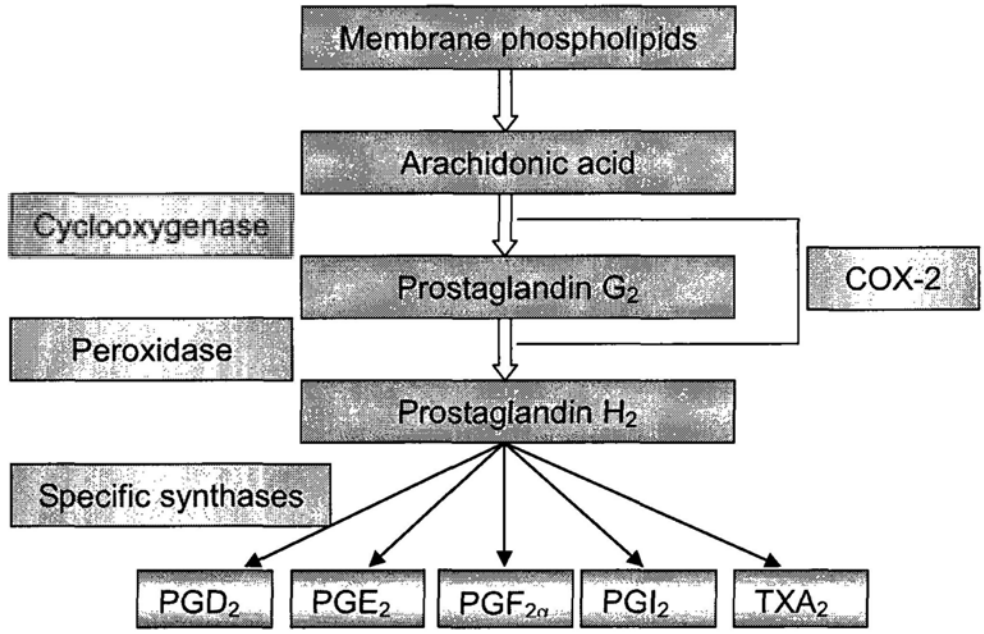


Figure 1.1 Prostaglandin biosynthesis.

1.2.2 Prostaglandin E_2 and E-series of prostaglandin (EP) receptors

Among all prostanoids, PGE_2 has been found to play a crucial role in promoting cancer promotion. For instance, PGE_2 levels are elevated in various human cancers including colon, lung, and esophageal squamous cell carcinoma (Lau et al., 1987; Morgan, 1997; Gupta and Dubois, 2000). Moreover, PGE_2 promoted intestinal adenoma growth (Wang et al., 2004) and reversed NSAID-induced adenoma regression in APC^{min} mice (Hansen-Petrik et al., 2002). Also, PGE_2 significantly enhanced carcinogen-induced colon tumor incidence and multiplicity in rats (Kawamori et al., 2003). Furthermore, COX-2 selective inhibitors have been found to reduce tumor multiplicity in carcinogen-induced esophageal tumors in rats by reducing PGE_2 levels (Li et al., 2001; Li et al., 2002; Stoner et al., 2005). Inhibition of PGE_2 by monoclonal antibody has also been demonstrated sufficient to retard the growth of transplantable lung tumors *in vivo* (Stolina et al., 2000). In addition to PGE_2, to our knowledge, the only other COX derived prostaglandin implication in oncogenesis is TXA_2, which was reported to promote angiogenesis (Pradono et al., 2002; Wang and DuBois, 2006).

Despite the anti-cancer properties of NSAIDs and COX-2 selective inhibitors, their uses as chemoprophylactic or therapeutic agents have been hampered by the potential cardiovascular side effects (Wang et al., 2005). The potential inhibition of endothelial cell derived COX activity and subsequent PGI_2 production may promote platelet aggregation and result in an increased risk of coronary thrombosis and stroke (Mukherjee et al., 2001). Moreover, PGI_2 also exerts protective effects on

cardiomyocytes against oxidative damage (Adderley and Fitzgerald, 1999). Therefore, it becomes attractive to develop inhibitors of PGE_2 signaling that do not inhibit production of other prostanoids, such as the antithrombotic PGI_2. Given that only PGE_2 appears to be procarcinogenic, it is hoped that selective inhibition of PGE_2 signaling may have advantages over COX-2 selective inhibition. It therefore represents a more suitable target for long-term usage.

Prostaglandin E_2 exerts its biological functions by acting on a group of G-protein-coupled receptors (GPCRs). There are four GPCRs responding to PGE_2 designated subtypes EP1, EP2, EP3, and EP4 and multiple splicing variants of the subtype EP3 (Sugimoto and Narumiya, 2007). The EP receptor subtypes exhibit differences in signal transduction (Figure 1.2). The EP1 receptors mediate signaling events by activation of phospholipase C and elevation of cytoplasmic signaling intermediates including inositol triphosphate, diacylglycerol and Ca^{2+}. The EP2 and EP4 receptors coupled to the stimulation of cAMP/protein kinase A (PKA) signaling through the sequential activation of $G_{\alpha s}$ and adenylate cyclase. The EP3 receptors are unique in their ability to couple to multiple G proteins. Their major signaling pathway is inhibition of adenylate cyclase via G_i. Apart from activation of G_i subunits, signaling via EP3 receptors can also activate G_s leading to cAMP production (Dey et al., 2006; Sugimoto and Narumiya, 2007).

Cumulative evidence suggests that PGE_2 promotes tumor growth by binding its receptors and activating signaling pathways which control cell proliferation, migration, apoptosis, and/or angiogenesis. To date, all four subtypes of EP receptors

have been individually knocked out in mice and various phenotypes have been reported for each subtype, which facilitates the characterization of each EP receptor subtype in tumorigenesis. In this respect, EP1- and EP4- receptor-deficient mice were resistant to carcinogen-induced aberrant crypt foci formation in the colon (Watanabe et al., 1999; Mutoh et al., 2002). Disruption of the EP2 receptor also decreases the number and size of intestinal polyps, the intensity of angiogenesis, and vascular endothelium growth factor expression in APC^{min} mice (Sonoshita et al., 2001; Seno et al., 2002). Moreover, EP3 receptor-knockout mice develop less tumor-associated blood vessels due to the reduction of vascular endothelium growth factor expression (Amano et al., 2003). In addition to studies from knockout mice, studies using EP receptor agonists and antagonists have also helped in uncoupling the diverse function of PGE_2 signaling involving distinct EP receptors. For example, the chemopreventive effects of selective EP1 antagonist (ONO-8711) have been demonstrated in breast cancer development, where the administration of ONO-8711 was reported to delay the onset of carcinogen-induced breast tumor in rats (Kawamori et al., 2001). Conversely, treatment with EP1 receptor agonist (ONO-DI-004) enhanced human cholangiocarcinoma cell growth and invasion, which were inhibited by the EP1 receptor small interfering RNA or antagonist ONO-8711 (Han and Wu, 2005). EP4 receptor antagonist (AH23848) significantly inhibited metastasis of breast cancer in mice. Of note, the antimetastatic activity was comparable to that of indomethacin (Kundu and Fulton, 2002; Fulton et al., 2006).

To date, much of the data regarding the role of EP receptors in cancer come

from animal models of colon cancer. Concerning esophageal squamous cell carcinoma, research in this area is much scant. A study, just published in 2009, showed that EP2 overexpression was observed in 43.4% (98/226) of human esophageal squamous cell carcinoma. Moreover, overexpression of EP2 was associated with worse prognosis, and correlated positively with T status. In addition, among those patients at earlier stages, EP2 overexpression significantly disclosed patients at high risks for poor prognosis (Kuo et al., 2009).

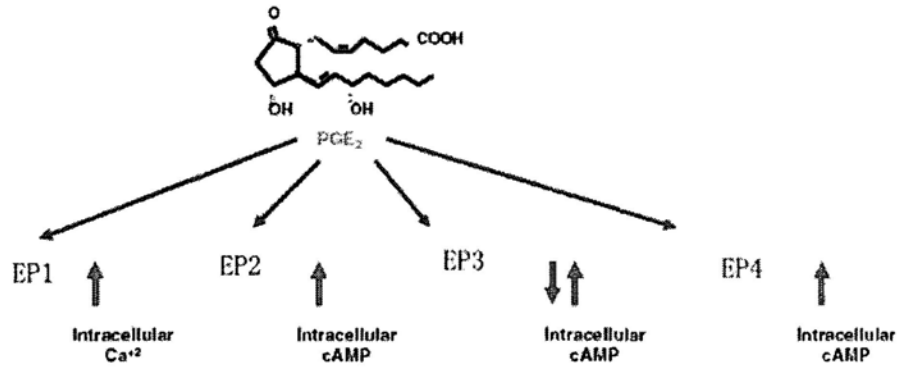


Figure 1.2 PGE_2-EP receptor signaling pathways.

1.3 PGE_2 and cell proliferation

1.3.1 Mitogen-activated protein kinase (MAPK)

Mitogen-activated protein kinase (MAPK) pathways are evolutionarily conserved kinase modules that link extracellular signals to the machinery that controls fundamental cellular processes such as growth, proliferation, differentiation, migration and apoptosis. The activation of MAPKs requires phosphorylation of conserved tyrosine and threonine residues by dual specificity MAPK kinases (MAPKK), which in turn are activated by phosphorylation of two serine residues by upstream MAPKKKs. Three main families of MAPKs exist in mammalian species, grouped by their structures and functions: the extracellular signal-regulated protein kinase 1 and 2 (Erk1/2), the p38 MAPK, and the c-Jun amino-terminal kinase/stress activated protein kinase (JNK/SAPK) (Figure 1.3) (Dhillon et al., 2007). In general, the Erk1/2 pathway is activated by growth factors, whereas the JNK/SAPK and p38 pathways are activated by cellular stress (Reddy et al, 2003).

The Erk pathway is implicated in multiple cellular processes like proliferation, differentiation and survival (Gerits et al., 2007). There is substantial evidence validating the importance of this pathway in cancer progression and in promotion of cancer growth (Shields et al., 2000; Roberts and Der, 2007). The activation of Erk1/2 induces proliferative signals that may contribute to normal and cancerous cell proliferation (Cowley et al., 1994; Mansour et al., 1994). Consistently, deregulation of the Erk pathway is a frequent event in many human types, including esophageal squamous cell carcinoma (Hoshino et al., 1999; Chattopadhyay et al., 2007).

Phosphorylation of Erk1/2 results in their translocation to the nucleus, where they stimulate the activities of many nuclear transcription factors, thus regulating gene expression. For example, activation of Erk1/2 leads to the stimulation of an early response gene c-fos expression by acting on transcription factors bound on the c-fos promoter, such as Elk-1 that is also known as ternary complex factor (Whitmarsh et al., 1996). In addition, activation of Erk pathway can also regulate protein expression at the post-translational level. For instance, the half-life of c-Myc oncoprotein increases markedly in mitogen-stimulated cells, and this stabilization depends on the activation of Erk pathway, which phosphorylates c-Myc protein on Serine 62, leading to increased protein stability (Sears et al., 1999; Sears et al., 2000; Turjanski et al., 2007).

Although phosphorylation of Erk1/2 has been demonstrated to mediate the mitogenic action of PGE_2 in other cancer cell types (Krysan et al., 2005; Cherukuri et al., 2007), whether it shows similar effects in human esophageal squamous cell carcinoma remains unclear.

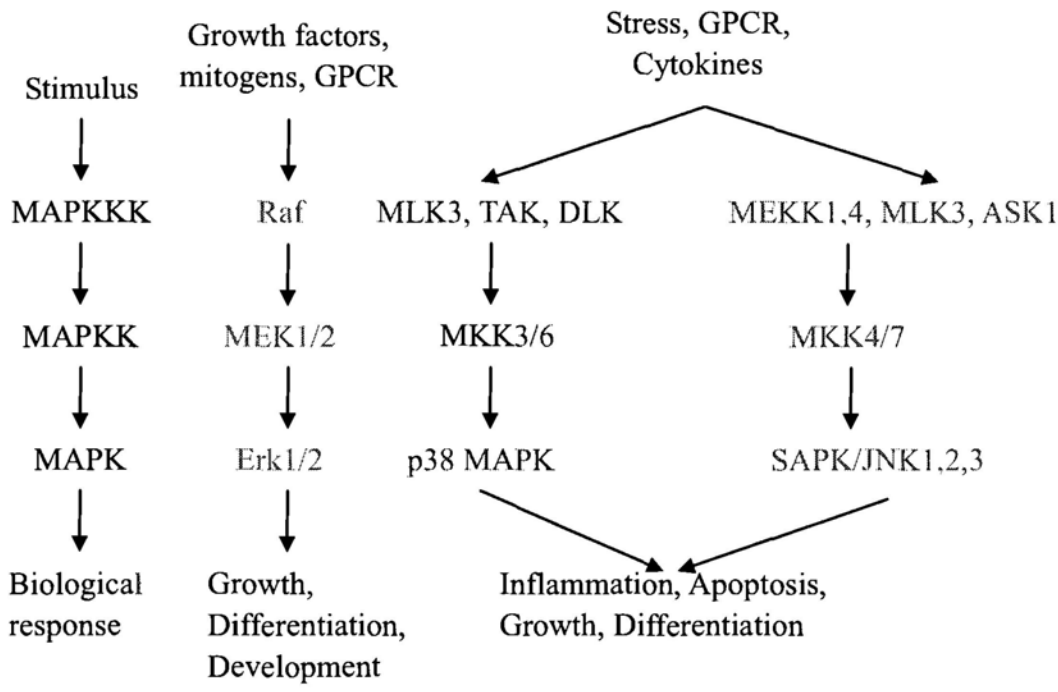


Figure 1.3 Schematic representation of mammalian mitogen-activated protein kinase (MAPK) signaling cascades. The three major MAPK subgroups comprise of extracellular-signal regulated kinase/mitogen activated protein kinase (ERK/MAPK), p38, c-Jun amino-terminal kinase/stress activated protein kinase (JNK/SAPK).

1.3.2 Activator protein 1 (AP-1)

The AP-1 (activator protein 1) transcription factor is composed of heterodimers of Fos (c-Fos, Fos B, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) or homodimers of Jun/Jun (Young et al., 1999). The activation of AP-1 is one of the earliest nuclear events induced by growth factors that stimulate Erk1/2 subgroup of MAPKs. As mentioned previously, the activation of Erk1/2 leads to an increased expression of c-Fos mRNA (Whitmarsh et al., 1996). Thereafter, c-Fos heterodimerizes with Jun proteins to form stable AP-1 dimer and trigger target genes transcription (Shaulian and Karin, 2002).

Fos and Jun proteins were first identified as the viral oncoproteins v-Fos and v-Jun in the Finkel-Biskis-Jinkins osteosarcoma virus and avian sarcoma virus 17, respectively (Vogt, 2002). Identification of c-Fos and c-Jun, the mammalian homologs of the retroviral oncoproteins, immediately linked AP-1 to cancer and neoplastic transformation (Verde et al., 2007). It comes no surprise that elevated AP-1 activity has been frequently documented in various types of human caner (Liu et al., 2002; Young et al., 2003). The regulation of cell proliferation by AP-1 might be of crucial importance for the multi-stage development of tumors (Park et al., 1999; Liu et al., 2002). In this regard, inhibition of Fos and Jun expression in mouse fibroblasts and erythroleukaemia cells by antisense RNA demonstrated their requirement for proliferation and cell cycle progression (Shaulian and Karin, 2001). Similarly, microinjection of antibodies against Fos and Jun prevents serum stimulated quiescent mouse fibroblasts from re-entering the cell cycle (Kovary and

Bravo, 1991). In addition, AP-1 blockade suppressed mitogenic signals from multiple different growth factors like IGF-1, EGF, heregulin-β, and bFGF, and inhibits the growth of breast cancer cells both *in vitro* and *in vivo* by reducing cell proliferation (Liu et al., 2002). With regard to human esophageal squamous cell carcinoma, whether AP-1 is involved in the mitogenic action of mitogens or growth factors is largely unknown.

1.3.3 c-Myc

The proto-oncogene *c-myc* ("cellular myomatosis oncogene"), originally identified as the cellular homologue to the viral oncogene (*v-myc*) of the avian myelocytomatosis retrovirus, encodes a transcription factor that is regarded essential for progression in human malignancies (Cole, 1986). Deregulated expression of c-Myc was subsequently demonstrated in up to 50% of all human cancers (Alitalo and Schwab, 1986; Ponzielli et al., 2005; Arvanitis and Felsher; 2006).

Amplification of *c-myc* and/or deregulated c-Myc expression has been documented in human esophageal squamous cell carcinoma (Lu et al., 1988; Mandard et al., 2000; Stoner and Gupta, 2001). Experimental and preclinical studies also showed a strong association between c-Myc overexpression and the progression of human esophageal squamous cell carcinoma. For example, overexpression of c-Myc plus Bcl-XL converted human immortalized primary esophageal epithelial cells into tumorigenic cancer cell lines whose molecular profiles resembled to those of esophageal squamous carcinomas (Kim et al., 2006). Moreover, suppression of

c-Myc expression by antisense RNA induced terminal differentiation and apoptosis of human esophageal squamous cell carcinoma cells *in vitro*. Heterotransplants of these cells with reduced c-Myc expression into the nude mice also revealed a substantial decrease in tumorigenicity and morphological changes characteristic of terminal differentiation and apoptosis (Zhao et al., 1995). In addition, downregulation of c-Myc by siRNA obviously decreased cell proliferation of esophageal squamous cell carcinoma *in vitro* (Tsuneoka et al., 2005).

c-Myc functions as a transcription factor by dimerizing with its partner protein Max and the dimer subsequently binds to E box sequence elements to activate the transcription of target genes. Max expression is ubiquitous and constitutive whereas c-Myc expression is tightly regulated at the transcriptional, post-transcriptional, and post-translational levels (Sears et al, 1999; Sears, 2004). The expression of c-Myc varies with proliferative states. In this connection, c-Myc expression is virtually undetectable in quiescent cells whilst it can be induced upon the addition of growth factors (Pelengaris et al, 2002). Deletion of *c-myc* by homologous recombination results in a dramatically reduced growth rate (Mateyak et al., 1997; de Alboran et al., 2001). Therefore, c-Myc is thought to contribute to tumorigenesis, at least in part, through unstrained cellular proliferation, which is a fundamental feature of cancer pathogenesis (Oster et al., 2002; Arvanitis and Felsher, 2006). Additionally, transgenic animal model also directly demonstrates that enforced c-Myc expression in either skin or hematopoietic lineages results in neoplastic phenotypes while deactivation of c-Myc triggers spontaneous regression of these neoplastic changes

(Felsher and Bishop, 1999; Pelengaris et al., 1999).

Although both the COX-2/PGE_2 pathway and c-Myc signaling play pivotal roles in the pathogenesis of human esophageal squamous cell carcinoma, the linkage between them in human esophageal malignancy remains obscure.

1.4 Drug resistance

1.4.1 Treatment of esophageal squamous cell carcinoma

Surgery alone remains the standard of care for the treatment of early-stage tumors confined to esophagus and paraesophageal region. For advanced disease, the failure of standard surgical therapy alone is due both to locoregional recurrence and to early systemic dissemination of disease (Ku and Ilson, 2007). Given the unsatisfactory results of surgery or radiotherapy alone and the demonstrated activity of chemotherapy in patients with advanced disease, strategies that combine these three treatment approaches including preoperative chemotherapy, either alone or with radiotherapy, and chemoradiotherapy without surgery have been used in attempts to enhance local control and improve prognosis (Wobst et al., 1998; Enzinger and Mayer, 2003; Stahl et al., 2005). Neoadjuvant chemotherapy utilizing the FAP regimen consisting of 5-FU (5-fluorouracil), cisplatin and adriamycin (doxorubicin) has been reported to be effective in the treatment of advanced esophageal carcinoma (Naritaka et al., 2004; Kosugi et al., 2005; Akita et al., 2006; Yano et al., 2006; Shimakawa et al., 2008). Compared with FP regimen consisting of 5-FU and cisplatin, FAP regimen exhibits much higher response rate and promising long-term outcomes for patients with highly advanced esophageal carcinoma (Kosugi et al., 2005).

In addition, over the past decade, a new understanding of tumor biology and genetics has enabled the development of novel approaches for the targeted treatment of esophageal squamous cell carcinoma. These targeted therapies are in

various phase I or II clinical trials, which include monoclonal antibodies (mAbs) and signal transduction/tyrosine kinase inhibitors (TKIs) for epiderma growth factor receptor (EGFR), mAbs to the HER-2/Neu receptor and vascular endothelial growth factor (VEGF) ligand, oral COX-2 inhibitors, and other novel drugs (Tew et al., 2005).

1.4.2 Cellular mechanisms of drug resistance

Chemotherapeutics are the most effective treatment for metastatic cancers. However, drug resistance remains a major obstacle to the success of chemotherapy in many cancers, which has been reported to cause treatment failure in over 90 % of patients with metastatic cancer (Longley and Johnston, 2005). Moreover, drug resistance has been described as the single most common reason for discontinuation of a drug (Hurley, 2002). Cancer cells can be intrinsically resistant prior to chemotherapy, or resistance may be acquired during treatment by cancer cells that are initially sensitive to chemotherapy (Kerbel et al., 1994). In the process of acquiring resistance, cancer cells not only become resistant to the drugs originally used to treat them, but may also become cross-resistance to a range of structurally dissimilar and functionally divergent chemotherapies. This phenomenon is known as multidrug resistance (MDR) (Wilson et al., 2006). The cytotoxic drugs that are most frequently associated with MDR are hydrophobic, amphipathic natural products,

such as the taxanes (paclitaxel, docetaxel), vinca alkaloids (vinorelbine, vincristine, vinblastine), anthracyclines (doxorubicin, daunorubicin, epirubicin), epipodophyllotoxins (etoposide, teniposide), topotecan, dactinomycin and mitomycin (Ambudkar et al., 1999; Krishna and Mayer, 2000; Thomas and Coley, 2003).

A number of different mechanisms have been proposed to account for the phenomenon of drug resistance, including: accelerated drug efflux, reduced drug uptake, activation of detoxifying systems, activation of DNA repair, and evasion of apoptosis. (Figure 1.4) (Gottesman et al., 2002).

Of note, an important issue of MDR is that cancer cells are genetically heterogenous. Although the process that results in uncontrolled cell proliferation in cancer favours clonal expansion, cancer cells that are exposed to anticancer drugs will be selected for their ability to survive and grow in the presence of cytotoxic drugs. Therefore, in any population of cancer cells that are exposed to chemotherapy, more than one mechanism of MDR can be present. This phenomenon has been called multifactorial multidrug resistance (Gottesman et al., 2002).

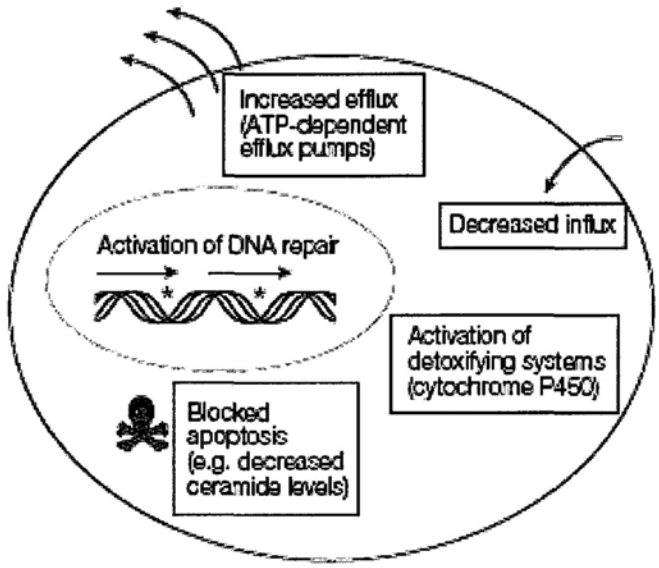


Figure 1.4 Overview of cellular mechanisms of drug resistance in cancer cells. Cancer cells become resistant to anticancer agents by several mechanisms. One way is to accelerate drug efflux by increasing the activity of efflux pump, such as ATP-binding cassette transporters. Alternatively, resistance can occur as a result of decreased drug influx. In cases in which intracellular drug accumulation is unchanged, activation of detoxifying enzymes and DNA repair enzymes can promote drug resistance. Finally, defective apoptotic signaling pathways can also contribute to the development of resistance of cancer cells to anticancer drugs. (From Gottesman et al., 2002. With permission from Nature Publishing Group.)

1.4.3 MDR mediated by ATP-binding cassette transporters

Resistance mechanisms are numerous and complex. Probably one of the most significant forms of resistance against the variety of currently used anti-cancer drugs is by the action of a group of membrane proteins which extrude cytotoxic molecules, keeping intracellular drug concentration below a cell-killing threshold. These membrane proteins belong to the ubiquitous super-family of ATP-binding cassette (ABC) transporters. In humans, 50 ABC transporters have been identified, and classified on the basis of their sequence homology and domain organization into seven distinct subfamilies: ABCA (12 members), ABCB (11 members), ABCC (13 members), ABCD (4 members), ABCE (1 member), ABCF (3 members) and ABCG (6 members).

To date, 13 ABC transporters (ABCA2, ABCB1, ABCB4, ABCB11, ABCC1-6, ABCC11-12, and ABCG2) have been associated with drug resistance and drug transport (Gillet et al., 2007).

1.4.4 MDR mediated by P-glycoprotein

P-glycoprotein (P-gp), the product of the ABCB1 (or MDR1) gene, is definitely the most thoroughly studied ABC transporter. Human P-gp is a large transmembrane glycoprotein with molecular weight approximately 170 kDa consisting of 1280 amino acids organized in two tandem repeats of 610 amino acids joined by a linker region of around 60 amino acids (Chen et al., 1986). The protein appears to have arisen by a gene duplication event, fusing two related half molecules, each consisting

of one transmembrane domain and one ATP-binding site (van Veen et al., 2000; Stavrovskaya, 2000; Hennessy and Spiers, 2007). Radioligand-binding data and functional transport studies show that there are anywhere from two (Raviv et al., 1990; Homolya et al., 1993; van Veen et al., 1998; Loo and Clarke, 1999; Shapiro et al., 1999) to at least four (Martin et al., 2000) substrate-binding sites within the transmembrane domains of P-gp. The ATP-binding sites of P-gp are associated with its ATPase activity. Upon substrate binding, ATPase activity is increased by 3-4-fold (Senior et al., 1995; Martin et al., 1997), and in some cases up to 20-fold (Ambudkar et al., 1992). Two ATP hydrolysis events are required for P-gp to transport one substrate molecule (Senior and Bhagat, 1998). Binding of substrate to the substrate-binding sites in the transmembrane regions stimulates the ATPase activity of P-gp, leading to a conformational change that releases substrate to the extracellular environment (Ramachandra, et al., 1998). Hydrolysis at the second ATP site seems to be required to 're-set' the transporter so that it can return to its original conformation and bind substrate again, completing one catalytic cycle (Sauna and Ambudkar, 2000; Gottesman et al., 2002).

P-gp is expressed constitutively in a broad variety of normal tissues with excretory function (small intestine, liver and kidney) and at blood-tissue barriers (blood-brain barrier, blood-testis barrier and placenta). The physiological function of this transporter is to prevent the uptake of toxic xenobiotics from the gut to the body, and to protect vital structures such as the brain, cerebrospinal fluid, testis, foetus and bone marrow against toxins that enter the body (Fromm, 2004; Hennessy and Spiers,

2007). Furthermore, the analysis of physiological functions of P-gp was greatly simplified by the generation of P-gp knockout mice. In this respect, mice lacking P-gps are normal and fertile as long as they are in a protected environment (Smit et al., 1993; Schinkel et al., 1994; Schinkel et al., 1997). These findings suggest that P-gp has no essential function in physiology other than defending the body against xenotoxins (Borst and Elferink, 2002).

Apart from its advantageous effects on the defense of the body against xenotoxins, P-gp exhibits severe disadvantage by conferring drug resistance to cancer cells. Indeed, P-gp was first described in cancer cells that were resistant to a variety of chemotherapeutic agents as a result of P-gp overexpression (Juliano and Ling, 1976). Cancers that are intrinsically resistant to chemotherapy such as renal, adenocorticoid, hepatocellular, pancreatic and colorectal carcinoma often express or overexpress P-gp (Fojo et al., 1987; Goldstein et al., 1989; Filipits, 2004). With respect to malignancies with only low or no baseline P-gp expression, such as acute myeloid leukemia (AML), breast cancer and small cell lung cancer, P-gp expression has been reported to be induced by chemotherapy (Trock et al., 1997; Mechetner et al., 1998; Filipits, 2004). The most uniform associations between P-gp expression and clinical drug resistance have been reported in AML. P-gp expression occurs in about one-third of AML patients at the time of diagnosis and more than 50% at relapse and correlates with a reduced complete remission rate, shorter duration of survival and higher incidence of refractory disease (Leith et al., 1999; Han et al., 2000; van den Heuvel-Eibrink et al., 2000; Gottesman et al., 2002; Filipits, 2004).

The correlations between P-gp expression and drug resistance have also been suggested in solid tumors (Chan et al., 1990; Gregorcyk et al., 1996; Chan et al., 1997). According to a meta-analysis of studies examining P-gp expression in breast cancer, P-gp expression increased after therapy and was associated with a greater likelihood of treatment failure (Trock et al., 1997).

The most intriguing characteristic of P-gp is its highly promiscuous substrate specificity. It recognizes and transports a plethora of structurally diverse compounds, which explains the cross-resistance to several chemically different compounds, the hallmark of MDR phenotype. The anti-cancer agents that have been reported to be substrates of P-gp include anthracyclines (doxorubicin, daunorubicin, epirubicin), vinca alkaloid (vinblastine, vincristine, vinorelbine), taxanes (paclitaxel, docetaxel), epipodophyllotoxins (etoposide, teniposide), topotecan (Perez-Tomas, 2006; Hennessy and Spiers, 2007).

1.4.5 Strategies to circumvent P-gp-mediated MDR

Downregulation of P-gp expression by gene silencing approaches and inhibition of P-gp activity by pharmacological inhibitors are two major strategies for effectively reversing P-gp-mediated drug resistance in human cancers. In the former, molecular approaches utilizing hammerhead ribozymes (Nagata et al., 2002; Wang et al., 2003; Qia et al, 2005), antisense oligonucleotides (Efferth and Volm, 1993; Quattrone et al., 1994; Alahari et al, 1998; Astriab-Fisher et al., 2000;

Ramachandran and Wellham, 2003; Ren et al., 2004), and RNA interference (Yague et al., 2004; Chen et al., 2006; Shi et al., 2006) have been used to decrease P-gp expression. In this respect, partial and even complete reversals of the MDR phenotype have been reported. Much of recent work has focused on RNA interference, which is based on a sequence specific gene silencing induced by double-stranded RNAs (Izquierdo, 2005). Several studies have showed that RNA interference is a valid approach to circumvent P-gp-mediated drug resistance. In this regard, some *in vitro* and *in vitro* studies showed a complete reverse of the MDR phenotype by using RNA interference to knockdown P-gp expression (Yague et al., 2004; Chen et al., 2006; Shi et al., 2006). Although molecular approaches like RNA interference have great potential in the circumvention of P-gp-mediated drug resistance, numerous limitations remain to be overcome. Problems we need to resolve include achieving longevity of siRNA expression and a sufficient degree of knockdown to obtain a therapeutic effect (Modok, et al., 2006).

Here, we focus on the second strategy mentioned above. Inhibiting P-gp by pharmacological agents as a way of reversing MDR has been extensively studied for around three decades. A large number of agents have been identified and observed to inhibit the transport activity of P-gp including calcium channel blockers (e.g. verapamil), steroidal agents (e.g. aldosterone), protein kinase C inhibitors (e.g., safingol), immunosuppressive drugs (e.g. cyclosporin A), antibiotics (e.g. erythromycin), anti-arrhythmic agents (e.g. quinidine) etc (Hennessy and Spiers, 2007). From a pharmacological perspective, P-gp function may be inhibited as a

result of competitive and non-competitive antagonism. Competitive inhibitors like verapamil act as substrates to compete with cytotoxic agent for transportation by P-gp, limiting the efflux of the cytotoxic agent and increasing its intracellular concentration. Conversely, non-competitive inhibitors like tariquidar bind with high affinity to P-gp but are not themselves substrates, which prevent ATP hydrolysis and transport of cytotoxic agent out the cell, resulting in an increased intracellular concentration (Figures 1.5 and Figure 1.6) (Thomas and Coley, 2003).

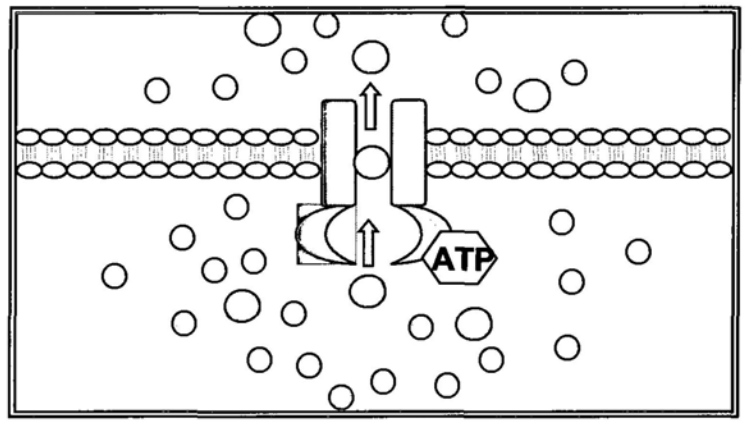


Figure 1.5 Competitive inhibition of the P-gp transporter. Competitive inhibitors compete as substrates with the anticancer drugs for transport by the drug efflux pump. The small balls represent molecules of anticancer drugs; the large balls represent molecules of competitive inhibitors.

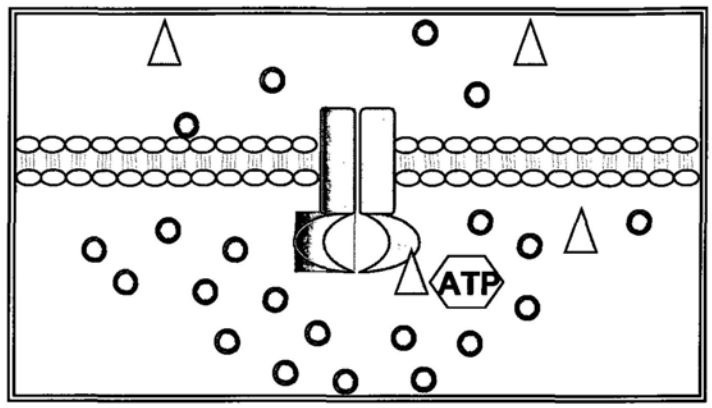


Figure 1.6 Non-competitive inhibition of the P-gp transporter. Non-competitive inhibitors bind with P-gp but are not themselves substrates. This binding induces a conformational change of P-gp, thereby preventing ATP hydrolysis and transport of the anticancer drugs to extracellular environment. The small balls represent molecules of anticancer drugs; the triangles represent molecules of non-competitive inhibitors.

1.4.6 Role of COX-inhibitors on P-gp-mediated MDR

Apart from their chemotherapeutic and chemoprophylactic actions, non-steroidal anti-inflammatory drugs (NSAIDs), including cyclooxygenase-2 (COX-2) selective inhibitors, have been implicated in circumvention of MDR when combining with conventional chemotherapeutic agents (Arunasree et al., 2008; Singh et al., 2008; de Groot et al., 2007; Patrignani et al., 2005; Duffy et al., 1998). Inhibition of P-gp expression by COX-inhibitors has been reported to contribute to this phenomenon.

A causal link between COX-2 and P-gp expression has been reported. In this study, rat COX-2 cDNA was transferred into renal rat mesangial cells via adenovirus. The enforced expression of COX-2 caused enhancement in P-gp expression and functional activity. The COX-2 selective inhibitor NS398 was able to block the COX-2-mediated increase in P-gp expression and function in infected cells, again reinforcing the link between COX-2 and P-gp (Patel et al., 2002). Moreover, compound that increases P-gp expression in isolated mouse brain capillaries from COX-2-expressing wide-type mouse failed to increase P-gp expression in brain capillaries from COX-2-null mice (Bauer et al., 2008). Apart from the results based on molecular biological approaches by which COX-2 was overexpressed or silenced, pharmacological antagonism by using COX-inhibitors also provides evidence that COX-2 may play an important role in regulating P-gp expression. In this regard, COX-inhibitors have been reported to down-regulate

P-gp expression in a variety of cancers, such as chronic myelogenous leukemia, breast cancer, hepatocellular carcinoma, medullary thyroid carcinoma, and childhood ependymoras (Arunasree et al., 2008; Zatelli et al., 2007; Fantappie et al., 2007; Puhlmann et al., 2005; Kim et al., 2004).

In addition to COX-2 dependent mechanism, COX-2 independent mechanisms for COX-inhibitors have also been suggested. For example, COX-2 selective inhibitors, indomethacin heptyl ester and nimesulide, and a non-selective COX-inhibitor, naproxen, reduced P-gp expression in differentiated Caco-2 cells in which COX-2 protein was undetectable by the Western blot analysis (Zrieki et al., 2008).

Till now, there is no report on the effects of COX-inhibitors on cytotoxic action of anti-cancer drugs in human esophageal squamous cell carcinoma. In view of the chemotherapeutic and chemoprophylactic potentials of NSAIDs, including COX-2 selective inhibitors, in human esophageal squamous cell carcinoma (Zimmermann et al., 1999; Corley et al., 2003; Stoner et al., 2005), it is worthwhile to investigate the possible benefit of combining NSAIDs and COX-2 selective inhibitors with conventional chemotherapy. Moreover, there is no definitive conclusion on the mechanisms by which COX-inhibitors enhance P-gp expression and function. In the present study, we addressed these issues in vitro by studying the cytotoxic effects of NSAIDs, COX-1 and COX-2 selective inhibitors in combination with doxorubicin on human esophageal squamous cell carcinoma cells.

1.5 Aims of the present study

Cumulative evidence has shown that overexpression of COX-2 and elevation of its product PGE_2 are implicated in human esophageal squamous cell carcinoma (Morgan, 1997; Zimmermann et al., 1999; Shamma et al., 2000; Zhi et al, 2006;). Epidemiological study also reported that regular or occasional use of aspirin or other NSAIDs is associated with a lower risk for esophageal squamous cell carcinoma (Corley et al., 2003; Altorki, 2003). However, the mechanism(s) by which PGE_2 exerts its deleterious effects in this malignant disease is still obscure. In addition, although COX-inhibitors have been demonstrated to overcome multidrug resistance in some cancer cells (Arunasree et al., 2008; Singh et al., 2008; de Groot et al., 2007; Patrignani et al., 2005; Duffy et al., 1998), research in this area is much rare in relation to human esophageal squamous cell carcinoma.

Therefore, the aims of the present study are:

1. To investigate the expression profile of COX-1, COX-2, EP1, EP2, EP3, and EP4 receptors in human esophageal squamous cell carcinoma cell lines;
2. To determine the effects of PGE_2 on cell proliferation of human esophageal squamous cell carcinoma cells and to explore which EP receptor(s) mediate(s) the mitogenic action of PGE_2;
3. To elucidate the molecular mechanism by which PGE_2 exerts its mitogenic action on human esophageal squamous cell carcinoma cells;
4. To investigate the effects of COX-inhibitors on cytotoxic effects of

chemotherapeutic drug (doxorubicin);

5. To elucidate the mechanisms by which NSAID indomethacin and COX-2 selective inhibitor SC-236 enhance the cytotoxic action of doxorubicin.

Chapter 2

Materials and Methods

2.1 Chemicals

2.1.1 Reagents and drugs

Name	*Company*	*Catalog number*
40 % Acrylamide	Bio-Rad	161-0140
Agarose	Invitrogen	15510-019
α-Cholic acid	Sigma	C1129
Ammonium persulfide	Bio-Rad	161-0700
Aprotinin	Sigma	A1153
2% Bis-acrylamide	Bio-Rad	161-0142
Biodegradable scintillation fluid	GE Healthcare	NBCS104
Bisindolylmaleimide I	Calbiochem	203290
Blue/Orange loading dye	Promega	G1881
Bovine serum albumin (BSA)	Sigma	A3350
Bromophenol blue	Sigma	B8026
Butaprost	Cayman	13740
(±)-15-deoxy-16S-hydroxy-17-cyclobutyl PGE_1 methyl ester		
Calcium chloride ($CaCl_2$)	BDH	10070
Chloroform	Merck	2442
Curcumin	Sigma	C7727
D-Glucose	BDH	28450

Dimethyl sulphoxide (DMSO)	Sigma	D8418
3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl -2H-tetrazolium bromide (MTT)	Sigma	M2128
Dipotassium hydrogen orthophosphate (K_2HPO_4)	BDH	104363A
Disodium hydrogen orthophosphate (Na_2HPO_4)	BDH	102494C
Dithiothreitol (DTT)	US Biochemicals	US15397
Doxorubicin	Sigma	D1515
Ethanol	Merck	986
Ethidium bromide	Sigma	E8751
Ethylene diamine tetraacetic acid (EDTA)	Sigma	ED-2S2
Fetal bovine serum (FBS)	Invitrogen	10437-028
Forskolin	Sigma	F3917
Glycerol	Sigma	G8773
Glycine	Bio-Rad	160-0717
Ham F-12	Invitrogen	21700-026
Hybond C nitrocellulose membrane	GE Healthcare	RPN203G
Hydrochloride acid (HCl)	Merck	9970
1- Methyl-3-isobutylxanthine (IBMX)	Sigma	I7018
Indomethacin	Sigma	I7378
1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-		

3-indoleacetic acid		
Isopropanol	Merck	9634
Leupeptin	Sigma	L7920
MEM	Invitrogen	41500-034
2-Mercaptoethanol	Sigma	M3148
Methanol	Merck	6009
Nimesulide	Sigma	N1016
N-(4-Nitro-2-phenoxyphenyl)methanesulfonamide		
NS398	Calbiochem	123653-11-2
N-(2-Cyclohexyloxy-4-nitrophenyl)methanesulfonamide		
ONO-AE3-208	ONO	none
2-(2-(2-methyl-2-naphth-1-ylacetylamino)-phenylmethyl)-benzoic acid		
ONO-AE3-240	ONO	none
2-[2-{[4-methyl-2-(1-naphthyl)pentanoyl]amino}-4-(1H-pyrazol-1-ylmethyl)benzyl]benzoic		
ONO-DI-004	ONO	none
17S-17,20-dimethyl-2,5-ethano-6-oxo PGE_1		

Paraffin (Tissue Prep)	Fisher Scientific	T565
Penicillin G	Sigma	P3032
Pepstatin A	Sigma	P5318
Phenylmethylsulphonyl fluoride (PMSF)	Sigma	P7626
Phorbol 12-myristate 13-acetate (PMA)	Sigma	P1585
Potassium chloride (KCl)	BDH	295944B
Potassium dihydrogen orthophosphate (KH_2PO_4)	BDH	102034B
PGE_1 alcohol	Cayman	13020
Prostaglandin E_2 (PGE_2)	Sigma	P0409
Protein A agorose	Upstate	16-156
Protein G agorose	Upstate	16-266
Ready-Load™ 1 kb DNA ladder	Invitrogen	12308-011
Recombinant human epidermal growth factor (EGF)	Sigma	E9644
Ro-31-8425 2-[8-(Aminomethyl)-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide, HCl	Calbiochem	557514
RPMI 1640	Invitrogen	23400-021
SC236 (4-[5-(4-Chlorophenyl)-3-(trifluoromethyl)	Pharmacia	565605CB

-1H-pyrazol-1-yl]benzenesulfonamide)		
SC560 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)- 3-trifluorom-ethylpyrazole	Cayman	703430
SeeBlueTM prestained standards	Invitrogen	LC5625
Sodium carbonate (Na_2CO_3)	BDH	102405Y
Sodium chloride (NaCl)	BDH	10241AP
Sodium dihydrogen orthophosphate (NaH_2PO_4)	BDH	301324Q
Sodium dodecyl sulfide (SDS)	Sigma	L3771
Sodium fluoride (NaF)	Sigma	S7920
Sodium hydrocarbonate ($NaHCO_3$)	BDH	10247V
Sodium hydroxide (NaOH)	BDH	102524X
Sodium orthovanadate (Na_3VO4)	Sigma	S6508
Streptomycin sulphate	Sigma	S9137
Sulprostone 16-Phenoxy-ω-17,18,19,20-tetranor- prostaglandin E_2-methylsulfonylamide	Cayman	14765
SYBR GreenER qPCR superMix	Invitrogen	11761-500
TBE (Tis-borate-EDTA) buffer	Bio-Rad	161-0733
TEMED (N,N,N',N'-Tetramethyl-ethylenediamine)	Bio-Rad	161-0801

Transfectamin 2000	Invitrogen	11668-027
Trichloroacetic acid (TCA)	Sigma	T9159
Tris/Glycine buffer	Bio-Rad	161-0771
Tris/Glycine/SDS buffer	Bio-Rad	161-0772
Triton X-100	Sigma	T8787
TRIZOL Reagent	Invitrogen	15596-026
Trypsin EDTA	Invitrogen	25200-056
Tween 20	Sigma	P1379
U0126 1,4-Diamino-2,3-dicyano-1,4-bis (o-aminophenylmercapto)butadiene ethanolate	Sigma	U120
Verapamil 5-[*N*-(3,4-Dimethoxyphenylethyl)methyla mino]-2-(3,4-dimethoxyphenyl)-2-isopropy lvaleronitrile hydrochloride	Sigma	V4629

2.1.2 Antibodies, plasmids, siRNA, radioisotopes and commercial kits

Name	Company	Catalog number
Alexa Fluor 488 goat anti-mouse IgG(H+L)	Invitrogen	A11029
AKT antibody	Cell Signaling	9272
β-Actin antibody	Sigma	A5441
Cyclic adenosine monophosphate (cAMP) immunoassay kit	GE Healthcare	RPN225
c-Fos antibody	Cell Signaling	2250
c-Myc antibody	Santa Cruz	SC-42
c-Myc siRNA	Santa Cruz	SC-29226
Control siRNA	Qiagen	1027280
Cyclooxygenase-1 (COX-1) antibody	Santa Cruz	SC-1752
Cyclooxygenase-2 (COX-2) antibody	Cayman	160112
COX-2 siRNA	Qiagen	SI00301525
Dual-Luciferase Reporter Assay System	Promega	E1910
ECL Western blot detection kit	GE Healthcare	RPN2106
EGFR antibody	Santa Cruz	SC-03
EGFR antibody	Upstate	06-129
EP1 receptor antibody	Cayman	101740
EP2 receptor antibody	Cayman	101750
EP2 receptor siRNA	Qiagen	SI02757580

EP3 receptor antibody	Cayman	101760
EP4 receptor antibody	Cayman	101775
ERK antibody	Cell Signaling	9102
ERK1 siRNA	Santa Cruz	SC-29307
ERK2 siRNA	Santa Cruz	SC-35335
Fluorescent-labeled RNA duplex	Invitrogen	2013
Goat anti-Mouse IgG-HRP conjugate	Zymed	81-6520
Goat anti-Rabbit IgG-HRP conjugate	Zymed	62-6120
[^{3}H]Thymidine	GE Healthcare	TRK328
Max antibody	Cell Signaling	4732
MRP1 antibody	Santa Cruz	SC-18835
NF-κB p65 antibody	Santa Cruz	SC-372
Nuclear Extraction Kit	Cayman	10009277
p38 antibody	Cell Signaling	9217
P-Glycoprotein antibody	Santa Cruz	SC-55510
Phospho-c-Myc (Ser 62) antibody	Abcam	Ab51156
Phospho-AKT (Ser 473) antibody	Cell Signaling	9271
Phospho-AKT (Thr 308) antibody	Cell Signaling	9275
Phospho-ERK1/2 antibody	Cell Signaling	9101
Phospho-p38 antibody	Cell Signaling	9215
Phospho-SAPK/JNK antibody	Cell Signaling	9251
Phospho-tyrosine antibody	Upstate	05-321

Platinum PCR Supermix	Invitrogen	11306-016
Prostaglandin E_2 (PGE_2) Enzyme Immunoassay Kit	Assay Designs	900-001
pAP-1(PMA)-luc plasmid	Clonetech	631906
pRL-TK plasmid	Promega	E2241
Protein Assay Kit	Bio-Rad	500-0006
Rabbit Anti-Goat IgG-HRP Conjugate	Zymed	81-1620
Rabbit Anti-Sheep IgG-HRP Conjugate	Upstate	12-342
Thermoscript RT-PCR system	Invitrogen	11146-016

2.2 Cell Culture

Human esophageal squamous cell lines, HKESC-1, HKESC-2 and HKESC-3, were kindly provided by Prof. G. Srivastava (Department of Pathology, The University of Hong Kong). HKESC-1 and HKESC-2 were established from moderately differentiated human esophageal squamous cell carcinoma (Hu et al., 2000; Hu et al., 2002). HKESC-3 was established from a well differentiated human esophageal squamous cell carcinoma (Hu et al., 2002). Another two cell lines, KYSE150 and EC109, were established from poorly differentiated human esophageal squamous-cell carcinoma. KYSE150 was purchased from the Japanese Collection of Research Biosources (Osaka, Japan). EC109 was provided by Cancer Institute Chinese Academy of Medical Sciences (Beijing, China). HKESC-1, HKESC-2 and HKESC-3 were maintained in MEM medium, KYSE150 was maintained in Ham F-12 medium, and EC109 was maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

2.3 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium Bromide (MTT) Assay.

Cell viability was assessed by MTT assay, which depends on the ability of viable cells to reduce the MTT to a colored formazan product. In brief, cells (10^4 cells per well) were seeded in 96-well microculture plates overnight for attachment, and then incubated for 24 h with different concentrations of doxorubicin in the

presence or in the absence of NSAID, specific COX-1 inhibitor, and specific COX-2 inhibitors. In the next step, MTT was added to each well, and the cells were further incubated for 3 h. The colored formazan product was determined photometrically at 570 nm in a multi-well plate reader (Bio-Rad).

2.4 [^{3}H]Thymidine Incorporation Assay

Cell proliferation was assessed as the amount of DNA synthesis by measuring the incorporation of [^{3}H]thymidine into DNA. Briefly, cells (4×10^4 cells per well) were seeded into 24-well plates overnight for attachment, then serum deprived for 24 h and stimulated with PGE_2 or selective EP receptor agonists for another 24 h. To study the effects of antagonists or inhibitors, cells were pretreated with specific antagonists or inhibitors for 1 h prior to treatment with PGE_2. In the next step, 0.5 μCi/ml [^{3}H]thymidine (GE Healthcare, Arlington Heights, IL) was added to each well, and the cells were further incubated for another 4 h. The amount of DNA synthesized was measured by liquid scintillation spectrometry with a beta-counter (Beckman Instruments, Fullerton, CA). The final concentration of vehicle did not exceed 0.2% (v/v) in cell culture medium, which showed no effects on cell proliferation.

2.5 Conventional and Real-Time Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from esophageal squamous-cell carcinoma cells using

TRIzol reagent (Invitrogen, Carlsbad, CA). Two micrograms of total RNA was used to generate the first strand of cDNA by reverse transcription using the ThermoScript^{TM} RT-PCR system (Invitrogen) in accordance with the manufacture's instructions. Specific primers (Table 2.1) were designed to screen the expression of EP1, EP2, EP3, and EP4. Conditions for PCR were 95°C for 5 min, 35 cycles of 94°C for 30 sec, 55-60°C (see below) for 30 sec and 72°C for 1 min. The final extension step was at 72°C for 10 min. The annealing temperature was 58°C for EP1 receptor, 55°C for EP4 receptor, 60°C for EP2, EP3 receptors and β-actin. A negative control which was the PCR reaction without prior reverse transcription was included to exclude PCR amplification of genomic DNA. The PCR products were electrophoresed on 1.2% (W/V) agarose gels containing 0.5 μg/ml ethidium bromide. Gel photographs were then analyzed in a multianalyzer (Bio-Rad, Hercules, CA).

For quantitation of mRNA expression, real-time PCR was performed with an iQ^{TM} Multicolor Real-Time PCR Detection System (Bio-Rad) using the SYBR GreenER^{TM} qPCR Supermix (Invitrogen) as recommended by the manufacturer. Real-time PCR was performed using the specific primer pairs as shown in Table 2.2. PCR conditions were 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s for 45 cycles. The mRNA expression was calculated using the comparative threshold cycle (C_T) method and normalized against expression of β-actin.

2.6 Western blot Analysis

Cells were lysed in radioimmunoprecipitation assay buffer [50 mM Tris-HCl

(pH 7.5), 150 mM sodium chloride, 0.5 % α-cholate acid, 0.1 % SDS, 2 mM EDTA, 1% Triton X-100 and 10% glycerol], containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM NaF, 1 mM Na_3VO_4). After sonication for 30 s on ice and centrifugation for 15 min at 14,000 g at 4°C, the supernatant was collected and protein concentration was determined by assay kit (Bio-Rad) using bovine serum albumin (BSA) as standard. Figure 2.1 shows the standard curve for BSA concentration with a range of 0-8 μg/ml.

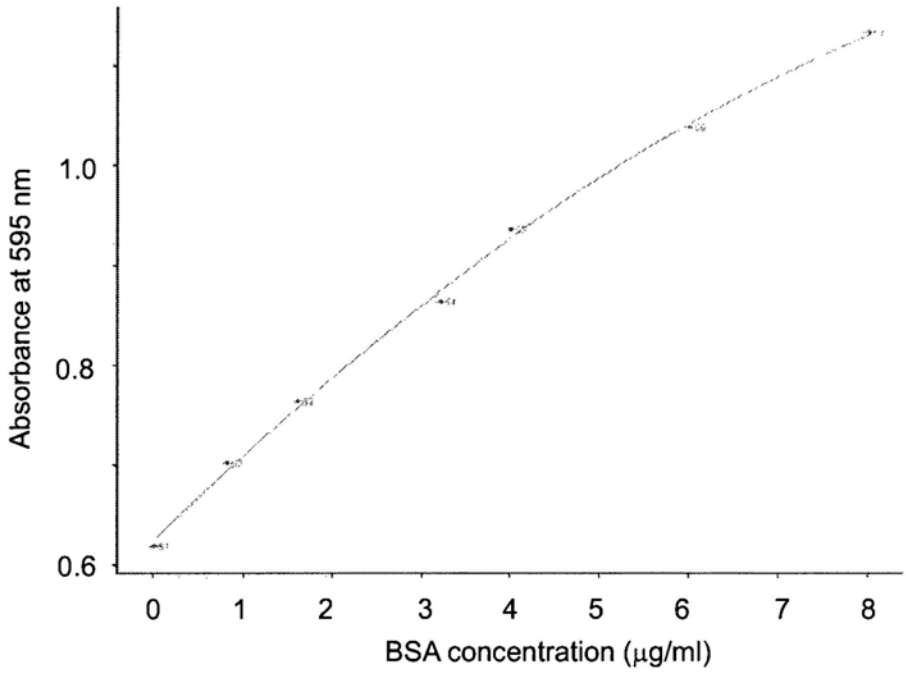


Figure 2.1 Standard curve for protein concentration using BSA as standard. (S1: 0; S2: 0.8; S3: 1.6; S4: 3.2; S5: 4; S6: 6; and S7: 8 μg/ml)

Equal amount of protein (50 μg/lane) were resolved with SDS-polyacrylamide gel electrophoresis, and transferred to Hybond C nitrocellulose membranes (GE Healthcare). The membranes were probed with primary antibodies overnight at 4°C

and incubated for 1 h with secondary peroxidase-conjugated antibody at room temperature. Chemiluminescent signals were then developed with Lumiglo reagent (Cell signaling Technology Inc., Danvers, MA) and detected by the ChemiDoc XRS documentation system (Bio-Rad).

2.7 Immunoprecipitation

Cells were harvested in radioimmunoprecipitation assay buffer containing proteinase and phosphatase inhibitors used in Western blot analysis. Protein extracts were clarified by centrifugation. Supernatant with equal concentrations of proteins were gently rocked with Max or EGFR antibody at 4°C for 1 h. 100 μl of washed Protein G agarose bead slurry (Upstate) were then added to capture immunocomplexes. Protein G-bound immunocomplexes were washed five times with ice-cold PBS and processed for SDS-PAGE and Western blot analysis.

2.8 Nuclear and Cytosolic Extracts

Nuclear and cytosolic extracts were isolated by using the Nuclear Extraction Kit (Cayman) according to the manufacturer's recommendations. Briefly, cells were harvested in 500 μl ice-cold Hypotonic Buffer and incubated on ice for 15 min allowing cells to swell. Then 50 μl of 10% Nonide P-40 was added. After centrifugation for 30 s at 14,000 g at 4 °C, the supernatants containing the cytosolic fraction were collected. The pellets were resuspended in 50 μl ice-cold Extraction Buffer with protease and phosphatase inhibitors. After centrifugation at 14,000 g for

10 min at 4 °C, the supernatants containing the nuclear fraction were collected. The extracted nuclear and cytosolic fractions were then subjected to Western blot analysis.

2.9 Immunofluorescence

Cells grown on petri-dish were fixed with 2 % (v/v) paraformaldehyde for 30 min and then made permeable with ice-cold methanol in freezer for 10 min. The cells were then covered with 10 % (v/v) goat serum (Invitrogen) for 30 min at room temperature to block nonspecific absorption of antibodies to the cells. After this procedure, cells were incubated with primary antibody against c-Myc at 4 °C overnight. Cells were then probed with Alexa Fluor 488 goat anti-mouse secondary antibody (Invitrogen) and incubated at room temperature for 2 h. Finally cells were incubated with 4-6-diamidino-2-phenylindole (DAPI) at room temperature for 10 min. Fluorescent signals were detected using a Nikon ECLIPSE TE2000-E confocal microscope. The immunofluorescence intensities of c-Myc were quantified using the EZ-C1 3.20 image analysis software (Nikon).

2.10 Determination of c-Myc Protein Stability

HKESC-1 cells were treated with 100 μg/ml protein synthesis inhibitor cycloheximide for 30 min prior to PGE_2 stimulation in order to establish the translational block before mitogenic stimulation to the cells. Whole-cell lysates were then extracted at each time point for Western blot analysis.

2.11 RNA Interference

Cells were transiently transfected with small interference RNA (siRNA) oligonucletides by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For each transfection, 20 pmol target-specific siRNA (Erk1, Erk2, EP2, c-Myc and COX-2) or control siRNA were added to each well and incubated at 37 °C for 6 h. The transfection efficiency was optimized by fluorescein-labeled double-stranded RNA (Invitrogen). The efficacy of target genes depletion was verified by Western blot analysis.

2.12 Luciferase Reporter Gene Transcription Assay

Transient transfection with pAP-1 (PMA)-TA-Luc luciferase reporter plasmid (Clontech Laboratories, Mountain View, CA) was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In brief, HKESC-1 cells were transfected with a 10:1 ratio of the pAP-1 (PMA)-luc plasmid and pRL-TK plasmid (Promega, Madison, WI). Cell lysates were then subjected to dual-luciferase reporter assay system, and luciferase activities were measured with a Lumat LB9501 luminometer (Berthold Company, Wildbad, Germany). Firefly luciferase activities were normalized to *Renilla* luciferase activity for transfection efficiency.

2.13 PGE_2 Assay

The measurement of PGE_2 in the cell culture medium was carried out by using

the Correlate-EIA Prostaglandin E_2 Enzyme Immunoassay kit from Assay Designs (Ann Arbor, MI) according to the manufacturer's instruction. Briefly, cells were plated in a 24-well microculture plates in the presence of 10 % serum. At confluence, fresh medium containing 1 % serum in the presence or in the absence of tested NSAID, specific COX-1 inhibitor, or selective COX-2 inhibitors was added. Cells were incubated in medium containing tested compounds for 24 h, after which supernatants were collected for PGE_2 measurement. The PGE_2 level was expressed as pg/ml per μg of protein. Figure 2.2 shows the standard curve for PGE_2 level with a range of 39.1-2500 pg/ml.

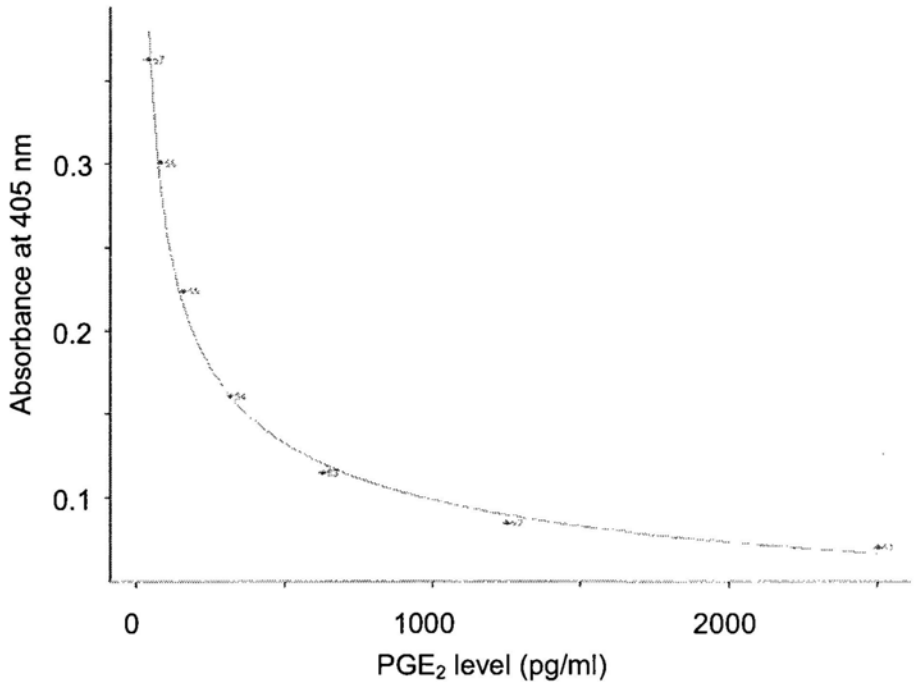


Figure 2.2 Standard curve for PGE_2 level. (S1: 2500; S2: 1250; S3: 625; S4: 313; S5: 156; S6: 78.1; and S7: 39.1 pg/ml)

2.14 Cyclic AMP assay

Intracellular cyclic AMP assay was performed according to the manufacturer's instructions (Amersham Corporation). Briefly, 1×10^6 cells were treated with PGE_2, butaprost, forskolin for 10 minutes in the presence of phosphodiesterase inhibitor IBMX (100 μM) to prevent the breakdown of cAMP. The cAMP level was then measured using a non-acetylation EIA procedure. The cAMP level was expressed as picomoles per milligram of protein. Figure 2.3 shows the standard curve for cAMP level with a range of 12.5-3200 fmol/well.

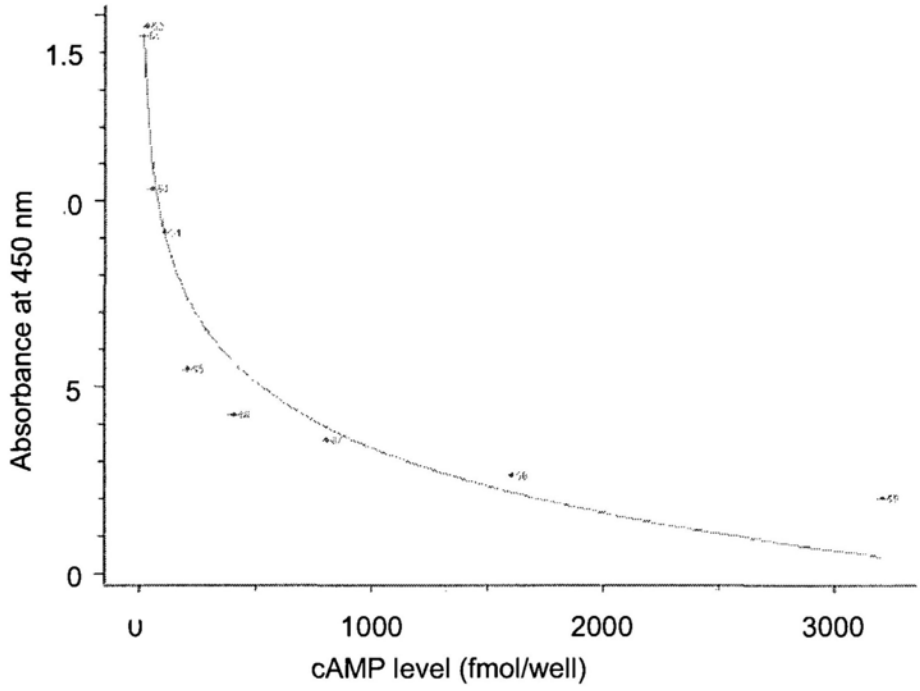


Figure 2.3 Standard curve for cAMP level. (S1: 12.5; S2: 25; S3: 50; S4: 100; S5: 200; S6: 400; S7: 800; S8: 1600; and S9: 3200 fmol/well)

2.15 Doxorubicin Accumulation Assay

Cells were seeded in petri-dish and incubated overnight for attachment after which doxorubicin was added. Cells were incubated in medium containing

doxorubicin for 6 h. Subsequently, the culture medium was removed and cells were washed three times with PBS. Cells were fixed in 4 % paraformaldehyde for 15 min at room temperature, washed three times with PBS. Intracellular doxorubicin fluorescent signals were visualized by using a Nikon ECLIPSE TE2000-E confocal microscope. Doxorubicin fluorescence was excited with an argon laser at 488 nm, and the emission was collected through a 530 nm long-pass filter.

Flow cytometry was used to quantify the intracellular accumulation and retention of doxorubicin. Cells were washed twice in PBS, harvested after trypsin treatment, and washed twice again. Then the doxorubicin fluorescence was measured using Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA). Using the exitation with an argon laser at 488 nm, emission of 10,000 events per sample was detected on FL2 (575 nm).

2.16 P-gp ATPase Assay

Activity of P-gp ATPase in response to doxorubicin and COX-inhibitors was determined by Pgp-Glo^{TM} assay system (Promega, Madison, WI). Following the user protocol provided by the vender, the activity of P-gp ATPase was measured in the presence of verapamil (as a positive conference), doxorubicin, or tested COX-inhibitors. The luminescence of the sample reflects the ATP level in the sample, which is negatively correlated with the activity of P-gp ATPase and was recorded using the Wallac Victor 1420 multilabel counter (PerkinElmer, Monza, Italy). Test compound-treated activities are expressed as percentage of basal activity. By

comparing the basal activity to test compound-treated activities, the compounds can be ranked as stimulating, inhibiting or having no effect on basal P-gp ATPase activity.

2.17 Statistical Analysis

Results were expressed as mean $\pm$ SEM. Statistical analysis was performed with either an analysis of variance (ANOVA) followed by the Turkey's t-test or Student's t-test. P values less than 0.05 were considered statistically significant.

Table 2.1 Oligonucleotide sequence of primers used for RT-PCR

Name and Gene Bank Number	Primer sequence	Fragment size (bp)
EP1 receptor (NM_000955.2)	Forward: CCAATGCTGGTGTTGGTGGC Reverse: AGGGTGGGCTGGCTTAGTCG	314
EP2 receptor (NM_000956.2)	Forward: CCACCTCATTCTCCTGGCTA Reverse: CGACAACAGAGGACTGAACG	216
EP3 receptor (NM_000957.2)	Forward: CTTCGCATAACTGGGGCAAC Reverse: TCTCCGTGTGTGTCTTGCAG	300
EP4 receptor (NM_000958.2)	Forward: AGACGACCTTCTACACGC Reverse: GACGAATACTCGCACCAC	731
β-actin (NM_001101.3)	Forward: AACACCCCAGCCATGTACG Reverse: CGCTCAGGAGGAGCAATGA	623

Table 2.2 Oligonucleotide sequence of primers used for real-time PCR

Name and Gene Bank Number	Primer sequence	Fragment size (bp)
c-Fos	Forward: AGGGCTGGCGTTGTGA	149
(NM_005252.2)	Reverse: CGGTTGCGGCATTTGG	
FosB	Forward: CCAGCGGAACTACCAGT	135
(NM_006732.2)	Reverse: CTGCTGCTAGTTTATTTCGT	
Fra-1	Forward: GCATGTTCCGAGACTTCG	173
(NM_005438.3)	Reverse: ATGAGGCTGTACCATCCACT	
Fra-2	Forward: CCAAGACCTGGCGTGA	98
(NM_005253.3)	Reverse: CGGATGCGACGCTTCT	
c-Jun	Forward: CTGCGTCTTAGGCTTCTCC	112
(NM_002228.3)	Reverse: TCGCCCAAGTTCAACAA	
JunB	Forward: GTACCCGACGACCACCATC	116
(NM_002229.2)	Reverse: CGGTCTGCGGTTCCTCCTT	
JunD	Forward: CTTCGCTGCCGAACCTGTG	94
(NM_005354.4)	Reverse: CGTCTGTGGCTCGTCCTTGA	
c-Myc	Forward: AGGCTATTCTGCCCATTT	180
(NM_002467.3)	Reverse: TCGTAGTCGAGGTCATAGTTC	
β-actin	Forward: AGCACTGTGTTGGCGTACAG	115
(NM_001101.3)	Reverse: CTCTTCCAGCCTTCCTTCCT	

Chapter 3

Results and Discussion

3.1 PGE_2 promoted cell proliferation mainly through EP2 receptor subtype

3.1.1 Expression profile of COX-1, COX-2, and EP receptor subtypes in human esophageal squmous cell carcinoma cell lines

We determined the expression of EP1 to EP4 receptors expression in a panel of human esophageal squamous cell carcinoma cell lines (HKESC-1, HKESC-2, HKESC-3, EC109, and KYSE150). Results from RT-PCR showed that the tested five esophageal squamous cell carcinoma cell lines expressed mRNAs for all EP receptor subtypes (Figure 3.1). Furthermore, in the tested cell lines, the protein expression of EP receptors was confirmed by Western blot analysis in which specific EP receptors were detected at the anticipated molecular weight using EP receptor specific antibodies (Figure 3.2). The protein expression of COX-1 and COX-2 was also detected in these cell lines (Figure 3.2). HKESC-1 cells were thereafter elected as the working cell line for further analysis. In HKESC-1 cells, aside from the expression of EP receptors, we also investigated whether HKESC-1 cells could actively secrete PGE_2. In this regard, the basal release of PGE_2 was determined to be 2.27±0.02 pg/ml per μg total protein over 24 h.

3.1.2 PGE_2 or EP2 agonist butaprost increased HKESC-1 cell proliferation

To study the effect of PGE_2 on proliferation of esophageal squamous cell carcinoma cells, HKESC-1 cells were treated with PGE_2 at concentrations ranging from 0.1 to 10 μM. Results showed that PGE_2 at these concentrations significantly

increased HKESC-1 cell proliferation in a concentration-dependent manner (Figure 3.3). In the next step, we determined which EP receptor mediated the mitogenic effect of PGE_2 using selective EP receptor agonists or antagonists. Results showed that EP2 receptor agonist butaprost at the concentration of 25 μM substantially increased HKESC-1 cell proliferation to an extent similar to that of 10 μM PGE_2 while EP1 receptor agonist ONO-DI-004 and EP3/EP1 receptor agonist sulprostone at all concentrations tested only minimally stimulated HKESC-1 cell proliferation (Figures 3.4, 3.5, and 3.6). These data indicated that EP2 receptor, and to a lesser extent EP1 receptor, were involved in mediating the stimulatory of PGE_2. The involvement of EP3 and EP4 receptors was further excluded based on the finding that EP4/EP3 receptor agonist PGE_1 alcohol exhibited no effect on cell proliferation (Figure 3.7) whilst EP3 receptor antagonist ONO-AE3-240 and EP4 receptor antagonist ONO-AE3-208 failed to attenuate PGE_2-induced cell proliferation as shown in Figures 3.8 and 3.9.

3.1.3 Knockdown of EP2 receptor attenuated the mitogenic effect of PGE_2

As EP2 receptor agonist butaprost strongly increased HKESC-1 cell proliferation as compared with other agonists, the role of the EP2 receptor in PGE_2-induced cell proliferation was further investigated by RNA interference experiment. Using specific siRNA, down-regulation of EP2 receptor significantly attenuated PGE_2-induced proliferation in HKESC-1 cells (Figure 3.10A). We used fluorescein-labeled double-stranded RNA (dsRNA) oligomer to facilitate the assessment and optimization of transfection conditions. Results showed that the

siRNA transfection efficiency was more than 90% (Figure 3.10B). The efficacy of the EP2 receptor depletion was further verified by Western blot analysis in which the results showed that EP2 receptor siRNA successfully down-regulated EP2 receptor protein levels 24h post-transfection (Figure 3.10C).

3.1.4 Preliminary discussion and conclusion

Over-expression of COX-2 and the subsequent elevation of PGE_2 levels have been implicated in the pathogenesis of human esophageal squamous cell carcinoma (Zhi et al., 2006; Zimmermann et al., 1999; Morgan, 1997). Here we demonstrate that PGE_2 stimulates the proliferation of a human esophageal squamous cell carcinoma cell line HKESC-1 (Figure 3.3). These observations indicate that PGE_2 exerts its pro-carcinogenic effect in esophageal squamous cell carcinoma, at least in part, through direct stimulation of cell proliferation. In this connection, EP receptors have been reported to mediate the mitogenic effects of PGE_2 in different cell types (Fulton et al., 2006). In the present study, we show for the first time that all four EP receptor subtypes, namely, EP1 to EP4 receptors, are expressed in a panel of human esophageal squamous cell carcinoma cell lines (Figure 3.1 and 3.2). Further characterization by pharmacological and RNA interference approaches reveals that EP2 receptor mediates the mitogenic effect of PGE_2 in HKESC-1 cells, in which the EP2 receptor agonist butaprost mimics the mitogenic effect of PGE_2 whilst knockdown of EP2 receptor attenuates the PGE_2-induced proliferative response (Figure 3.4-3.10). In line with this finding, a recent clinical study reported by Kuo et

al. (2009) showed that EP2 overexpression was observed in human esophageal squamous cell carcinoma, which was associated with poor prognosis. Here we provide a direct evidence that EP2 receptor plays a predominant role in the mediation of the stimulatory effect of PGE_2 in esophageal squamous cell carcinoma. Indeed, the importance of EP2 receptor in PGE_2-induced cell proliferation has also been documented in a variety of cancers such as colon cancer, epidermoid carcinoma, and lung carcinoma (Castellone et al., 2005; Donnini et al., 2007; Han and Roman, 2004).

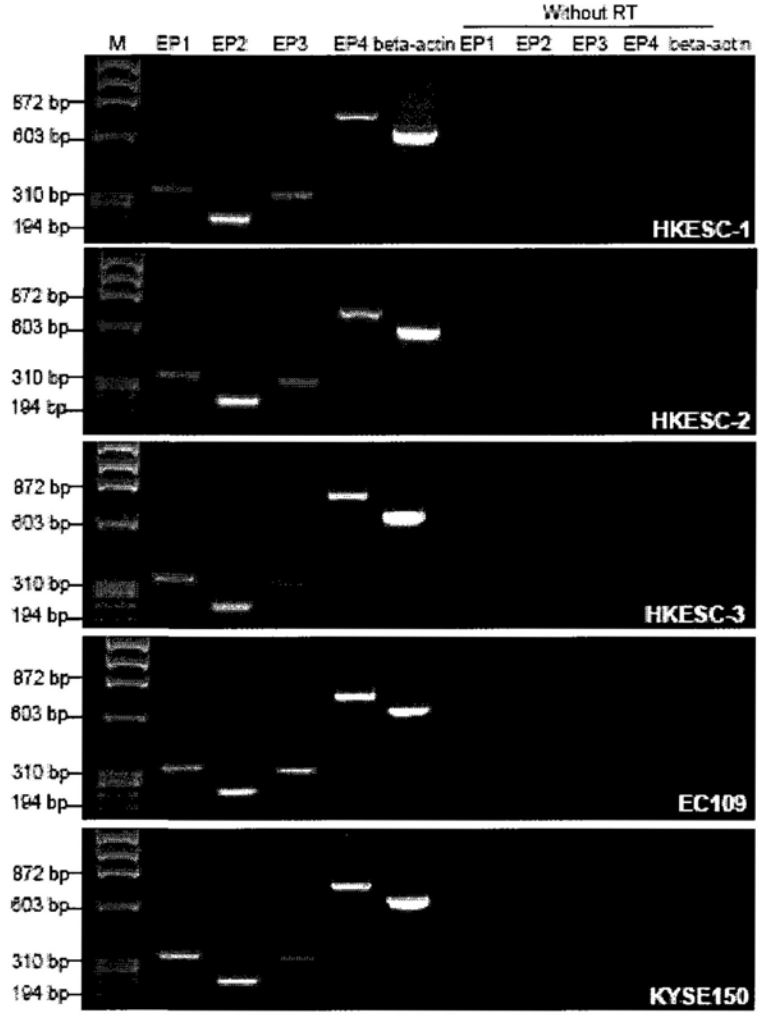


Figure 3.1 Expression of EP receptors in human esophageal squamous cell carcinoma cells. Results from RT-PCR revealed the transcripts of all four EP receptor subtypes, EP1 to EP4 receptors, were present in a panel of esophageal squamous-cell carcinoma cell lines (HKESC-1, HKESC-2, HKESC-3, EC109 and KYSE150). Direct PCR amplifications of mRNA without prior reverse transcription were used as negative control.

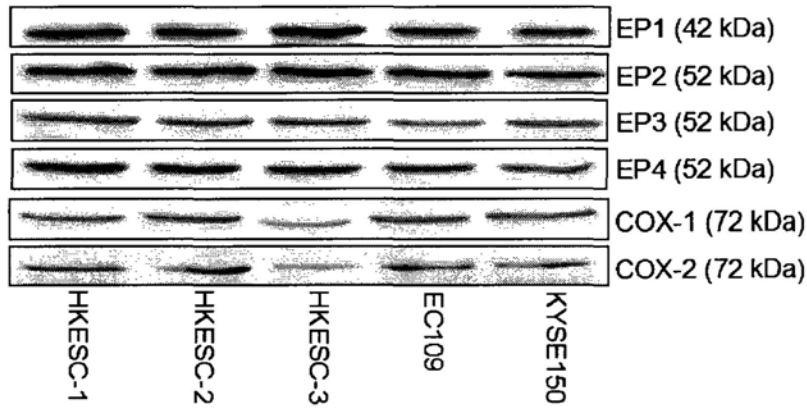


Figure 3.2 Protein expressions of four EP receptor subtypes, COX-1, and COX-2 in a panel of esophageal squamous cell carcinoma cell lines (HKESC-1, HKESC-2, HKESC-3, EC109, and KYSE150) verified by Western blot.

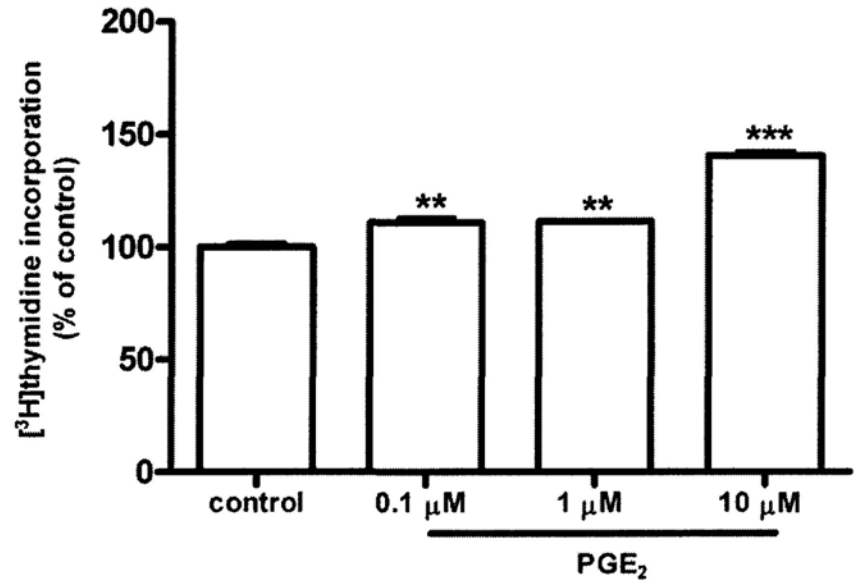


Figure 3.3 Effects of PGE_2 on cell proliferation of HKESC-1 cells. Cells were deprived of serum for 24 h and thereafter treated for another 24 h with PGE_2 at the indicated concentrations. Cell proliferation was then determined as the amount of DNA synthesis by [^{3}H]thymidine incorporation assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. **, $p<0.01$, ***, $p<0.001$ versus control group.

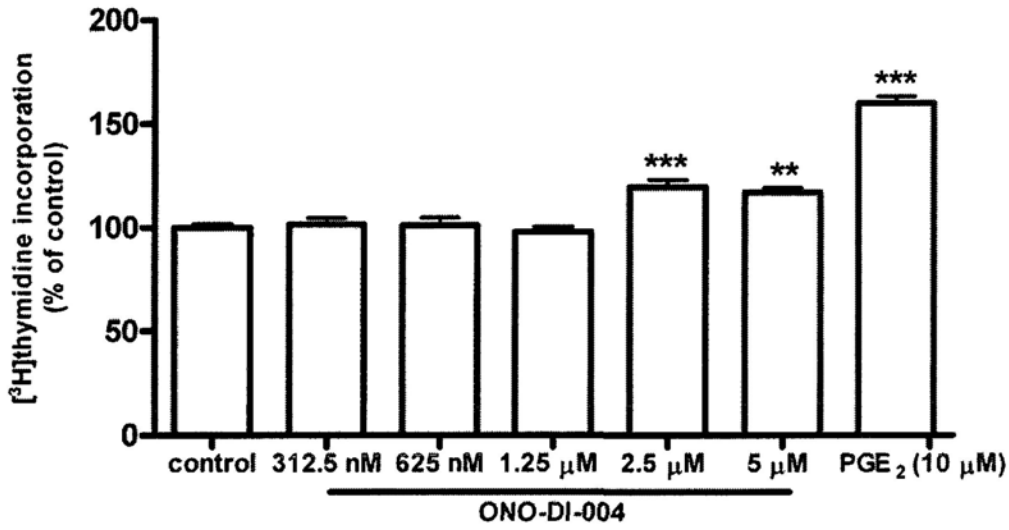


Figure 3.4 Effects of EP1 receptor agonist ONO-DI-004 on cell proliferation of HKESC-1 cells. Cells were deprived of serum for 24 h and thereafter treated for another 24 h with ONO-DI-004 at the indicated concentrations. Cell proliferation was then determined as the amount of DNA synthesis by [^{3}H]thymidine incorporation assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. **, $p<0.01$, ***, $p<0.001$ versus control group.

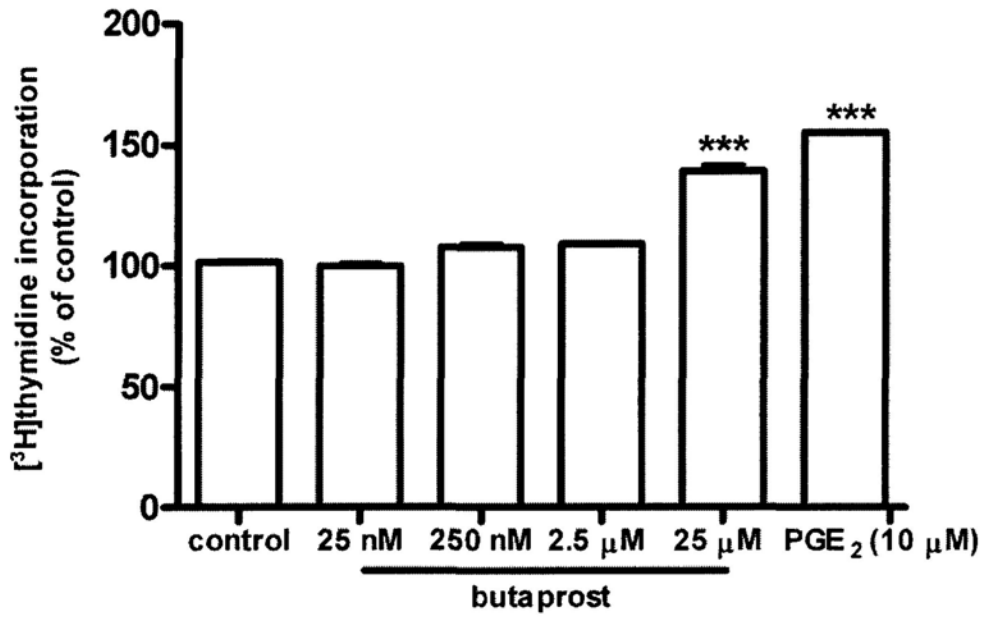


Figure 3.5 Effects of EP2 receptor agonist butaprost on cell proliferation of HKESC-1 cells. Cells were deprived of serum for 24 h and thereafter treated for another 24 h with butaprost at the indicated concentrations. Cell proliferation was then determined as the amount of DNA synthesis by [^{3}H]thymidine incorporation assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. ***, $p<0.001$ versus control group.

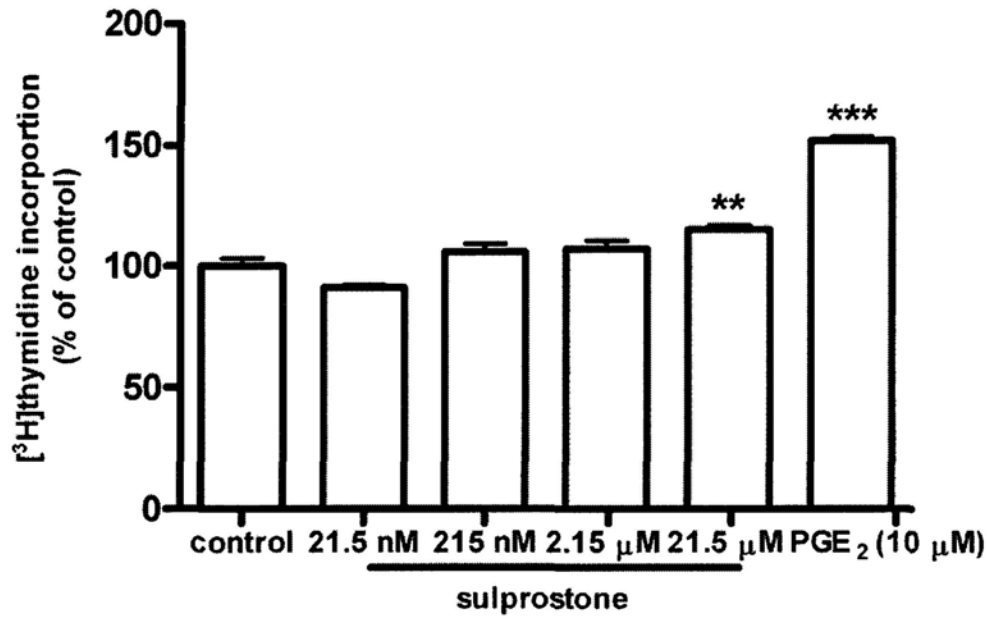


Figure 3.6 Effects of EP3/EP1 receptor agonist sulprostone on cell proliferation of HKESC-1 cells. Cells were deprived of serum for 24 h and thereafter treated for another 24 h with sulprostone at the indicated concentrations. Cell proliferation was then determined as the amount of DNA synthesis by [^{3}H]thymidine incorporation assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. **, $p<0.01$,***, $p<0.001$ versus control group.

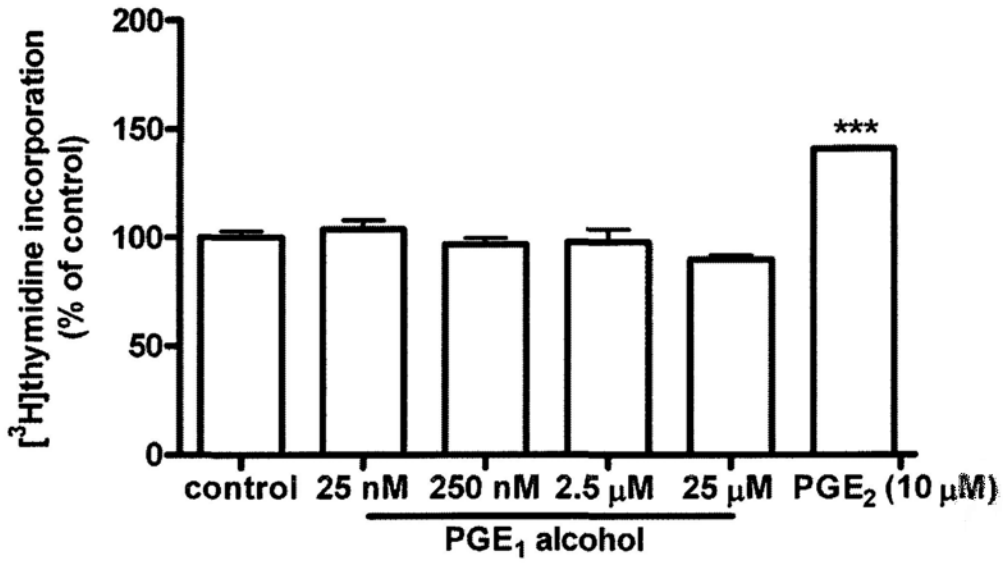


Figure 3.7 Effects of EP4/EP3 receptor agonist PGE_1 alcohol on cell proliferation of HKESC-1 cells. Cells were deprived of serum for 24 h and thereafter treated for another 24 h with PGE_1 alcohol at the indicated concentrations. Cell proliferation was then determined as the amount of DNA synthesis by [^{3}H]thymidine incorporation assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. ***, $p<0.001$ versus control group.

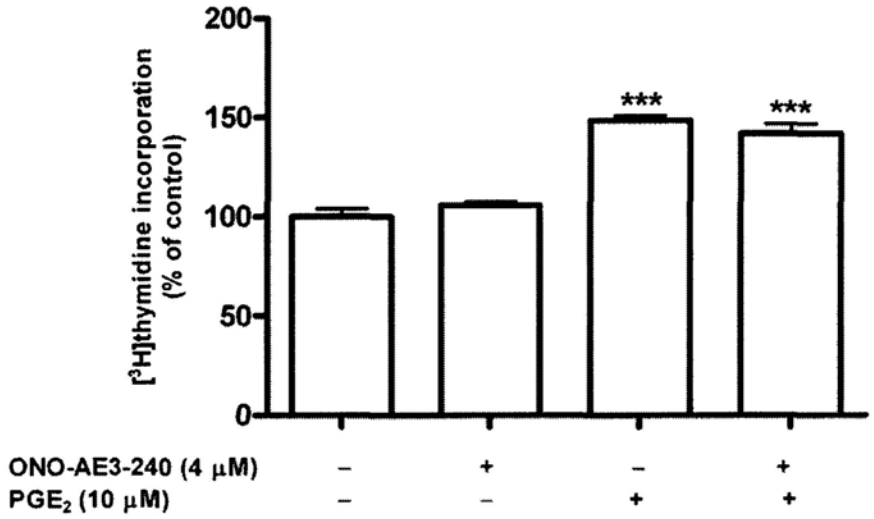


Figure 3.8 Effects of EP3 receptor antagonist ONO-AE3-240 on PGE_2-induced cell proliferation in HKESC-1 cells. Serum-deprived HKESC-1 cells were pretreated for 1 h with specific EP3 receptor antagonist ONO-AE3-240 before treatment with 10 μM PGE_2 for another 24 h. Cell proliferation was then determined as the amount of DNA synthesis by [^{3}H]thymidine incorporation assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. ***, $p<0.001$ versus control group.

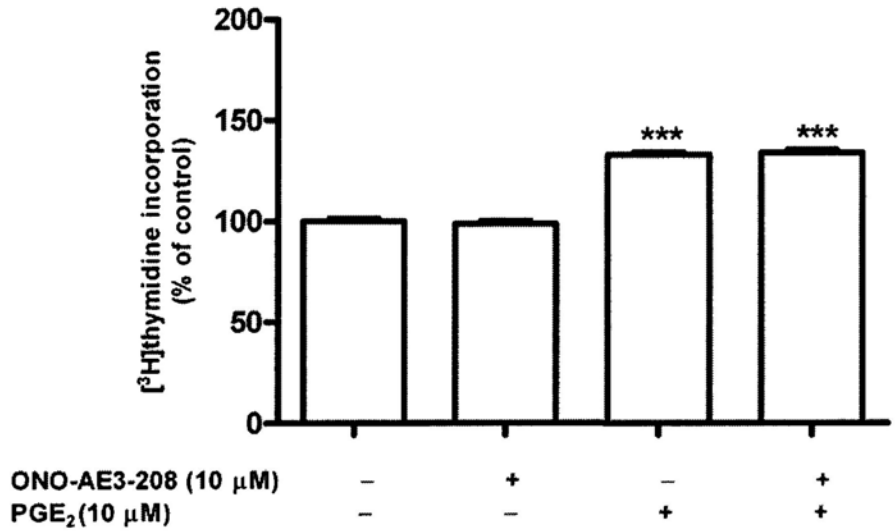
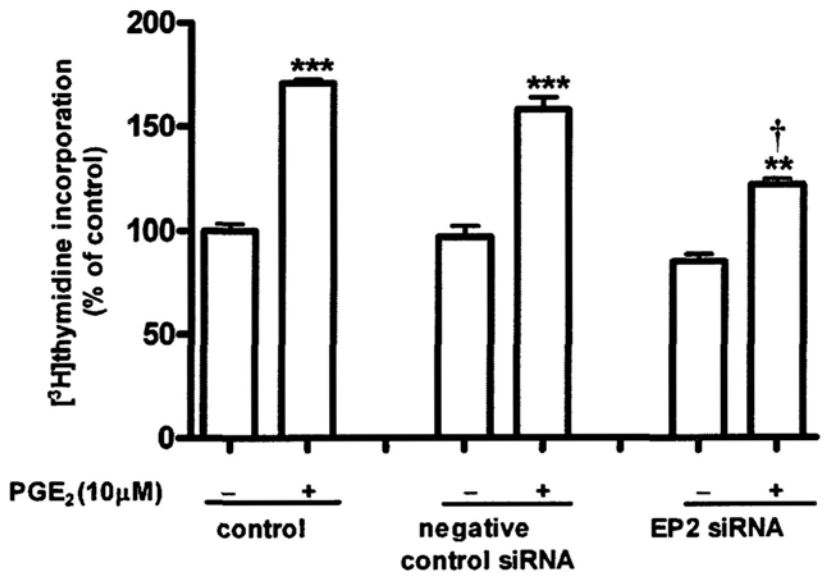
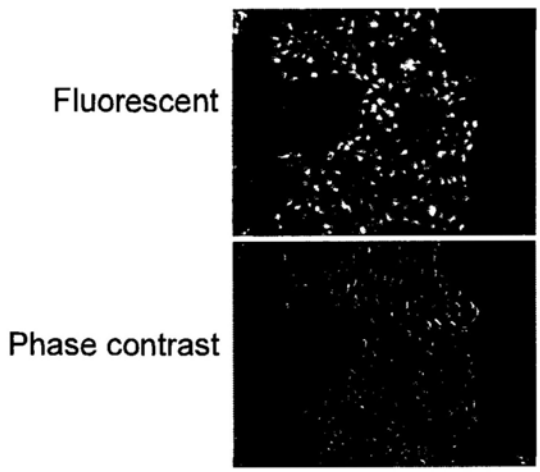


Figure 3.9 Effects of EP4 receptor antagonist ONO-AE3-208 on PGE_2-induced cell proliferation in HKESC-1 cells. Serum-deprived HKESC-1 cells were pretreated for 1 h with specific EP4 receptor antagonist ONO-AE3-208 before treatment with 10 μM PGE_2 for another 24 h. Cell proliferation was then determined as the amount of DNA synthesis by [^{3}H]thymidine incorporation assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. ***, $p<0.001$ versus control group.

A


200
150
100
50
0
[³H]thymidine incorporation (% of control)
***
***
†
**
PGE₂(10μM)
–
+
–
+
–
+
control
negative control siRNA
EP2 siRNA

B


Fluorescent
Phase contrast

C

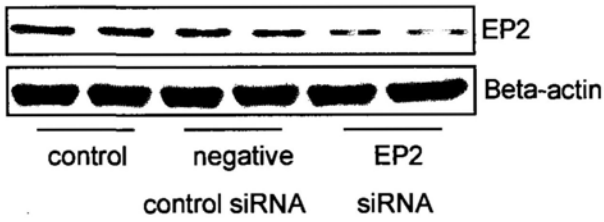

EP2
Beta-actin
control
negative control siRNA
EP2 siRNA

Figure 3.10 Effects of EP2 siRNA on PGE_2-induced cell proliferation in HKESC-1 cells. (A) After transfection with the EP2 receptor siRNA, HKESC-1 cells were treated with 10 μM PGE_2 for 24 h and examined for proliferation by [^{3}H]thymidine incorporation assay. (B) The transfection efficiency of siRNA was determined by transfection of fluorescein-labeled double stranded RNA oligomer in HKESC-1 cells. The result is the representative of three independent experiments. (C) The efficacy of the EP2 receptor depletion by EP2 receptor siRNA was further verified by Western blot analysis. Non-targeting siRNA was used as a control. β-actin was used to evaluate protein loading. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. **, $p<0.01$, ***, $p<0.001$ versus respective control group; †, $p<0.001$ versus PGE_2-treated group.

3.2 PGE_2 promoted cell proliferation via protein kinase C/extracellular signal-regulated kinase pathway

3.2.1 PGE_2 or EP2 receptor agonist increased Erk1/2 phosphorylation

As phosphorylation of protein kinase B (Akt) and Erk1/2 has been suggested to mediate the growth-promoting effect of PGE_2 in other cancer cell types (Krysan et al., 2005; Cherukuri et al., 2007; Han and Wu, 2005; Leng et al., 2003), we examined the direct effects with PGE_2 on the phosphorylation of these proteins. As shown in Figure 3.11, treatment of PGE_2 from 10 min to 30 min significantly stimulated the phosphorylation of Erk1/2, whereas it exerted no influence on the phosphorylation of Akt. Moreover, Western blot analysis revealed that the phosphorylation of p38 or JNK, members of the MAPK family in which Erk1/2 belongs, was not affected by PGE_2 treatment. To further examine whether Erk1/2 are involved in mediating the stimulatory effect of PGE_2 on cell proliferation, Erk1-siRNA and Erk2-siRNA were used to silence their expressions. It was demonstrated that knockdown of Erk1 or Erk2 protein expression significantly attenuated PGE_2-induced HKESC-1 cell proliferation (Figure 3.12A and 3.12B). Since EP2 receptor appeared to mediate the mitogenic effect of PGE_2, we also examined the effect of the EP2 receptor agonist butaprost on Erk1/2 phosphorylation. It was shown that butaprost at 25 μM also markedly increased Erk1/2 phosphorylation after 10 min treatment (Figure 3.13).

3.2.2 PGE_2-mediated Erk1/2 phosphorylation was abolished by protein kinase C inhibitors

It has been reported that PGE_2 can phosphorylate Erk1/2 through transactivation of epidermal growth factor receptor (EGFR) (Pai et al., 2002; Buchanan et al., 2003). Our results show that epidermal growth factor (EGF) notably increased phosphorylation of EGFR and Erk1/2 whilst PGE_2 exerted no effects on EGFR phosphorylation (Figure 3.14 and 3.15), suggesting that other mechanisms instead of EGFR activation may account for the activation of ERK by PGE_2 stimulation. Aside from EGFR, activated PKC can also activate the MAPK/Erk signaling via direct phosphorylation of Raf-1 or by indirect activation of small GTPase Ras to promote cell proliferation (Kolch et al., 1993; Schonwasser et al., 1998; Bhalla and Lyengar, 1999). In this study, we determined the involvement of PKC in PGE_2-induced Erk1/2 phosphorylation by pre-treating HKESC-1 cells with selective PKC inhibitor Ro-31-8425 or Bisindolylmaleimide I prior to PGE_2 treatment. Results showed that the proliferation and phosphorylated Erk of HKESC-1 cells induced by PGE_2 was significantly attenuated by Ro-31-8425 (Figure 3.16 and 3.17) and Bisindolylmaleimide I (Figure 3.18 and 3.19). These findings suggest the involvement of PKC in PGE_2-mediated Erk activation and cell proliferation.

3.2.3 Preliminary discussion and conclusion

MAPK cascades (Erk1/2, p38, and JNK) and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway are key signaling molecules involved in the regulation of cell

proliferation, survival and differentiation. It therefore comes as no surprise that deregulation of these signaling pathways frequently occurs in human cancer, including esophageal squamous cell carcinoma (Chattopadhyay et al., 2007; Li et al., 2007). Our results demonstrate that PGE_2 markedly increased the phosphorylation of Erk1/2, but not JNK or p38, in cultured esophageal squamous cell carcinoma cells (Figure 3.11). RNA interference-mediated down-regulation of Erk1 or Erk2 also attenuated the stimulatory effect of PGE_2 on cell proliferation (Figure 3.12A and 3.12B), suggesting that phosphorylation of Erk1/2 but not the other two members of MAPK cascades is required for the mitogenic effect of PGE_2. Intriguingly, activation of Erk1/2 has also been shown to up-regulate the activity of COX-2 (Chun et al., 2003), which has been observed aberrantly up-regulated in esophageal squamous cell carcinoma (Zhi et al., 2006; Zimmermann et al., 1999). It is therefore possible that COX-2-derived PGE_2 may enhance a positive feedback loop to stimulate cell proliferation in esophageal squamous cell carcinoma cells. Regulation of Erk1/2 activity by cAMP has been observed in some cell lines (Gerits et al., 2008). As EP2 receptor is a Gs protein coupled receptor, it may regulate Erk1/2 activity via cAMP pathway. In this respect, we investigated effects of cAMP on activation of Erk1/2 in HKESC-1 cells. Although forskolin, an adenylate cyclase activator, increased intracellular cAMP level more potently than EP2 agonist butaprost and PGE_2 (Figure 3.20), it did not influence Erk1/2 phosphorylation and cell proliferation (Figure 3.21 and 3.22). Thus, mechanism other than cAMP pathway, may be involved in EP2 receptor mediated HKESC-1 cell proliferation. Although PGE_2 has also been

reported to transactivate EGFR to induce Erk1/2 phosphorylation and cell proliferation in other cell types (Pai et al., 2002; Buchanan et al., 2003), our results indicate that PGE_2 exerted no effect on EGFR phosphorylation (Figure 3.15). To this end, PKC has been shown to activate Erk1/2 in an EGFR-independent manner by stimulation of Raf-1 or Ras (Kolch et al., 1993; Schonwasser et al., 1998; Bhalla and Lyengar, 1999). In this regard, we demonstrate that pre-treating the cells with PKC inhibitors could abrogate PGE_2-induced Erk1/2 phosphorylation and cell proliferation (Figure 3.16-3.19). This finding is consistent with the results reported by Krysan *et al.* demonstrating that PGE_2 induced Erk1/2 activation through PKC signaling in human non-small cell lung cancer cells (Krysan et al., 2005).

Apart from the MAPK cascade, Akt has been implicated in PGE_2-induced cholangiocarcinoma and hepatocellular carcinoma cell proliferation (Han and Wu, 2005; Leng et al., 2003). Akt becomes activated as a result of phosphorylation of Thr308 within the T loop of the catalytic domain and Ser473 located in a C-terminal, noncatalytic region of the enzyme, termed the "hydrophobic motif". In this regard, our results show that treating HKESC-1 cells with PGE_2 did not alter the expression of total Akt or its phosphorylation at Ser473. Furthermore, phosphorylated Akt at Thr308 was undetectable irrespective of the presence of PGE_2 (Figure 3.11), suggesting that Akt may not be involved in PGE_2-induced cell proliferation in esophageal squamous cell carcinoma cells.

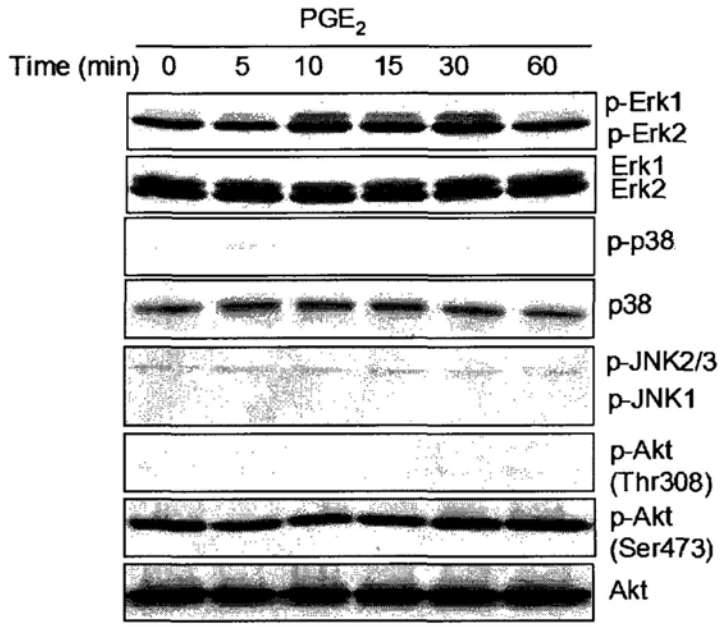


Figure 3.11 The stimulatory effect of PGE_2 on phosphorylation of Erk1/2 in HKESC-1 cells. Serum-deprived cells were lysed after 10 μM PGE_2 stimulation, and lysates were probed with phospho-Erk1/2, Erk1/2, phospho-p38, p-38, phospho-JNK, phospho-Akt (Thr308), phospho-Akt (Ser473), Akt antibodies, as indicated. Data shown are representative of three independent experiments.

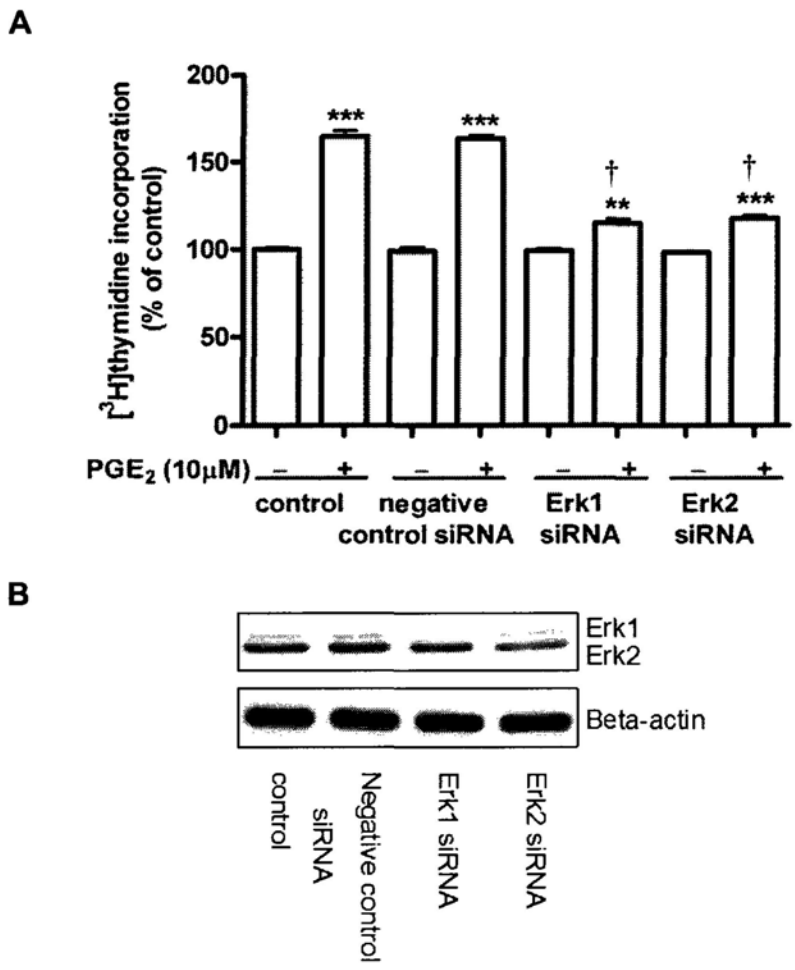


Figure 3.12 Effects of Erk1 siRNA and Erk2 siRNA on PGE_2-induced cell proliferation in HKESC-1 cells. (A) After transfection with Erk1- or Erk2-siRNA, cells were treated with 10 μM PGE_2 for 24 h and examined for proliferation by [^{3}H]thymidine incorporation assay. Non-targeting siRNA was used as a control. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. **, $p<0.01$, ***, $p<0.001$ versus respective control group; †, $p<0.001$ versus PGE_2-treated group. (B) The expressions of Erk1 and Erk2 after respective siRNA transfection were evaluated by Western blot analysis. Non-targeting siRNA was used as a control. β-actin was used to evaluate protein loading. Data shown are representative of three independent experiments.

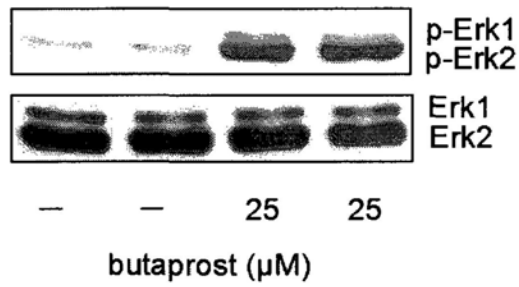


Figure 3.13 The effect of EP2 agonist butaprost on Erk1/2 phosphorylation in HKESC-1 cells. Serum-deprived cells were lysed after 25 μM butaprost stimulation for 10 min, and lysates were probed with phospho-Erk1/2 and Erk1/2 antibodies. Data shown are representative of three independent experiments.

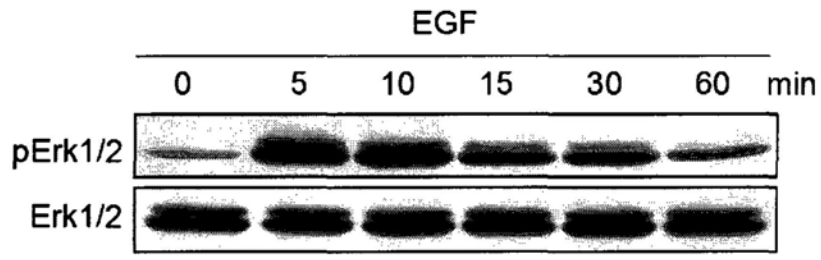


Figure 3.14 The stimulatory effect of EGF on phosphorylation of Erk1/2 in HKESC-1 cells. Serum-deprived cells were lysed after 10 ng/mL EGF stimulation, and lysates were probed with phospho-Erk1/2, Erk1/2 antibodies, as indicated. Data shown are representative of three independent experiments.

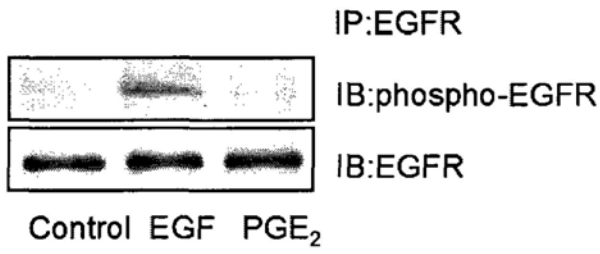


Figure 3.15 Effects of EGF and PGE_2 on EGFR activation in HKESC-1 cells. Serum-deprived cells were treated with EGF (10 ng/mL) or PGE2 (10 μM) for 10 min. Cells lysates were then prepared and subjected to immunoprecipitation (IP) with anti-EGFR antibody, followed by SDS-PAGE and immunoblotting (IB) with anti-phosphotyrosine antibody and anti-EGFR antibodies respectively. Immunoprecipitated tyrosine-phosphorylated EGFR (upper panel) and total EGFR (lower panel) were shown. Representative results from three independent experiments are shown.

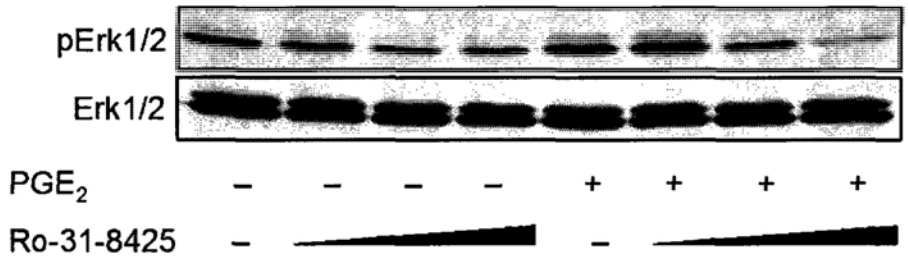


Figure 3.16 The effect of selective PKC inhibitor Ro-31-8425 on PGE_2-induced Erk1/2 activation in HKESC-1 cells. Serum-deprived cells were pretreated with selective PKC inhibitor Ro-31-8425 (300, 400 and 500 nM) for 30 min before treatment with 10 μM PGE_2 for an additional 10 min. Thereafter, cells were collected for determination of phosphorylated Erk1/2 and total Erk1/2 protein levels by Western blot analysis. Representative results from three independent experiments are shown.

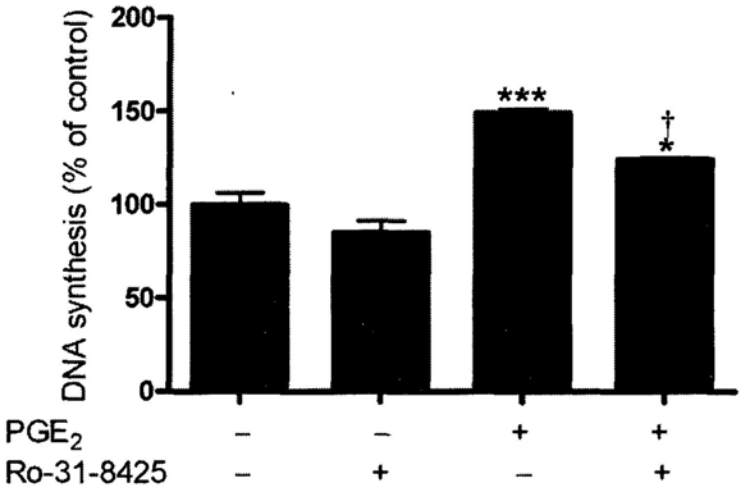


Figure 3.17 The effect of selective PKC inhibitor Ro-31-8425 on PGE_2-induced cell proliferation in HKESC-1 cells. Serum-deprived cells were pretreated with 400 nM Ro-31-8425 for 1 h before treatment with 10 μM PGE_2 for another 24 h. Cell proliferation then measured by [^{3}H]thymidine incorporation assay. *, $p<0.05$ and ***, $p<0.001$ versus the control group; †, $p<0.05$ versus PGE_2-treated group. Representative results from three independent experiments are shown.

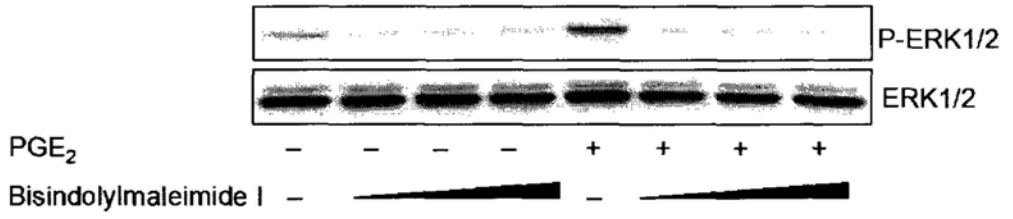


Figure 3.18 The effect of selective PKC inhibitor Bisindolylmaleimide I on PGE_2-induced Erk1/2 activation in HKESC-1 cells. Serum-deprived cells were pretreated with selective PKC inhibitor Bisindolylmaleimide I (62.5, 250 and 1000 nM) for 30 min before treatment with 10 μM PGE_2 for an additional 10 min. Thereafter, cells were collected for determination of phosphorylated Erk1/2 and total Erk1/2 protein levels by Western blot analysis. Representative results from three independent experiments are shown.

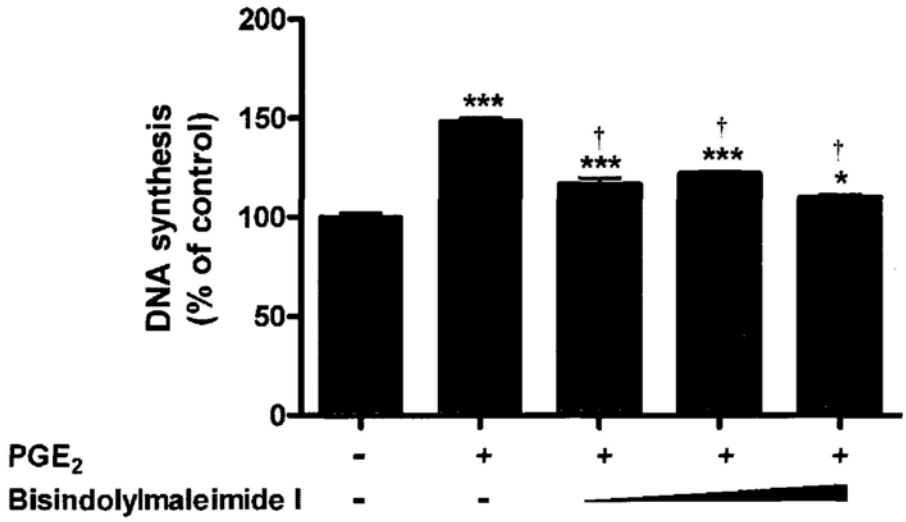


Figure 3.19 The effect of selective PKC inhibitor Bisindolylmaleimide I on PGE_2-induced cell proliferation in HKESC-1 cells. Serum-deprived cells were pretreated with Bisindolylmaleimide I (62.5, 250 and 1000 nM) for 1 h before treatment with 10 μM PGE_2 for another 24 h. Cell proliferation then measured by [^{3}H]thymidine incorporation assay. *, $p<0.05$ and ***, $p<0.001$ versus the control group; †, $p<0.05$ versus PGE_2-treated group. Representative results from three independent experiments are shown.

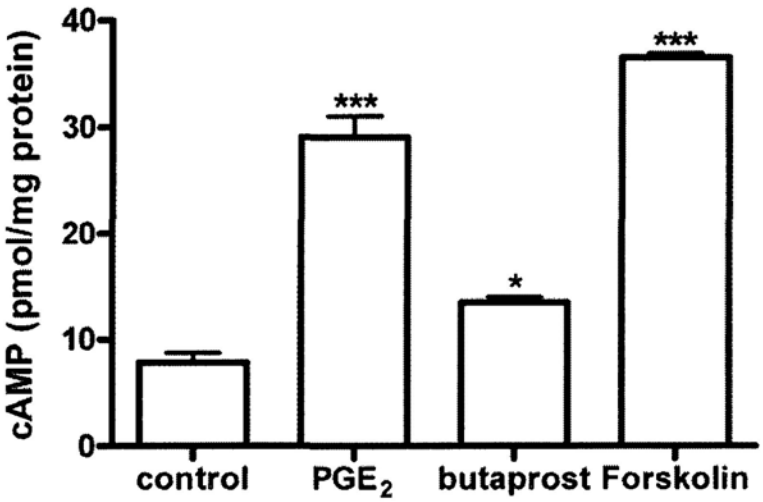


Figure 3.20 Effects of PGE_2 and EP2 receptor agonist butaprost on intracellular cyclic AMP production in HKESC-1 cells. Serum-deprived cells were treated with 10 μM PGE_2, 25 μM EP2 agonist butaprost, and 1 μM adenylate cyclase activator forskolin for 10 minutes in the presence of 100 μM phosphodiesterase inhibitor IBMX, then were lysed for intracellular cAMP assay. Forskolin was used as a positive control. Lysates from forskolin treated cells were 5-fold diluted before cAMP assay to avoid exceeding the upper detection limit of the test kit. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. *, $p<0.05$, ***, $p<0.001$ versus control group.

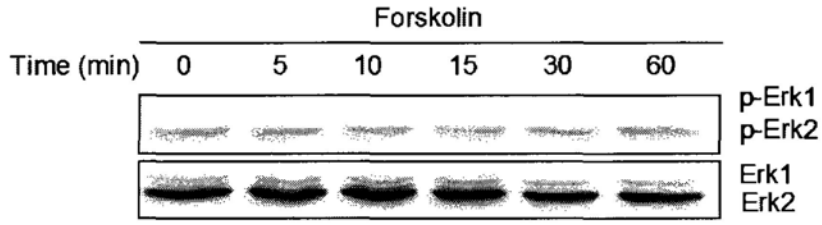


Figure 3.21 The effect of forskolin on Erk1/2 activation in HKESC-1 cells. Serum-deprived cells were lysated after 1μM forskolin stimulation, and lysates were probed with phopho-Erk1/2 and Erk1/2 antibodies. Data shown are representative of three independent experiments.

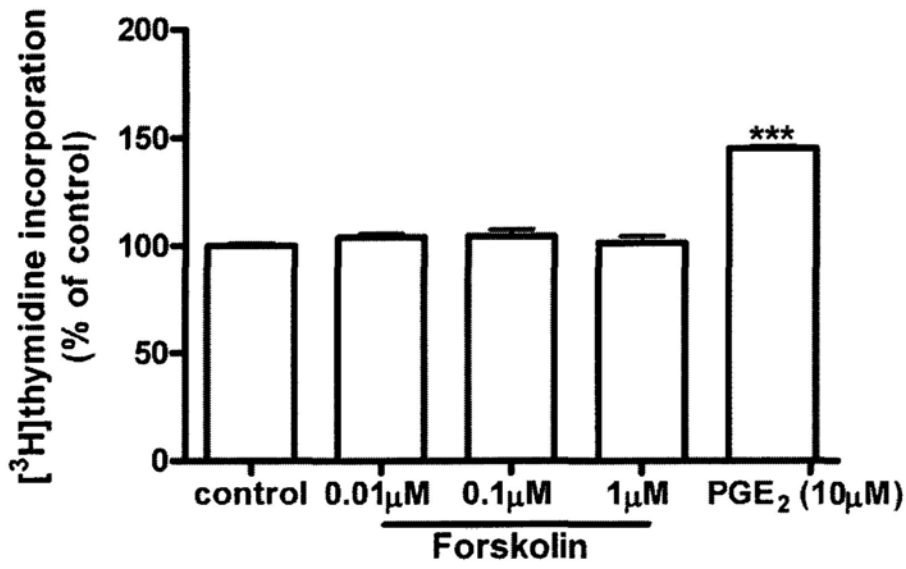


Figure 3.22 The effect of forskolin on cell proliferation in HKESC-1 cells. Serum-deprived cells were treated with forskolin for 24h at the indicated concentrations. Cell proliferation was then determined as the amount of DNA synthesized by [^{3}H]thymidine incorporation assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. ***, $p<0.001$ versus control group.

3.3 Activation of transcription factor activator protein-1 (AP-1) contributes to the mitogenic action of PGE_2

3.3.1 PGE_2 or butaprost up-regulated the mRNA expression of Fos and Jun family members

The data presented so far indicated that Erk1/2 phosphorylation participated, at least in part, in the mitogenic effect of PGE_2 on HKESC-1 cells. In this connection, the transcription factor activator protein-1 (AP-1), which consists of different members from the Fos and Jun families, has been reported to be induced upon Erk1/2 phosphorylation to mediate the effect on cell proliferation (Karin, 1995; Shaulian and Karin, 2002). We therefore measured the mRNA expression levels of these AP-1 components in HKESC-1 cells treated with or without PGE_2. As shown in Figure 3.23A and 3.23B, PGE_2 significantly increased the mRNA levels of c-Fos, FosB, Fra-1, c-Jun, and JunB, whereas it did not alter mRNA levels of Fra-2 or JunD. To this end, stimulating the cells with PGE_2 for 30 min resulted in a marked change in the expression of c-Fos, up to 18-fold increase compared with untreated cells. In parallel, EP2 receptor agonist butaprost significantly increased the mRNA levels of c-Fos, FosB, Fra-1, c-Jun, JunB, and JunD, whereas it showed no effects on Fra-2 mRNA level (Figures 3.24A and 3.24B). Similar to PGE_2 treatment, the change in c-Fos expression was the most prominent among the up-regulated genes, up to 10-fold increase compared with the control. We also observed that the time-course changes in FosB, Fra-1 and c-Jun mRNA levels between PGE_2 and butaprost treated cells were not exactly the same. The difference may be due to the fact that butaprost

is a highly selective EP2 receptor agonist whilst PGE_2 can activate all four EP receptor subtypes, EP1, EP3 and EP4 receptors may also involve in regulating the expression of these genes.

3.3.2 PGE_2-induced c-Fos protein expression was abolished by MEK inhibitor U0126

To further confirm the stimulatory effect of PGE_2 on c-Fos expression, we verified the up-regulation of c-Fos protein levels by Western blot analysis. Results showed that the expression level of c-Fos protein at basal condition was almost undetectable whilst it was dramatically elevated in response to PGE_2 treatment, reaching its peak level at 1 h post-treatment (Figure 3.25). In addition, MAPK/Erk kinase (MEK) inhibitor U0126 at the concentration of 1 μM completely abolished PGE_2-induced c-Fos expression (Figure 3.26). Similarly, EP2 receptor agonist butaprost but not EP1 receptor agonist ONO-DI-004, EP3/EP1 receptor agonist sulprostone, or EP4/EP3 receptor agonist PGE1 alcohol (Figure 3.27) markedly elevated c-Fos protein expression.

3.3.3 PGE_2- or butaprost-enhanced the transcriptional activity of AP-1 was abolished by MEK inhibitor U0126

As changes in the expression of AP-1 components might not exactly mirror the transcriptional activity of AP-1, we next determined AP-1 transcriptional activity in response to PGE_2 and butaprost treatment by dual-luciferase reporter assay. As

shown in Figure 3.28, PGE_2 or butaprost significantly increased AP-1 transcriptional activity. In this experiment, phorbol 12-myristate 13-acetate (PMA) was used as a positive control for AP-1 activity. In this respect, butaprost enhanced AP-1 transcriptional activity to an extent similar to that of PGE_2. MEK inhibitor U0126 also completely prevented the increase in AP-1 transcriptional activity induced by PGE_2. To further examine whether up-regulation of AP-1 transcriptional activity was required for the mitogenic effect of PGE_2, the AP-1 binding inhibitor curcumin (Guo et al., 2001) was used. To this end, curcumin significantly attenuated cell proliferation induced by PGE_2 (Figure 3.29).

3.3.4 Preliminary discussion and conclusion

Elevated AP-1 activity, which is associated with increased proliferation, has been frequently documented in various types of human cancer and is related to multi-stage development of tumors (Young et al., 2003; Liu et al., 2002). In mammalian cells, the AP-1 transcription factor is a heterodimeric complex that mainly comprises members of the Jun and Fos protein families, most of which belong to the category of immediate-early response genes and are promptly induced following growth factor stimulation (Karin, 1995).

AP-1 activity is predominantly governed by the MAPK cascade whose activation status is in turn influenced by extracellular stimuli such as growth factors, pro-inflammatory cytokines and UV radiation. In the context of cell proliferation, the most important mediator of growth factor is believed to be Erk1/2 whose

phosphorylation causes induction of c-Fos which subsequently heterodimerizes with Jun proteins to form stable AP-1 dimer (Shaulian and Karin, 2002). Concordantly, our study reveals that PGE_2 dramatically increased c-Fos expression and AP-1 transcriptional activity (Figures 3.25 and 3.28), both which can be abolished by the MEK inhibitor U0126 (Figure 3.26 and 3.28), suggesting that Erk1/2 phosphorylation is required for PGE_2-induced c-Fos expression and AP-1 activation. Above all, AP-1 binding inhibitor curcumin significantly attenuated PGE_2-induced cell proliferation (Figure 3.29), revealing that AP-1 activation is required for PGE_2-induced cell proliferation in esophageal squamous cell carcinoma. In parallel, the EP2 receptor agonist butaprost induces Erk1/2 phosphorylation (Figure 3.13), c-Fos expression (Figure 3.24 and 3.27), and AP-1 activity to a similar magnitude as PGE_2 exposure (Figure 3.28), indicating that EP2 receptor mediates the effects of PGE_2 on these parameters. This conclusion is substantiated by the fact that EP1 receptor agonist, EP3/EP1 receptor agonist, or EP4/EP3 receptor agonist shows minimal or no effect on c-Fos protein expression (Figure 3.27). To our knowledge, this is the first study to demonstrate the participation of Erk/AP-1 pathway in PGE_2-induced cell proliferation through EP2 receptor in human esophageal squamous cell carcinoma.

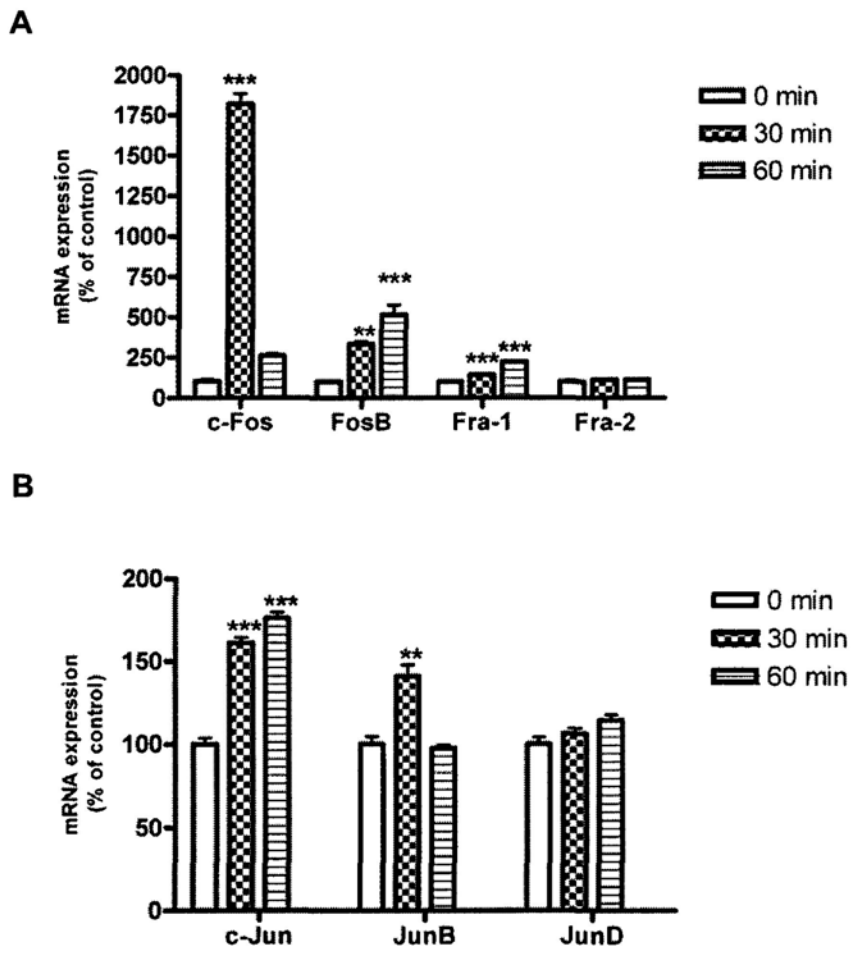


Figure 3.23 Effects of PGE_2 on the mRNA expression of members of Fos and Jun families in HKESC-1 cells determined by quantitative real-time PCR. The mRNA expression of members of (A) Fos and (B) Jun families (expressed as % of control) was up-regulated in response to treatment with 10 μM PGE_2 for 30 min and 60 min respectively. β-actin was used as an internal control for normalization. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. **, $p<0.01$, ***, $p<0.001$ versus respective control group.

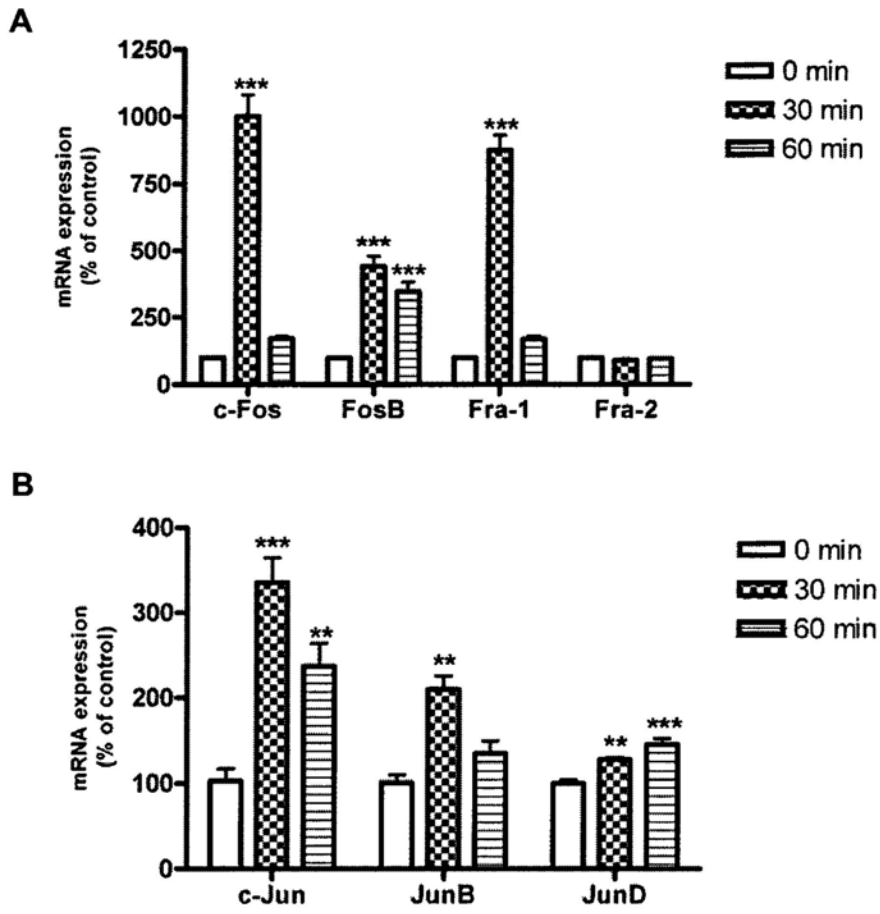


Figure 3.24 Effects of EP2 receptor agonist butaprost on the mRNA expression of members of Fos and Jun families in HKESC-1 cells determined by quantitative real-time PCR. The mRNA expression of members of (A) Fos and (B) Jun families (expressed as % of control) was up-regulated in response to treatment with EP2 receptor agonist butaprost (25 μM) for 30 min and 60 min. β-actin was used as an internal control for normalization. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. **, $p<0.01$, ***, $p<0.001$ versus respective control group.

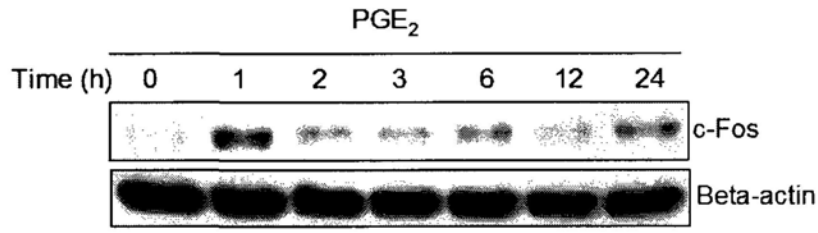


Figure 3.25 The effect of PGE_2 on c-Fos protein expression in HKESC-1 cells. Serum-deprived HKESC-1 cells were exposed to 10 μM PGE_2 and collected at 0, 1, 2, 3, 6, 12 and 24 h for the determination of c-Fos protein expression by Western blot analysis. β-actin was used to evaluate protein loading. Data shown are representative of three independent experiments.

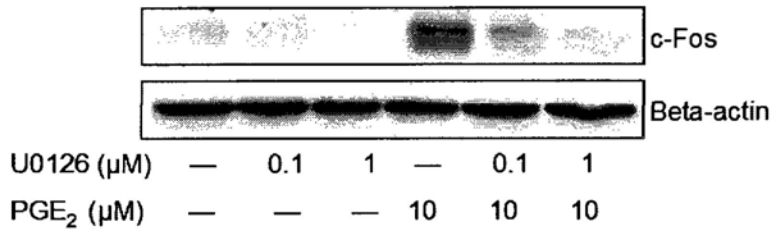


Figure 3.26 The effect of MEK inhibitor U0126 on PGE_2-induced c-Fos protein expression in HKESC-1 cells. Cells were pre-treated with MEK inhibitor U0126 at indicated concentrations for 1 h prior to treatment with 10 μM PGE_2 for another 1 h. Cells were thereafter collected for determination of c-Fos protein level by Western blot analysis. β-actin was used to evaluate protein loading. Data shown are representative of three independent experiments.

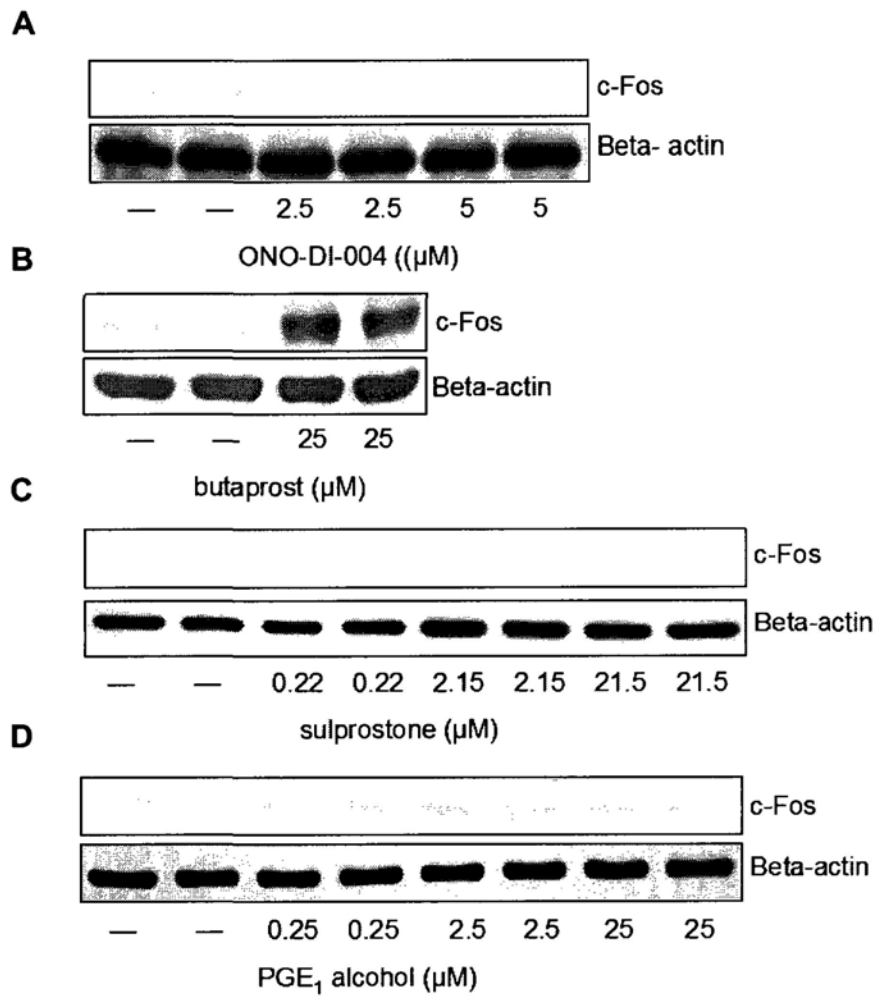


Figure 3.27 Effects of EP receptor agonists on c-Fos protein expression in HKESC-1 cells. Protein expression of c-Fos in response to (A) EP1 receptor agonist ONO-DI-004, (B) EP2 receptor agonist butaprost, (C) EP3/EP1 receptor agonist sulprostone or (D) EP4/EP3 receptor agonist PGE_1 alcohol treatment was determined by Western blot analysis. Serum-deprived cells were collected after 1 h treatment with respective EP receptor agonists. β-actin was used to evaluate protein loading. Data shown are representative of three independent experiments.

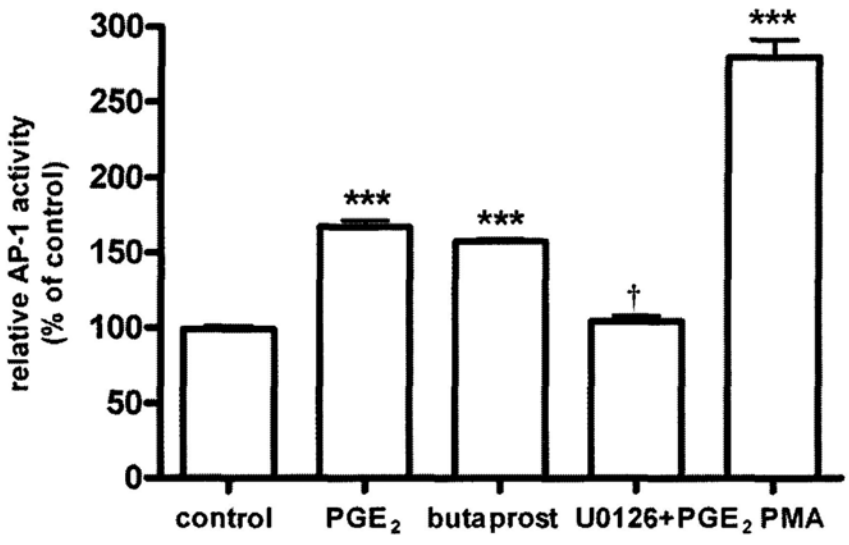


Figure 3.28 MEK inhibitor U0126 abolished PGE_2-induced AP-1 transcriptional activity. Cells were transfected with a ratio 10:1 of the pAP-1 (PMA)-luc plasmid and pRL-TK plasmid. After exposure to 10 μM PGE_2 or 25 μM butaprost for 6 h, cells were collected for determination of AP-1 activity. For investigating the role of Erk1/2 phosphorylation in PGE_2-induced AP-1 activation, cells were pretreated with 1 μM MEK inhibitor U0126 for 1 h prior to treatment with 10 μM PGE_2 for another 6 h. pAP-1 (PMA)-luc luciferase activities were normalized by pRL-TK luciferase activities for transfection efficiency. PMA at 100 nM was used as a positive control. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. ***, $p<0.001$ versus respective control group, †, $p<0.001$ versus PGE_2-treated group.

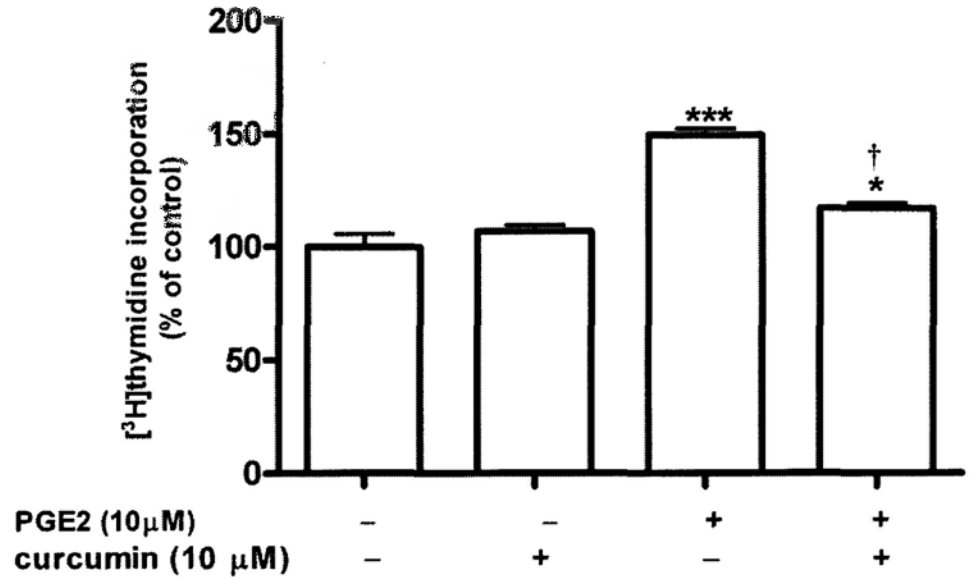


Figure 3.29 Involvement of AP-1 activation in PGE_2-induced cell proliferation in HKESC-1 cells. Serum-deprived cells were pre-treated with 10 μM curcumin for 1 h prior to treatment with 10 μM PGE_2 for another 24 h. Cell proliferation was then examined by [^{3}H]thymidine incorporation assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. *, $p<0.05$, ***, $p<0.001$ versus respective control group; †, $p<0.001$ versus PGE_2-treated group.

3.4 Upregulation of oncoprotein c-Myc expression contributes to the mitogenic action of PGE_2

3.4.1 PGE_2 induced c-Myc mRNA and protein expression in HKESC-1 cells

We determined the direct effect of PGE_2 on c-Myc expression at the mRNA and protein levels in cultured human esophageal squamous cell carcinoma cells (HKESC-1) by real-time PCR and Western blot, respectively. Results showed that PGE_2 significantly increased c-Myc mRNA expression from 1 h to 3 h treatment and then declined to basal level (Figure 3.30A). Protein level was obviously elevated from 30 min treatment and declined to basal level from 12 h treatment (Figure 3.30B). The stimulatory effect of PGE_2 on c-Myc expression was also observed in another esophageal squamous cell carcinoma cell line, HKESC-2 (Figure 3.31). The induction of c-Myc protein by PGE_2 was further confirmed by immunofluorescence assay. Cells treated with PGE_2 for 30 min exhibited much stronger fluorescent signaling for c-Myc than those in untreated cells. Moreover, fluorescent signal showed that the expression of c-Myc protein was confined to the nucleus (Figure 3.30C).

3.4.2 Knockdown of c-Myc attenuated the mitogenic effect of PGE_2

We previously demonstrated that PGE_2 directly increased HKESC-1 cell proliferation at concentrations ranging from 0.1 to 10 μM (Figure 3.3). Since c-Myc expression was elevated in response to PGE_2 treatment, the role of c-Myc in PGE_2-induced cell proliferation was further investigated by RNA interference

experiments. Using specific siRNA, down-regulation of c-Myc significantly lowered the basal proliferation and attenuated PGE_2-induced proliferation in HKESC-1 cells (Figure 3.32A). The efficacy of c-Myc depletion was further verified by the Western blot analysis, in which c-Myc siRNA effectively down-regulated c-Myc protein levels 24 h post-transfection (Figure 3.32B).

3.4.3 PGE_2 induced c-Myc phosphorylation on Serine 62 and increased c-Myc protein stability

c-Myc is a short-lived protein and its expression levels can be regulated at the post-translational levels by modulating the protein stability (Sears, 2004). We therefore determined whether PGE_2 stabilized c-Myc protein by monitoring c-Myc degradation in the presence of cycloheximide, a protein synthesis inhibitor. As shown in Figure 3.33, the steady-state level of c-Myc declined rapidly in the control group but decayed at a much slower pace in the presence of PGE_2. Two phosphorylation sites at the N-terminus, Serine 62 and Threonine 58, exerted opposing effects on c-Myc protein stability. Phosphorylation on Serine 62 stabilizes c-Myc whereas phosphorylation on Threonine 58 destabilizes it (Sears, 2004). In this regard, our results indicated that PGE_2 treatment substantially increased the phosphorylation of c-Myc on Serine 62 from 30 min to 1 h (Figure 3.34).

3.4.4 MRK inhibitor abolished PGE_2-induced c-Myc expression

Serine 62 of c-Myc is a target of Erk. We previously reported that PGE_2

significantly increased the phosphorylation of Erk1/2 from 10 min to 30 min (Figure 3.11). In the present study, we found that the MAPK/Erk kinase (MEK) inhibitor U0126 at the concentration of 10 nM completely abolished PGE_2-induced c-Myc expression, suggesting the protein stability of c-Myc was under the control of Erk pathway (Figure 3.35).

3.4.5 PGE_2 increased c-Myc-Max complex formation

Max associates with c-Myc and is required for c-Myc to bind DNA and activate transcription (Blackwood and Eisenman, 1991). Immunoprecipitation and Western blot experiments were therefore performed to determine whether PGE_2 facilitates the formation of heterodimer between c-Myc and Max. Unlike c-Myc, protein level of Max is quite stable in response to PGE_2 treatment (Figure 3.36). However, PGE_2 obviously enhanced the association between c-Myc and Max (Figure 3.37).

3.4.6 EP2 receptor mediates PGE_2-induced c-Myc expression

PGE_2 exerts its effects by acting on four different G-protein-coupled EP receptors, designated as EP1, EP2, EP3 and EP4. Our previous studies show human esophageal squamous cell carcinoma cells expressed all four EP receptor subtypes (Figures 3.1 and 3.2). In order to reveal which receptor(s) mediate PGE_2-induced c-Myc expression, selective agonists for respective EP receptor subtypes were utilized. As shown in Figure 3.38A-C, the EP2 receptor agonist butaprost significantly increased c-Myc expression, whereas the EP3/EP1 agonist sulprostone

or EP4/EP3 agonist PGE_1 alcohol showed no effects, indicating that EP2 receptor mediated PGE_2-induced c-Myc expression in HKESC-1 cells. In addition to pharmacological methods, EP2 siRNA was utilized to further examine whether EP2 receptor mediated PGE_2-induced c-Myc expression. It was demonstrated that knockdown of EP2 receptor attenuated PGE_2-induced c-Myc expression (Figure 3.38D). The efficacy of EP2 receptor depletion was further verified by our previous study, in which EP2 receptor siRNA successfully down-regulated EP2 receptor protein levels (Figure 3.10C).

3.4.7 Preliminary discussion and conclusion

It is well-established that human esophageal squamous cell carcinomas frequently overexpress COX-2 and produce high levels of PGE_2 (Zhi et al., 2006; Morgan, 1997; Zimmermann et al., 1999). In this study, we showed that PGE_2 increases c-Myc expression through activation of EP2 receptor, which is responsible, at least in part, for the mitogenic action of PGE_2. Mechanistically, we found that PGE_2-stimulated c-Myc expression involves activation of Erk signaling pathway and the subsequent stabilization of c-Myc protein. To our knowledge, this is the first study reporting that c-Myc is required for the mitogenic action of PGE_2 in human esophageal squmous cell carcinoma cells.

c-Myc is a nuclear transcription factor, which operates in a heterodimeric complex with Max to bind to E-Box motifs in DNA, thereby transcriptionally regulating numerous target genes involved in diverse cellular programs (Sears et al.,

1999; Sears, 2004; Lutz et al., 2002). In the present study, PGE_2 obviously increased c-Myc expression both at mRNA and protein levels (Figure 3.30). The association between c-Myc and Max is also enhanced in response to PGE_2 treatment (Figure 3.37), which suggests an increase in functional c-Myc protein. Considering that the Max protein is quite stable in response to PGE_2 treatment, the increased association between c-Myc and Max may be due to an overall increase in c-Myc protein expression. Moreover, down-regulation of c-Myc by specific siRNA lowers the basal cell proliferation and PGE_2-induced cell proliferation (Figure 3.32). As the cell proliferation was determined under growth restricted conditions, it is anticipated that c-Myc siRNA slightly but significantly decreased basal cell proliferation. These findings strongly suggest that c-Myc is involved in PGE_2-induced cell proliferation in esophageal squamous cell carcinoma.

c-Myc protein is a highly unstable protein, which exhibits an extremely short half-life, around 30 min in proliferating cells (Hann and Eisenman, 1984). It has been reported that the half-life of c-Myc increases markedly in mitogen-stimulated cells, and this stabilization depends on the MAPK/Erk pathway which phosphorylates c-Myc protein on Serine 62, leading to increased protein stability (Sears et al., 1999; Sears et al., 2000). Given that PGE_2 exerts mitogenic action and increases phosphorylation of Erk in esophageal squamous cell carcinoma cells, we determined whether post-translational control of c-Myc protein levels contribute to PGE_2-induced c-Myc expression. Our results show that PGE_2 increases phosphorylation of c-Myc on Serine 62 (Figure 3.34). In accord with these data,

pre-treating the cells with MEK inhibitor U0126 abrogates PGE_2-induced c-Myc expression (Figure 3.35). Moreover, the rate of c-Myc protein degradation is markedly slowed down by PGE_2 in the presence of protein synthesis inhibitor cycloheximide (Figure 3.33). These findings indicate that PGE_2-induced c-Myc protein expression can be attributed, at least in part, to the increased protein stability through activation of Erk pathway.

Our study has demonstrated that PGE_2 stimulates cell proliferation predominantly through activation of EP2 receptor. In line with this finding, EP2 receptor agonist also increases c-Myc expression to a similar extent to that of PGE_2 whilst activation of other EP receptor subtypes shows no influence on c-Myc expression (Figure 3.38).

In summary, this study reveals for the first time that PGE_2 up-regulates c-Myc via the EP2/Erk pathway to stimulate cell proliferation in human esophageal squamous cell carcinoma cells. These findings provide a novel mechanism for the carcinogenic actions of COX-2/PGE_2 in human esophageal squamous cell carcinoma.

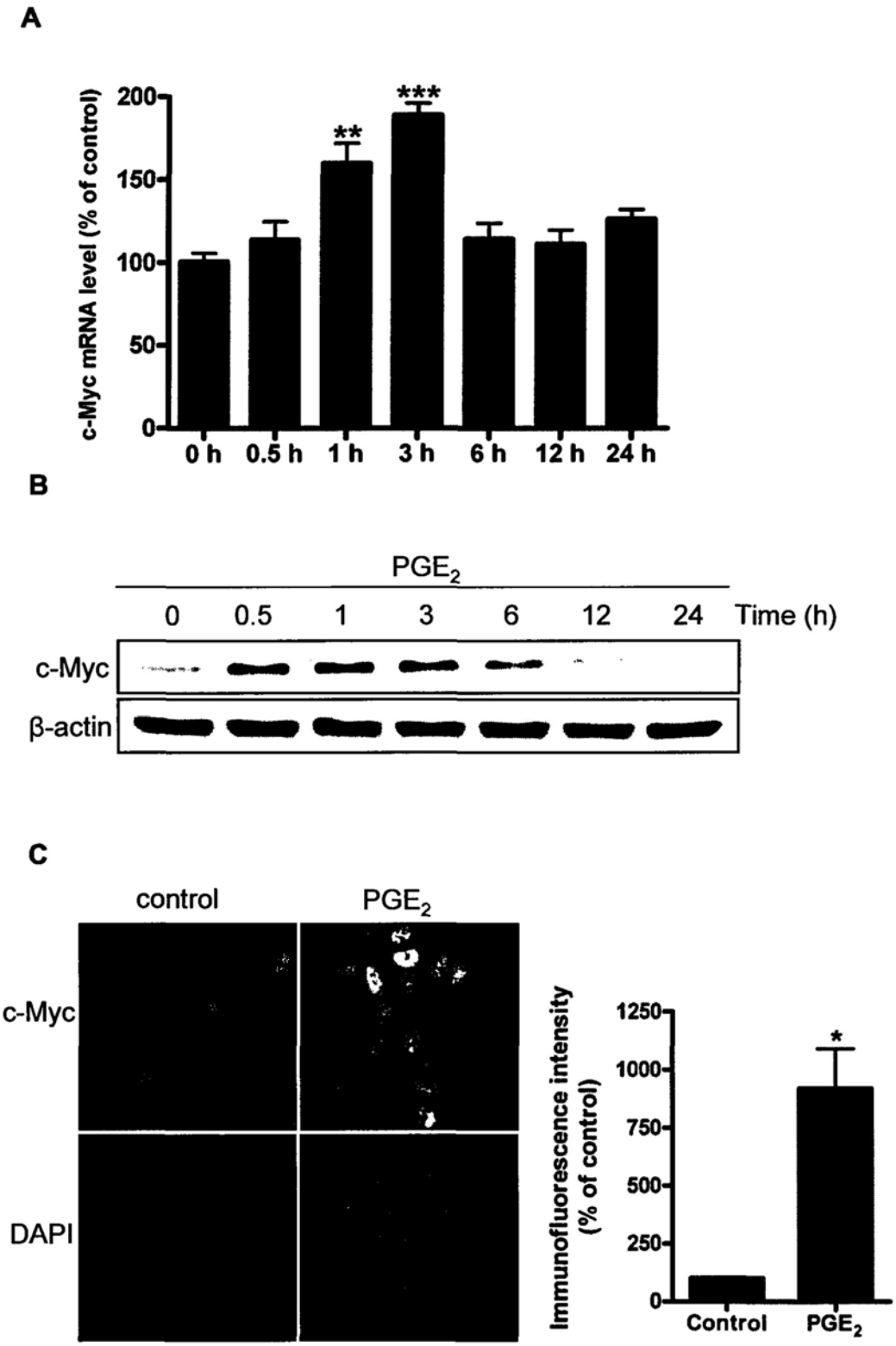

A
c-Myc mRNA level (% of control)
200
150
100
50
0
**
***
0 h
0.5 h
1 h
3 h
6 h
12 h
24 h
B
PGE2
0
0.5
1
3
6
12
24
Time (h)
c-Myc
β-actin
C
control
PGE2
c-Myc
DAPI
Immunofluorescence intensity (% of control)
1250
1000
750
500
250
0
*
Control
PGE2

Figure 3.30 The upregulation of c-Myc by PGE_2 treatment in HKESC-1 cells. Cells were deprived of serum for 24 h and thereafter treated with PGE_2 as indicated time points. (A) Real-time PCR revealed that treating HKESC-1 cells with PGE_2 (10 μmol/L) significantly increased the mRNA expression of c-Myc from 1 h to 3 h. **, $p<0.01$ and ***, $p<0.001$ versus the control group. Representative results from three independent experiments are shown. (B) PGE_2 (10 μmol/L) substantially increased c-Myc protein expression determined by Western blot analysis. β-actin was used to evaluate protein loading. Representative results from three independent experiments are shown. (C) HKESC-1 cells treated with PGE_2 (10 μM) for 30 min exhibited stronger nuclear florescent signaling for c-Myc as determined by immunofluorescence staining. The immunofluorescence intensities of c-Myc protein were expressed as the mean percentage of control ± SEM of three independent experiments in which at least 100 fields from at least 10 independent petri-dishes were counted from each experiment. *, $p<0.05$ versus the control group.

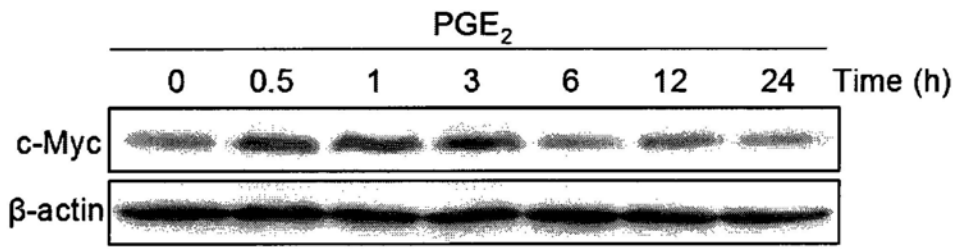


Figure 3.31 The upregulation of c-Myc by PGE_2 treatment in HKESC-2 cells. PGE_2 (10 μM) substantially increased c-Myc protein expression determined by Western blot analysis. β-actin was used to evaluate protein loading. Data shown are representative of three independent experiments.

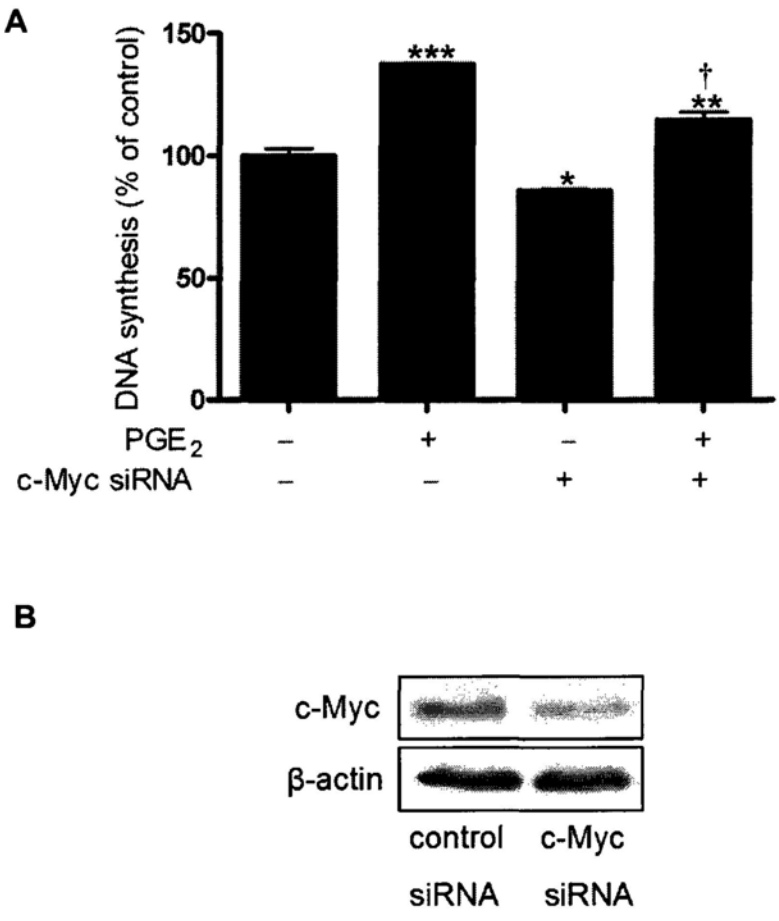


Figure 3.32 Effects of c-Myc on the proliferation of HKESC-1 cells. (A) After transfection with the c-Myc siRNA, HKESC-1 cells were treated with 10 μM PGE_2 for 24 h and examined for proliferation by [^{3}H]thymidine incorporation assay. *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$ versus the control siRNA-transfected group; †, $p<0.001$ versus the control siRNA-transfected group treated with PGE_2. (B) The efficacy of the c-Myc depletion by specific siRNA was verified by Western blot analysis. Non-targeting siRNA was used as control siRNA. β-actin was used to evaluate protein loading. Representative results from three independent experiments are shown.

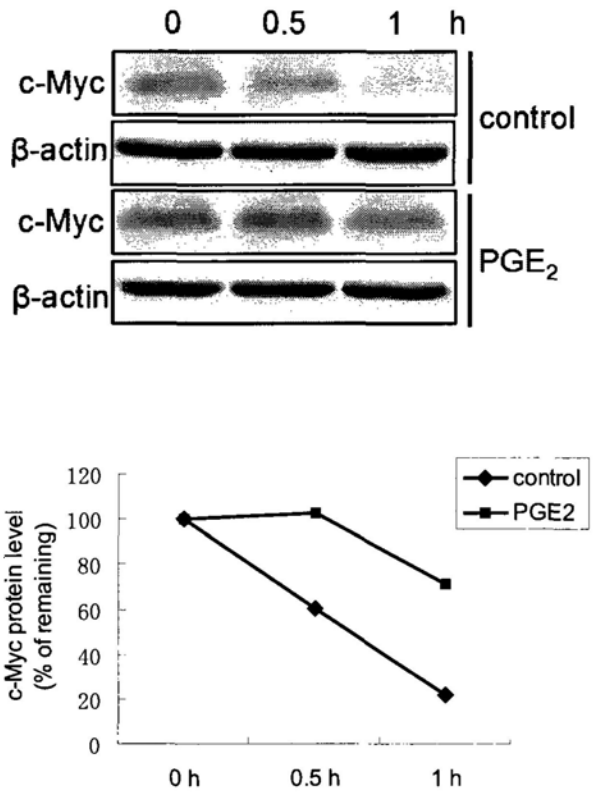


Figure 3.33 Alteration of protein stability in PGE_2-induced c-Myc expression. Serum-deprived HKESC-1 cells were incubated with protein synthesis inhibitor cycloheximide (100 μg/ml) for 30 min and stimulated by addition of PGE_2 (10 μM). Protein was collected from cells at various times after addition of PGE_2 and analyzed by Western blot. β-actin was used to evaluate protein loading. Representative results from three independent experiments are shown.

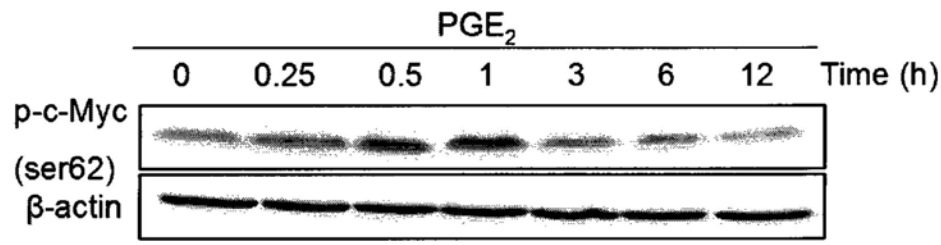


Figure 3.34 The effect of PGE_2 on c-Myc phosphorylation on Serine 62 in HKESC-1 cells. Serum-deprived HKESC-1 cells were exposed to 10 μM PGE_2 as indicated time point for the determination of phosphorylated c-Myc on Serine 62 expression by Western blot analysis. β-actin was used to evaluate protein loading. Representative results from three independent experiments are shown.

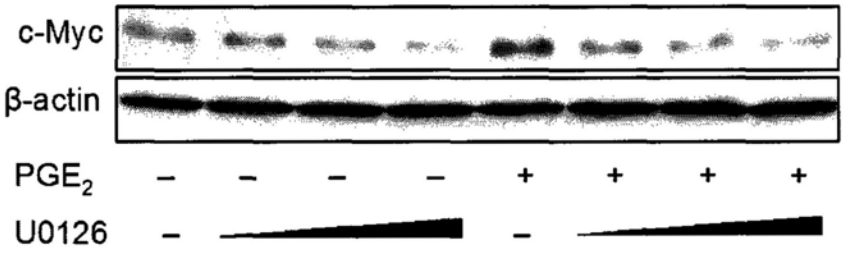


Figure 3.35 The effect of MEK inhibitor U0126 on PGE_2-induced c-Myc protein expression in HKESC-1 cells. HKESC-1 cells were pretreated with the MEK inhibitor U0126 (10, 100 and 1000 nM) for 30 min before treatment with 10 μM PGE_2 for an additional 30 min. Thereafter, cells were harvested for determination of c-Myc protein level by Western blot analysis. β-actin was used to evaluate protein loading. Representative results from three independent experiments are shown.

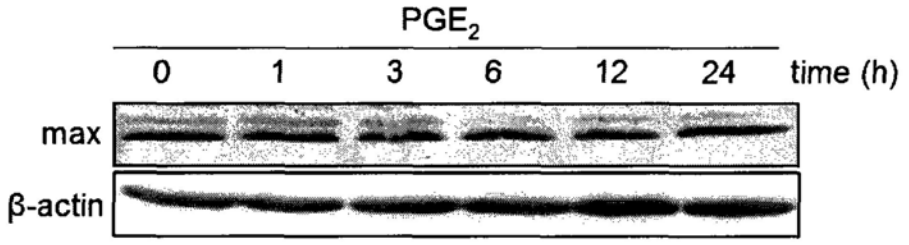


Figure 3.36 The effect of PGE_2 on Max protein expression in HKESC-1 cells. Serum-deprived cells were treated with 10 μM PGE_2 at different time points as indicated, then cells were harvested for Western blot analysis. β-actin was used to evaluate protein loading. Representative results from three independent experiments are shown.

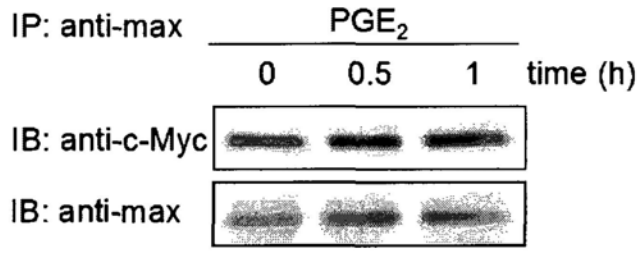


Figure 3.37 The effect of PGE_2 on the association between Max and c-Myc in HKESC-1 cells. Serum-deprived cells were stimulated with PGE_2 at 10 μM. Cells lysates were then prepared and subjected to immunoprecipitation (IP) with anti-Max antibody, followed by SDS-PAGE and immunoblotting (IB) with anti-c-Myc and anti-Max antibodies respectively. Immunoprecipitated c-Myc (upper panel) and Max (lower panel) were shown. Representative results from three independent experiments are shown.

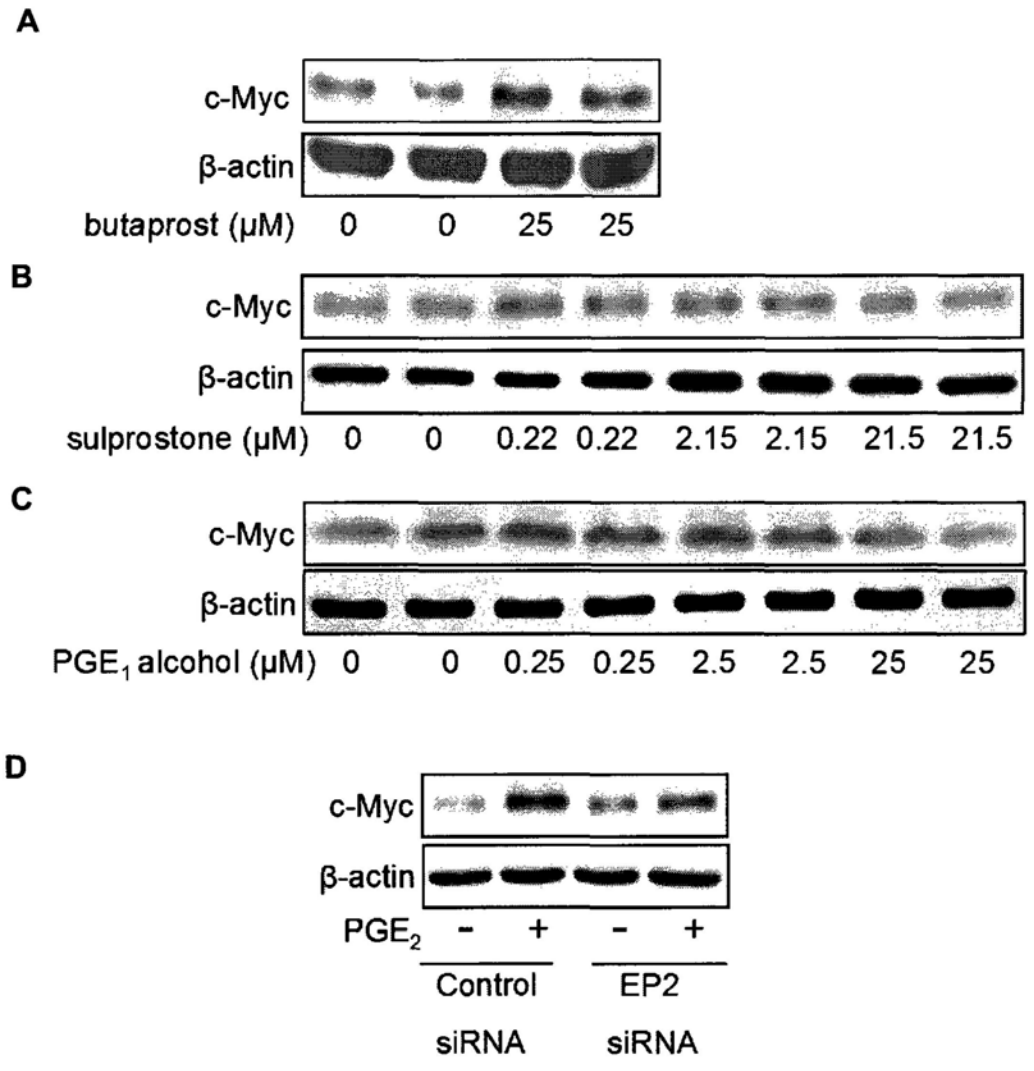

A
c-Myc
β-actin
butaprost (μM) 0 0 25 25
B
c-Myc
β-actin
sulprostone (μM) 0 0 0.22 0.22 2.15 2.15 21.5 21.5
C
c-Myc
β-actin
PGE1 alcohol (μM) 0 0 0.25 0.25 2.5 2.5 25 25
D
c-Myc
β-actin
PGE2 - + - +
Control siRNA
EP2 siRNA

Figure 3.38 Effects of different EP receptor agonists on c-Myc protein expression in HKESC-1 cells. Serum-deprived cells were treated with EP2 receptor agonist butaprost (A), EP3/EP1 agonist sulprostone (B), or EP4/EP3 agonist PGE_1 alcohol (C) for 30 min, and then cells were harvested for the determination of c-Myc protein expression by Western blot analysis. (D) After transfection with the EP2 siRNA, serum-deprived cells were treated with 10 μmol/L PGE_2 for 30 min and examined for c-Myc protein expression by Western blot analysis. Non-targeting siRNA was used as control siRNA. β-actin was used to evaluate protein loading. Representative results from three independent experiments are shown.

3.5 Indomethacin and COX-2 selective inhibitor SC236 enhanced the cytotoxic action of doxorubicin

3.5.1 Indomethacin and SC236 enhanced doxorubicin-induced cytotoxicity both in HKESC-1 and HKESC-2 cells

To study whether COX-inhibitors could enhance the cytotoxic action of doxorubicin, HKESC-1 cells (Figures 3.39-3.43) and HKESC-2 cells (Figures 3.44-3.48) were treated with doxorubicin (0-10 μM) in the absence or presence of the indomethacin (20 μM), COX-2 selective inhibitors SC236 (10 μM), NS398 (10 μM) and nimesulide (10 μM), or COX-1 selective inhibitor SC560 (20 μM). Doxorubicin decreased cell viability dose-dependently both in HKESC-1 and HKESC-2 cells with EC_{50} of around 1.2 μM and 0.8 μM, respectively. Co-treatment of HKESC-1 cells with indomethacin or SC236, both of which alone had no effect on cell viability, decreased the EC_{50} of doxorubicin from 1.2 μM to 0.4 μM and 0.6 μM, respectively. Similarly, indomethacin and SC236 also enhanced the cytotoxic action of doxorubicin in HKESC-2 cells, which decreased the EC_{50} from 0.8 μM to 0.3 μM and 0.4 μM, respectively. In contrast, COX-1 selective inhibitor SC560 and the other two tested COX-2 selective inhibitors NS398 and nimesulide exerted no effect on the action of doxorubicin in both cell lines. In order to determine the minimal effective concentrations of indomethacin and SC236, HKESC-1 cells were incubated with various concentrations of indomethacin or SC236 in combination with doxorubicin at the concentration of 1.25 μM, which was around its EC_{50} for HKESC-1 cells. Both indomethacin and SC236 at the concentration as low as 2.5 μM significantly

enhanced the cytotoxic action of doxorubicin (Figure 3.49 and 3.50). Similarly, these two COX-inhibitors using the same concentration significantly sensitized HKESC-2 cells to the cytotoxic action of doxorubicin (0.8 μM, the EC_{50} for HKESC-2 cells) (Figure 3.51 and 3.52).

3.5.2 Indomethacin and SC236 increased intracellular accumulation and retention of doxorubicin in HKESC-1 cells

Doxorubicin is an auto-fluorescent compound, which enables the visualization of its presence by confocal microscopy. Figure 3.53A showed the fluorescence of intracellular accumulated doxorubicin in HKESC-1 cells after incubation for 6 h with 1.25 μM doxorubicin in the absence or presence of 20 μM indomethacin or 10 μM SC236. Fluorescent signals in cells co-treated with doxorubicin and indomethacin or SC236 were much stronger than those in cells treated with doxorubicin alone, indicating both indomethacin and SC236 were able to increase the intracellular accumulation of doxorubicin. For the doses tested (1.25, 5, and 20 μM), the minimal concentration of indomethacin to significantly increase intracellular accumulation of doxorubicin was 5 μM (Figure 3.53B). In parallel, different doses (0.625, 2.5, and 10 μM) of SC236 were examined. The minimal concentration of SC236 to significantly increase doxorubicin accumulation was 2.5 μM (Figure 3.53C). In contrast, fluorescent signals in cells co-treated with doxorubicin and SC560 (20 μM), nimesulide (10 μM) or NS398 (10 μM) were similar to those in cells treated with doxorubicin alone (Figure 3.54).

In relation to intracellular retention of doxorubicin, Figure 3.55A showed the results of HKESC-1 cells incubated for 6 h with 1.25 μM doxorubicin followed by incubation for another 6 h in normal growth medium or in medium containing 20 μM indomethacin or 10 μM SC236. Results showed that only a small amount of previously accumulated doxorubicin left in cells after withdrawal of doxorubicin for 6 h. In contrast, when cells treated with indomethacin or SC236 contained much more doxorubicin than those that were incubated in normal growth medium after doxorubicin withdrawal. These findings indicated that both indomethacin and SC236 increased the intracellular retention of doxorubicin in esophageal cancer cells. Again for the doses tested (1.25, 5, and 20 μM), the minimal concentration of indomethacin to significantly increase intracellular retention of doxorubicin was 5 μM (Figure 3.55B). In parallel, for the doses tested (0.625, 2.5, and 10 μM), the minimal concentration of SC236 to significantly affect doxorubicin retention was 2.5 μM (Figure 3.55C). Unlike indomethacin and SC236, SC560, nimesulide or NS398 failed to augment doxorubicin retention in cells (Figure 3.56).

3.5.3 PGE_2 failed to reverse the enhancing effect of indomethacin or SC236 on the cytotoxicity of doxorubicin in HKESC-1 cells

As the enhancing effect of indomethacin or SC236 on the cytotoxicity of doxorubicin may be due to COX-2 inhibition, we therefore determined whether the major COX-2 end product PGE_2 could reverse the observed effects. The basal PGE_2 level released by HKESC-1 cells was 332 pg/ml, or around 10^{-3} μM (date not shown).

Moreover, it has been reported that PGE_2 at the concentration of 3 μg/ml (8.5 μM) neutralized the enhancing effect of the COX-2 selective inhibitor meloxicam on the cytotoxic action of doxorubicin in acute myeloid leukemia HL-60 cells (Puhlmann et al., 2005). Therefore, PGE_2 at concentrations ranging from 10^{-3} to 10 μM were employed in our study. As shown in Figures 3.57 and 3.58, PGE_2 alone did not influence the cytotoxic action of doxorubicin, moreover, it also failed to reverse the enhancing effect of indomethacin or SC236 on cytotoxicity.

The involvement of PGE_2 in the enhancing effect of indomethacin or SC236 was further excluded by the findings that SC560, NS398 and nimesulide suppressed PGE_2 release from HKESC-1 cells to a similar extent to that of indomethacin or SC236 (Figure 3.59A), however, unlike indomethacin or SC236, these three COX-inhibitors failed to enhance the cytotoxic action of doxorubicin on cancer cells. In addition, indomethacin at three tested concentrations (1.25, 5, and 20 μM) significantly decreased PGE_2 release to a similar extent in which there was no significant difference among these concentrations (Figure 3.59B). However, indomethacin at concentrations of 5 and 20 μM, not 1.25 μM, enhanced the cytotoxicity of doxorubicin. In parallel, SC236 at three tested concentrations (0.625, 2.5, and 10 μM) significantly reduced PGE_2 release to a similar extent (Figure 3.59C). However, SC236 only at the concentrations of 2.5 and 10 μM, not 0.625 μM, enhanced the cytotoxic action of doxorubicin. These findings further suggest that PGE_2 is not involved in the enhancing effect of indomethacin and SC236.

3.5.4 Down-regulation of COX-2 expression was unable to enhance doxorubicin-induced cytotoxicity in HKESC-1 cells

Since only SC236 among the three tested COX-2 inhibitors showed enhancing effect on doxorubicin-induced cytotoxicity, the direct role of COX-2 in this action was further investigated by RNA interference experiments. The efficacy of COX-2 depletion by COX-2 siRNA was verified by Western blot analysis (Figure 3.60A). Moreover, down-regulation of COX-2 expression substantially decreased PGE_2 level as shown in Figure 3.60B. In relation to the role of COX-2 in the cytotoxic action of doxorubicin, Figure 3.60C showed that down-regulation of COX-2 and subsequent reduction of PGE_2 level was unable to enhance doxorubicin-induced cytotoxicity, indicating that inhibition of COX-2 is not responsible for the enhancing effect of indomethacin or SC236.

3.5.5 Preliminary discussion and conclusion

A growing body of evidence has demonstrated that NSAIDs and COX-2 selective inhibitors enhance the cytotoxic action of certain chemotherapeutic drugs in a variety of cancer cells (Draper et al., 1997; Duffy et al., 1998; Roller et al., 1999; Awara et al. 2004; O'Connor et al., 2004; Zatelli et al., 2005; Puhlmann et al., 2005; Zatelli et al., 2007; Zrieki et al., 2008). In agreement with these findings, our results showed that indomethacin and the COX-2 selective inhibitor SC236 sensitized human esophageal squamous cell carcinoma cells (HKESC-1 and HKESC-2) to the cytotoxic action of doxorubicin. Similar enhancing effect of indomethacin and

SC236 was also observed in a human gastric adenocarcinoma cell line TMK1 (Figures 3.61 and 3.62). Although both compounds are COX inhibitors, we present evidence that their enhancing effect on the cytotoxicity of doxorubicin is COX-independent. First of all, unlike SC236, COX-2 selective inhibitors NS398 and nimesulide and COX-1 selective inhibitor SC560 showed no influence on cytotoxic action of doxorubicin, although all of these tested COX-inhibitors suppressed PGE_2 production to a similar extent. Moreover, exogenous supplementation of COX product PGE_2 failed to reverse the enhancing effect of indomethacin or SC236. In addition to pharmacological approach, siRNA-mediated knockdown of COX-2 also showed no effect on the cytotoxic action of doxorubicin, which provides the direct evidence for a COX-2-independent mechanism.

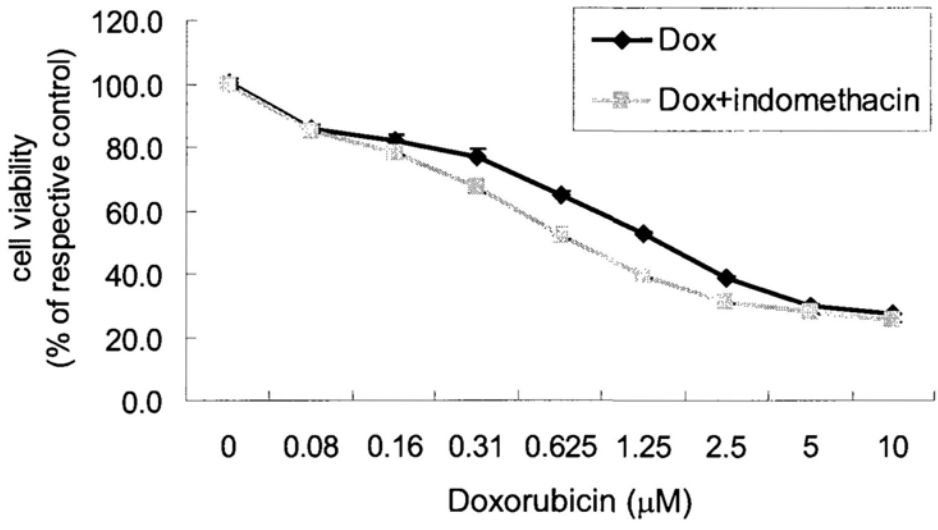


Figure 3.39 Effects of indomethacin on doxorubicin-induced cytotoxicity in HKESC-1 cells. Cells were treated for 24 h with doxorubicin (0-10 μM) alone, or in combination with indomethacin (20 μM). Cell viability was then determined by MTT assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate.

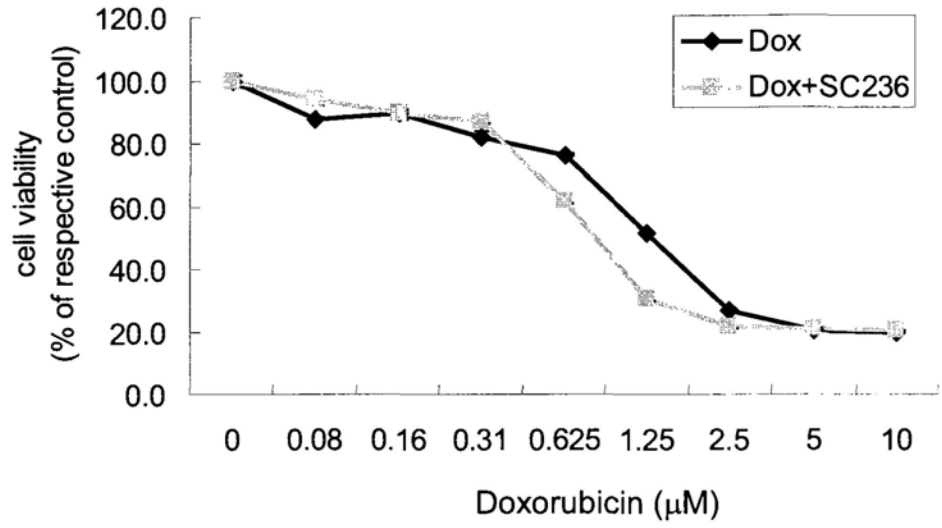


Figure 3.40 Effects of SC236 on doxorubicin-induced cytotoxicity in HKESC-1 cells. Cells were treated for 24 h with doxorubicin (0-10 μM) alone, or in combination with SC236 (10 μM). Cell viability was then determined by MTT assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate.

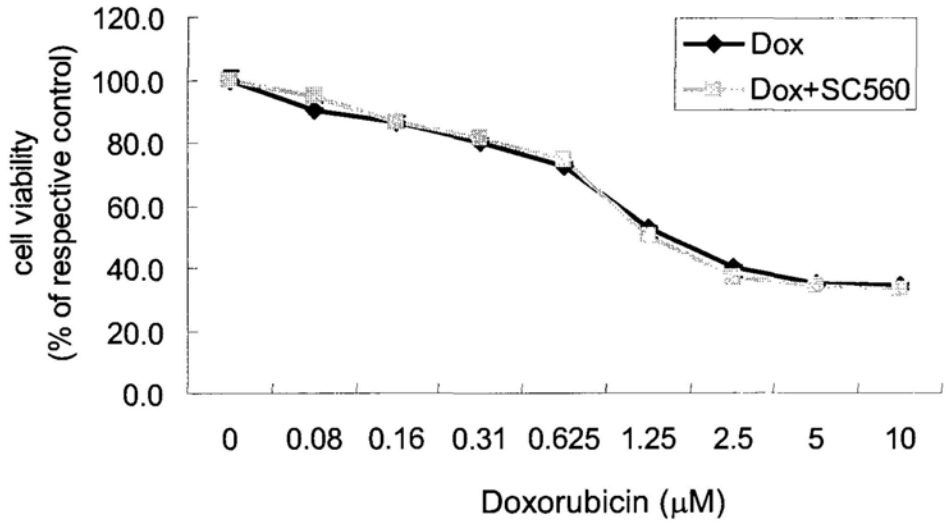


Figure 3.41 Effects of SC560 on doxorubicin-induced cytotoxicity in HKESC-1 cells. Cells were treated for 24 h with doxorubicin (0-10 μM) alone, or in combination with SC560 (20 μM). Cell viability was then determined by MTT assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate.

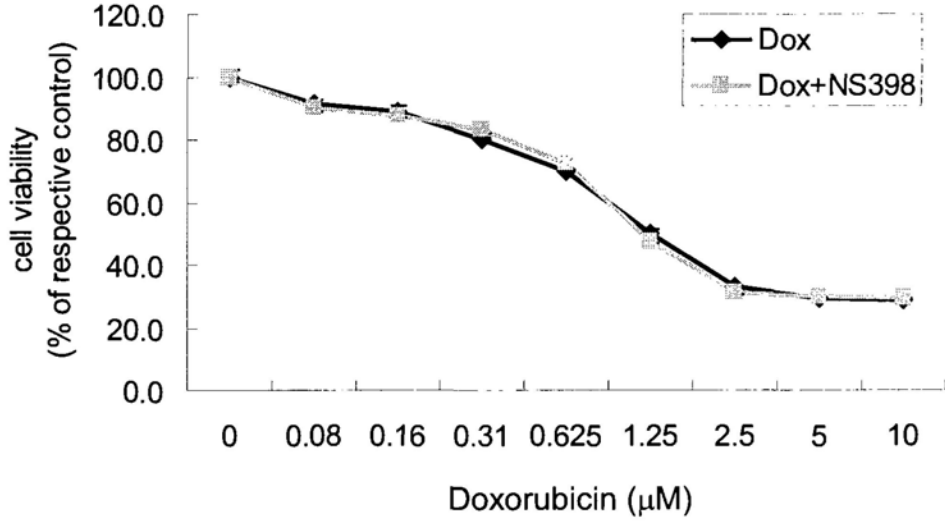


Figure 3.42 Effects of NS398 on doxorubicin-induced cytotoxicity in HKESC-1 cells. Cells were treated for 24 h with doxorubicin (0-10 μM) alone, or in combination with NS398 (10 μM). Cell viability was then determined by MTT assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate.

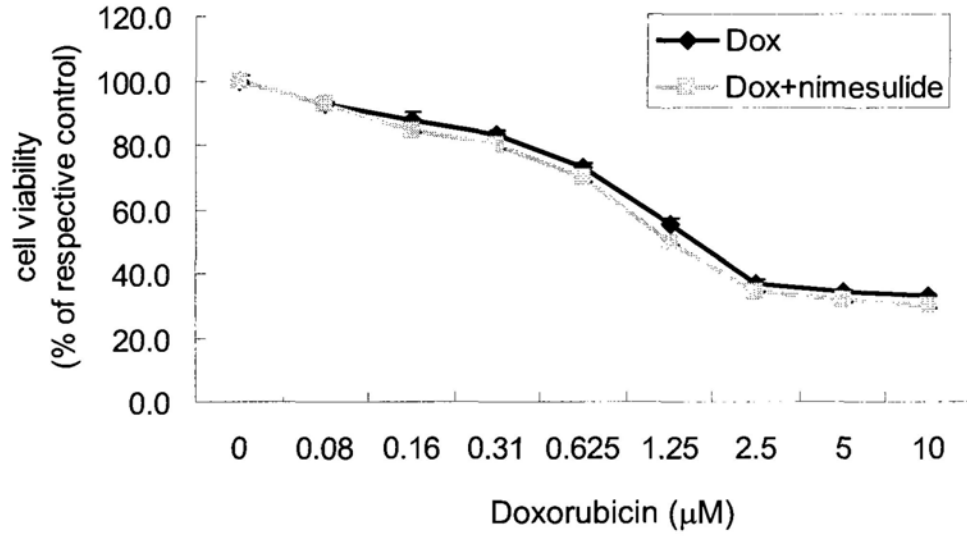


Figure 3.43 Effects of nimesulide on doxorubicin-induced cytotoxicity in HKESC-1 cells. Cells were treated for 24 h with doxorubicin (0-10 μM) alone, or in combination with nimesulide (10 μM). Cell viability was then determined by MTT assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate.

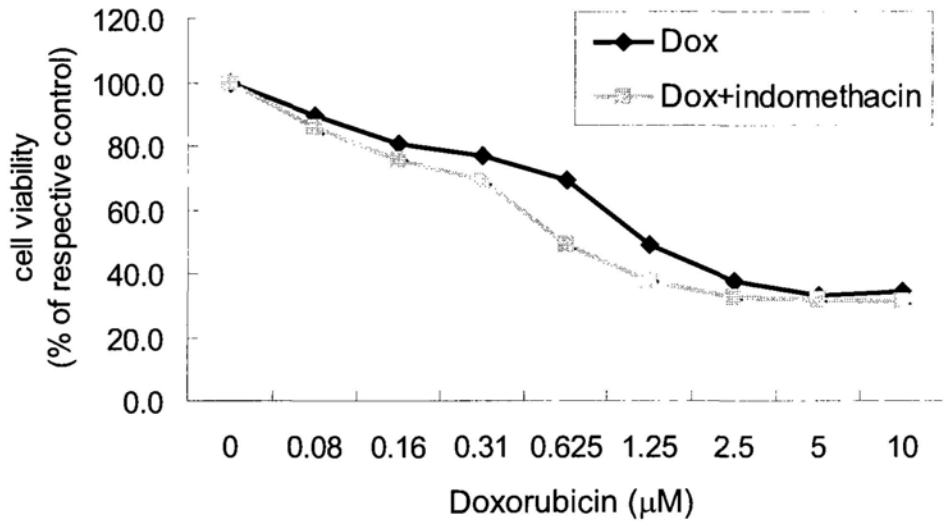


Figure 3.44 Effects of indomethacin on doxorubicin-induced cytotoxicity in HKESC-2 cells. Cells were treated for 24 h with doxorubicin (0-10 μM) alone, or in combination with indomethacin (20 μM). Cell viability was then determined by MTT assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate.

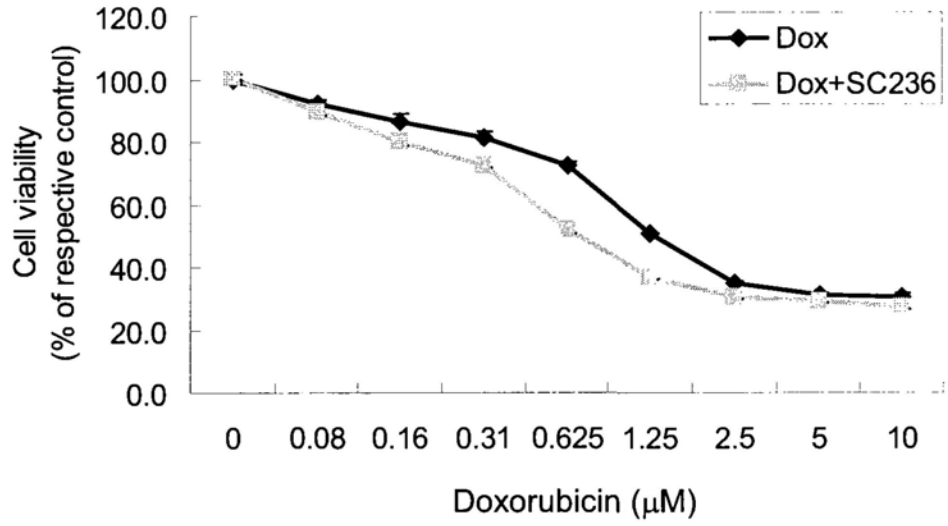


Figure 3.45 Effects of SC236 on doxorubicin-induced cytotoxicity in HKESC-2 cells. Cells were treated for 24 h with doxorubicin (0-10 μM) alone, or in combination with SC236 (10 μM). Cell viability was then determined by MTT assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate.

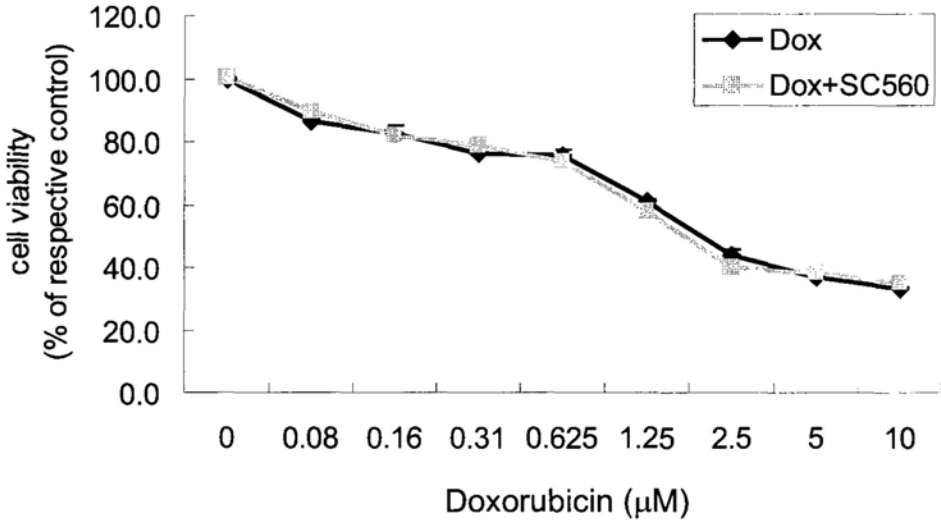


Figure 3.46 Effects of SC560 on doxorubicin-induced cytotoxicity in HKESC-2 cells. Cells were treated for 24 h with doxorubicin (0-10 μM) alone, or in combination with SC560 (20 μM). Cell viability was then determined by MTT assay. Data are presented as mean±SEM (n=3) of a representative experiment performed in triplicate.

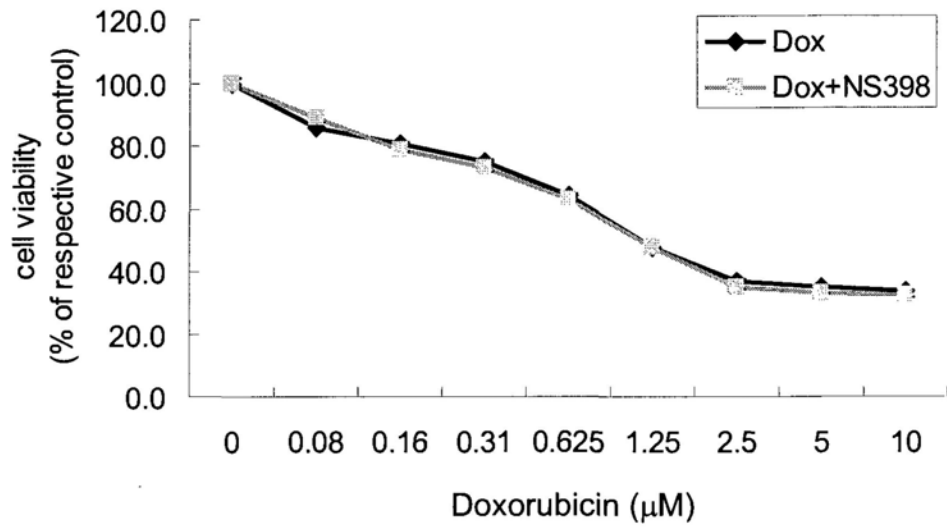


Figure 3.47 Effects of NS398 on doxorubicin-induced cytotoxicity in HKESC-2 cells. Cells were treated for 24 h with doxorubicin (0-10 μM) alone, or in combination with NS398 (10 μM). Cell viability was then determined by MTT assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate.

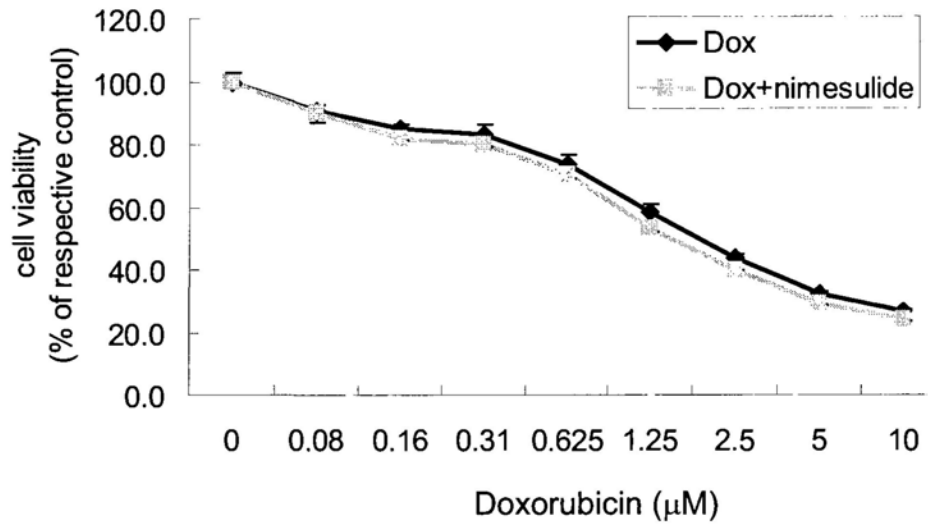


Figure 3.48 Effects of nimesulide on doxorubicin-induced cytotoxicity in HKESC-2 cells. Cells were treated for 24 h with doxorubicin (0-10 μM) alone, or in combination with nimesulide (10 μM). Cell viability was then determined by MTT assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate.

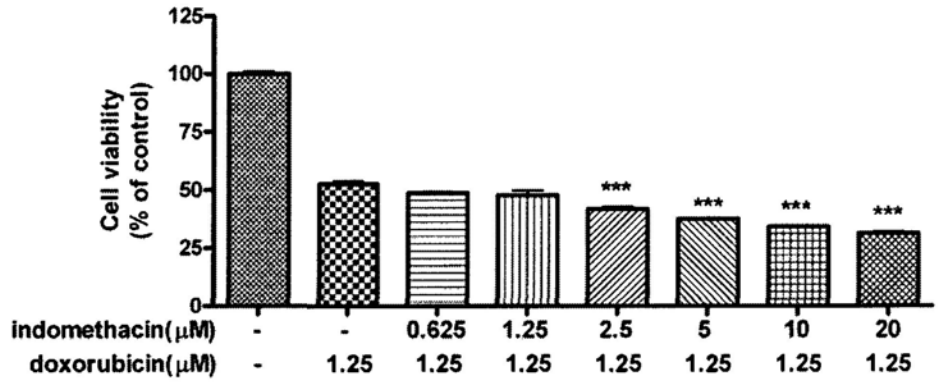


Figure 3.49 The minimal effective concentration of indomethacin to enhance cytotoxicity of doxorubicin in HKESC-1 cells. HKESC-1 cells were incubated with 1.25 μM doxorubicin under treatment of various concentrations of indomethacin for 24 h. Cell viability was then determined by MTT assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. ***, $p<0.001$compared with doxorubicin-treated group.

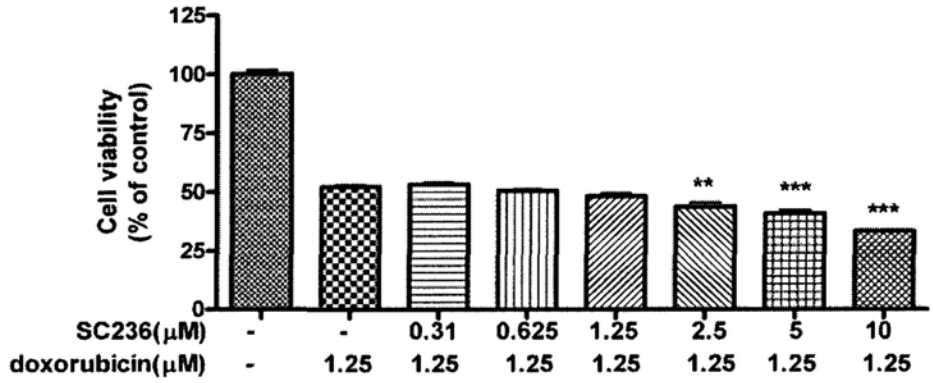


Figure 3.50 The minimal effective concentration of SC236 to enhance cytotoxicity of doxorubicin in HKESC-1 cells. HKESC-1 cells were incubated with 1.25 μM doxorubicin under treatment of various concentrations of SC236 for 24 h. Cell viability was then determined by MTT assay. Data are presented as mean±SEM (n=3) of a representative experiment performed in triplicate. **, $p<0.01$ and ***, $p<0.001$compared with doxorubicin-treated group.

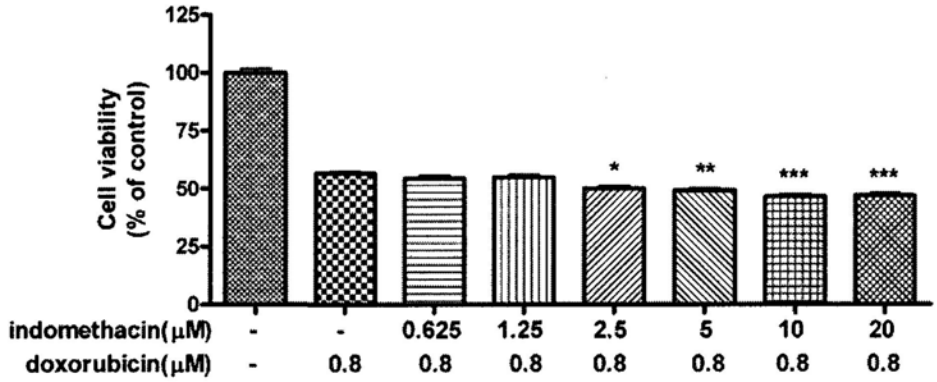


Figure 3.51 The minimal effective concentration of indomethacin to enhance cytotoxicity of doxorubicin in HKESC-2 cells. HKESC-2 cells were incubated with 0.8 μM doxorubicin under treatment of various concentrations of indomethacin for 24 h. Cell viability was then determined by MTT assay. Data are presented as mean ±SEM (n=3) of a representative experiment performed in triplicate. *, $p<0.05$, **, $p<0.01$, and ***, $p<0.001$compared with doxorubicin-treated group.

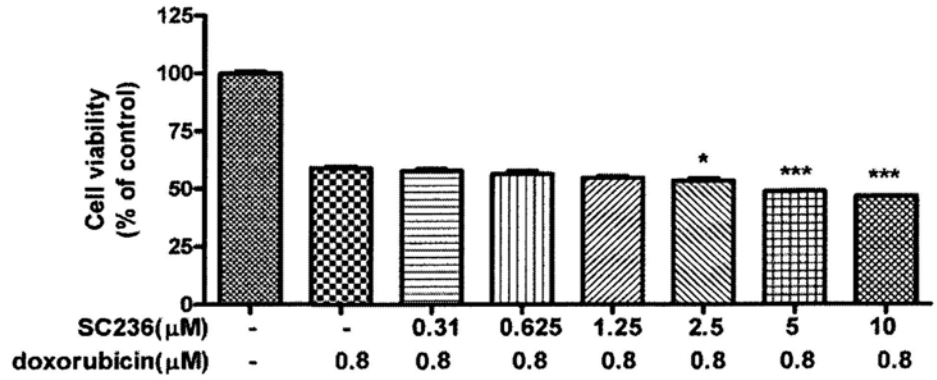
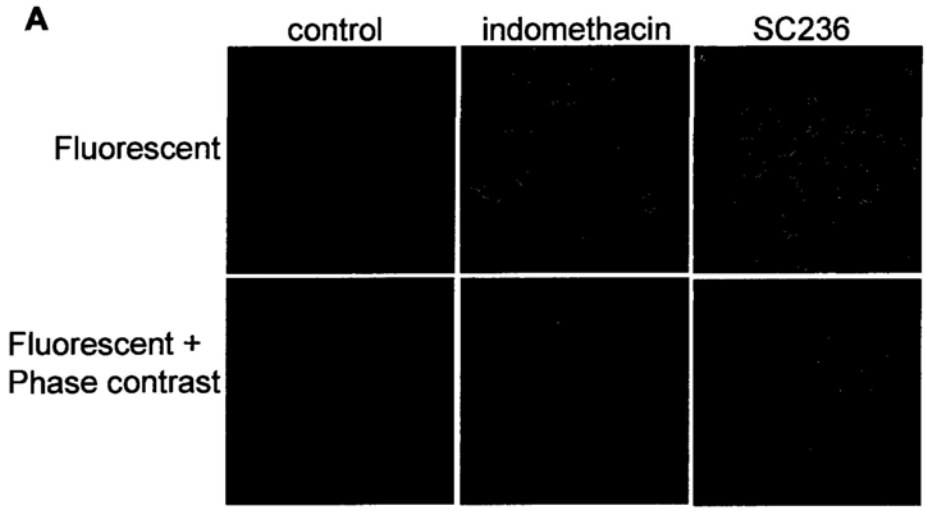
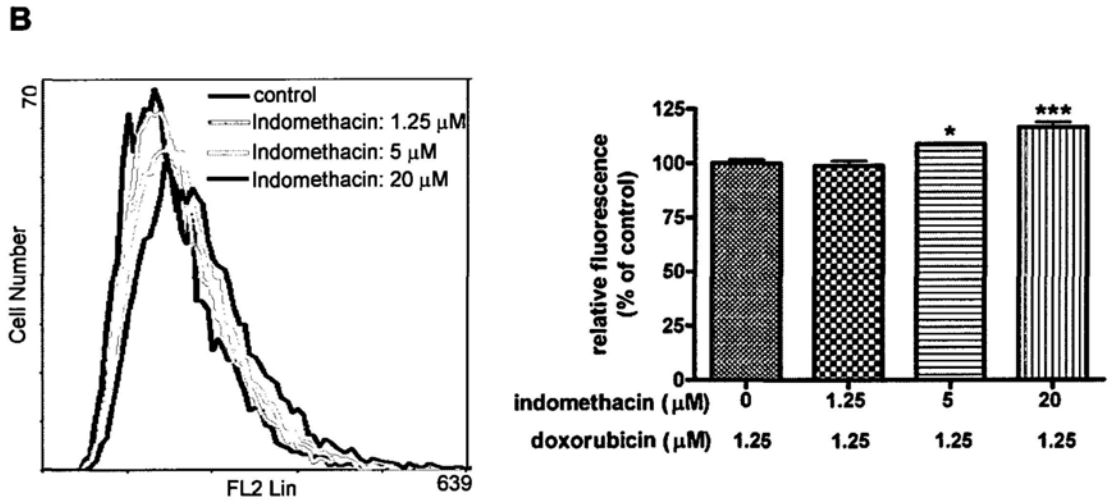


Figure 3.52 The minimal effective concentration of SC236 to enhance cytotoxicity of doxorubicin in HKESC-2 cells. HKESC-2 cells were incubated with 0.8 μM doxorubicin under treatment of various concentrations of SC236 for 24 h. Cell viability was then determined by MTT assay. Data are presented as mean±SEM (n=3) of a representative experiment performed in triplicate. *, $p<0.05$ and ***, $p<0.001$ compared with doxorubicin-treated group.


A
control
indomethacin
SC236
Fluorescent
Fluorescent +
Phase contrast


B
70
Cell Number
control
Indomethacin: 1.25 μM
Indomethacin: 5 μM
Indomethacin: 20 μM
FL2 Lin
639
relative fluorescence
(% of control)
125
100
75
50
25
0
*
***
indomethacin (μM) 0 1.25 5 20
doxorubicin (μM) 1.25 1.25 1.25 1.25

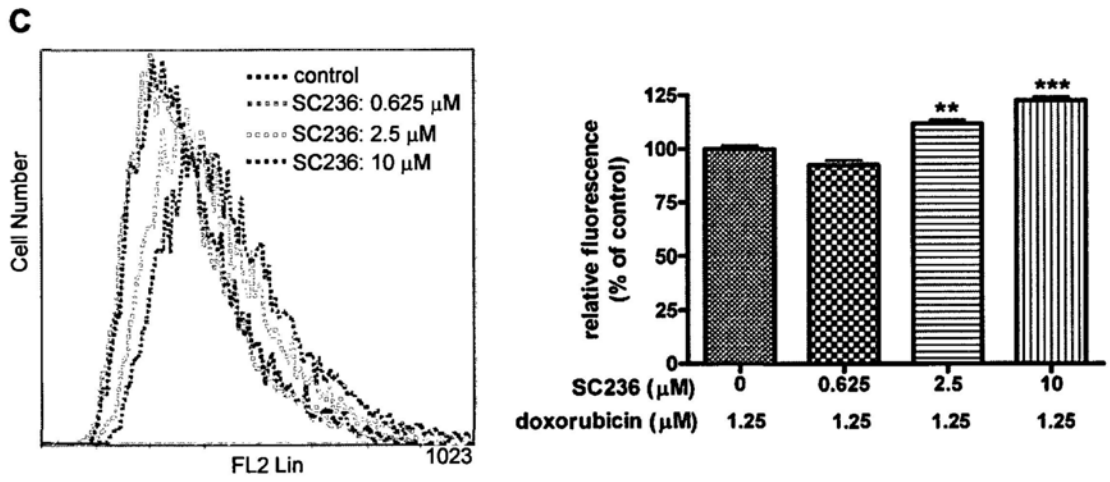

C
Cell Number
control
SC236: 0.625 μM
SC236: 2.5 μM
SC236: 10 μM
FL2 Lin
1023
relative fluorescence
(% of control)
125
100
75
50
25
0
**
***
SC236 (μM) 0 0.625 2.5 10
doxorubicin (μM) 1.25 1.25 1.25 1.25

Figure 3.53 Effects of indomethacin and SC236 on the intracellular accumulation of doxorubicin in HKESC-1 cells. (A) Shows fluorescent microscopic pictures of HKESC-1 cells that were incubated for 6 h in medium containing 1.25 μM doxorubicin alone, or in combination with 20 μM indomethacin or 10 μM SC236. Magnification: 400 ×. These results are representatives of three independent experiments. Histograms of the fluorescence intensity of doxorubicin in HKESC-1 cells under treatment of doxorubicin (1.25 μM) alone, or in combination with various concentrations of indomethacin (B) or SC236 (C). Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. *, $p<0.05$, **, $p<0.01$, and ***, $p<0.001$ compared with doxorubicin-treated group.

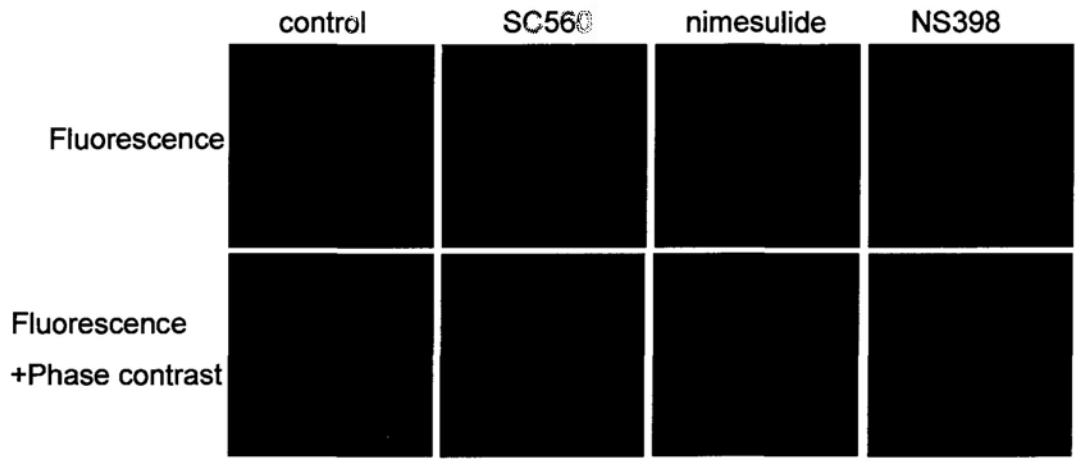
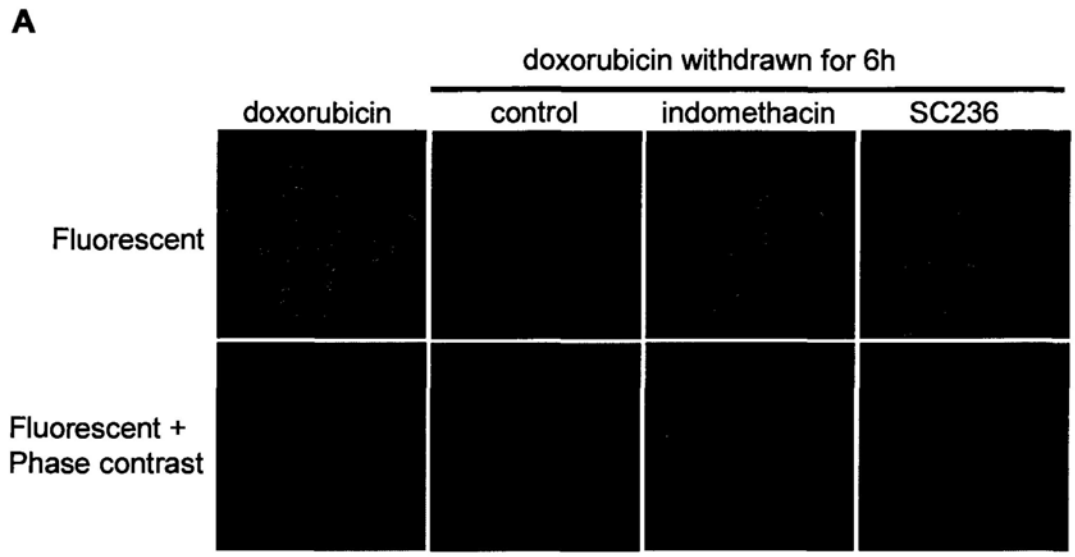
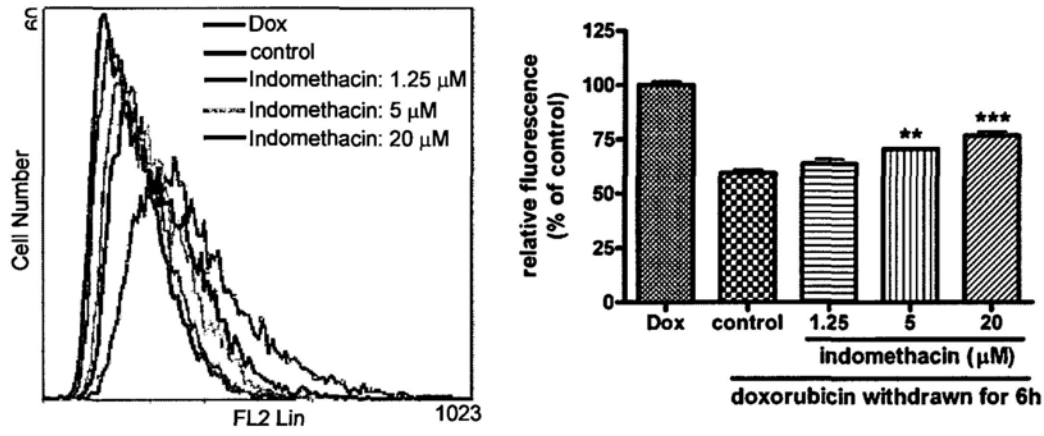


Figure 3.54 Effects of SC560, nimesulide and NS398 on the intracellular accumulation of doxorubicin in HKESC-1 cells. This figure shows fluorescent microscopic pictures of HKESC-1 cells that were incubated for 6 h in medium containing 1.25 μM doxorubicin alone, or in combination with 20 μM SC560, 10 μM nimesulide or 10 μM NS398. Magnification: 400×. These results are representatives of three independent experiments.


A
doxorubicin withdrawn for 6h
doxorubicin
control
indomethacin
SC236
Fluorescent
Fluorescent +
Phase contrast


B
Dox
control
Indomethacin: 1.25 μM
Indomethacin: 5 μM
Indomethacin: 20 μM
Cell Number
FL2 Lin
1023
relative fluorescence
(% of control)
125
100
75
50
25
0
**
***
Dox
control
1.25
5
20
indomethacin (μM)
doxorubicin withdrawn for 6h

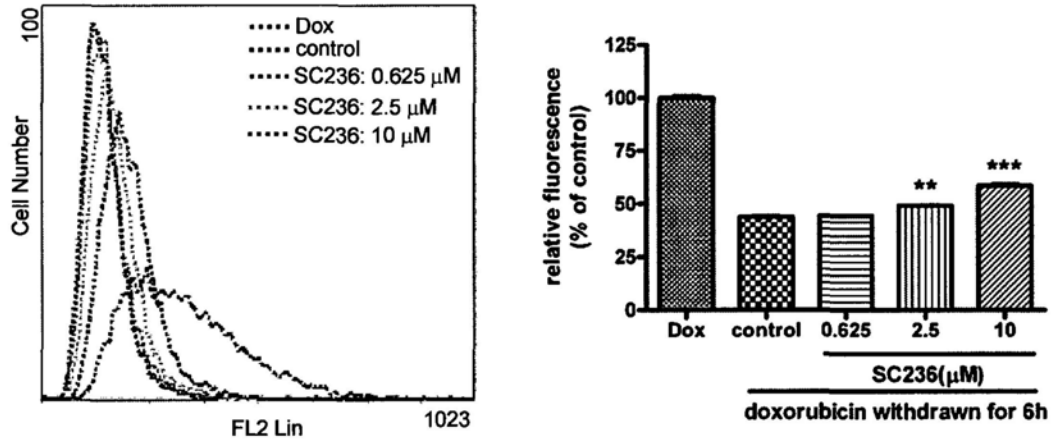

C
100
Dox
control
SC236: 0.625 μM
SC236: 2.5 μM
SC236: 10 μM
Cell Number
FL2 Lin
1023
relative fluorescence
(% of control)
125
100
75
50
25
0
**
***
Dox
control
0.625
2.5
10
SC236(μM)
doxorubicin withdrawn for 6h

Figure 3.55 Effects of indomethacin and SC236 on the intracellular retention of doxorubicin in HKESC-1 cells. (A) Shows fluorescent microscopic picture of HKESC-1 cells treated with 1.25 μM doxorubicin for 6 h, followed by 6 h incubation in normal growth medium or medium containing 20 μM indomethacin or 10 μM SC236. Magnification: 400×. These results are representatives of three independent experiments. Histograms of the fluorescence intensity of doxorubicin in HKESC-1 cells under treatment of doxorubicin (1.25 μM) for 6 h, followed by 6 h incubation in normal growth medium or medium containing various concentrations of indomethacin (B) or SC236 (C). Data are presented as mean±SEM (n=3) of a representative experiment performed in triplicate. **, $p<0.01$ and ***, $p<0.001$ compared with cells treated with doxorubicin for 6h followed by 6 h incubation in normal growth medium.

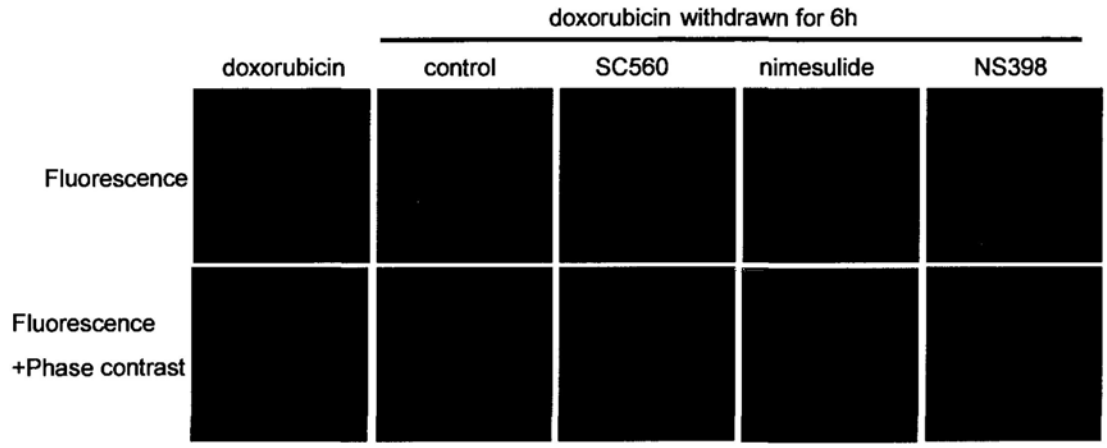


Figure 3.56 Effects of SC560, nimesulide and NS398 on the intracellular retention of doxorubicin in HKESC-1 cells. This figure shows fluorescent microscopic picture of HKESC-1 cells treated with 1.25 μM doxorubicin for 6 h, followed by 6 h incubation in normal growth medium or medium containing 20 μM SC560, 10 μM nimesulide or 10 μM NS398. Magnification: 400×. These results are representatives of three independent experiments.

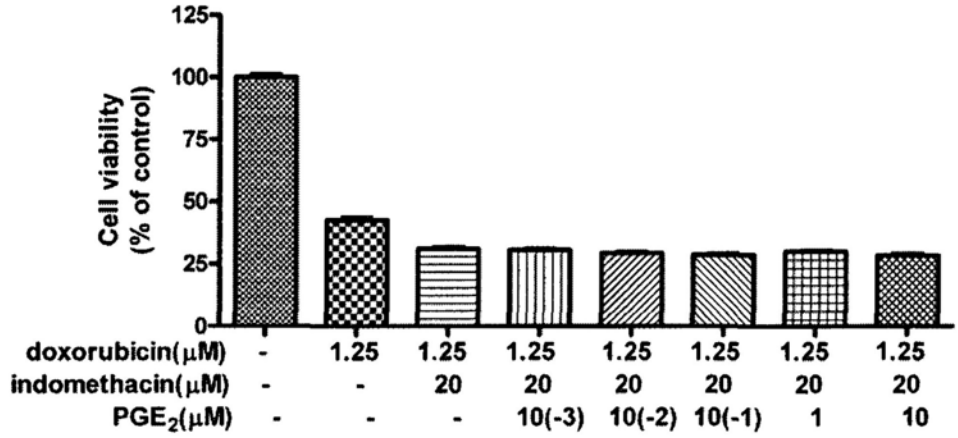


Figure 3.57 Effects of supplementation of PGE_2 on enhancement of doxorubicin cytotoxicity by indomethacin in HKESC-1 cells. Cells were treated with or without 1.25 μM doxorubicin in the absence or presence of PGE_2 (10^{-3} to 10 μM) and 20 μM indomethacin, alone or in combination for 24 h before determination of cell viability by MTT assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate.

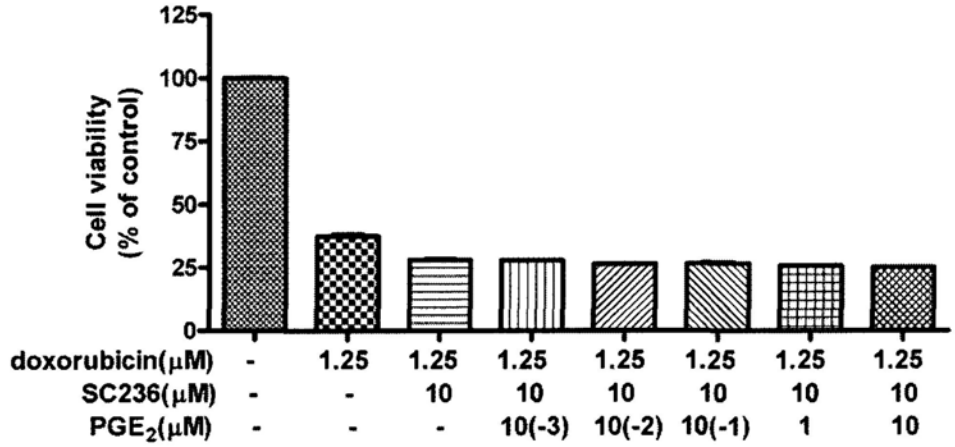
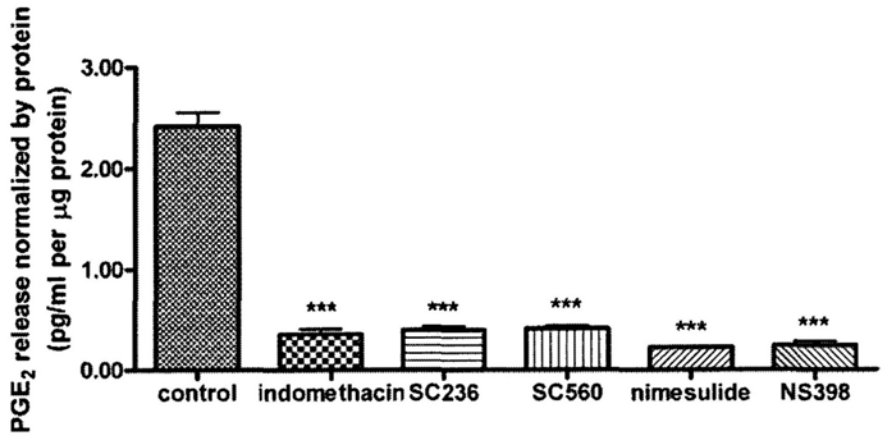
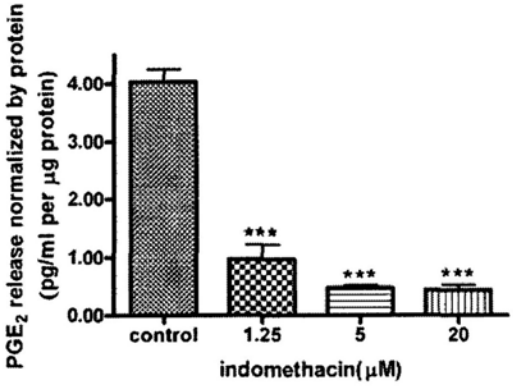


Figure 3.58 Effects of supplementation of PGE_2 on enhancement of doxorubicin cytotoxicity by SC236 in HKESC-1 cells. Cells were treated with or without 1.25 μM doxorubicin in the absence or presence of PGE_2 (10^{-3} to 10 μM) and 10 μM SC236, alone or in combination for 24 h before determination of cell viability by MTT assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate.

A


PGE2 release normalized by protein (pg/ml per μg protein)
3.00
2.00
1.00
0.00
***
control
indomethacin
SC236
SC560
nimesulide
NS398

B


PGE2 release normalized by protein (pg/ml per μg protein)
4.00
3.00
2.00
1.00
0.00
***
control
1.25
5
20
indomethacin(μM)

C

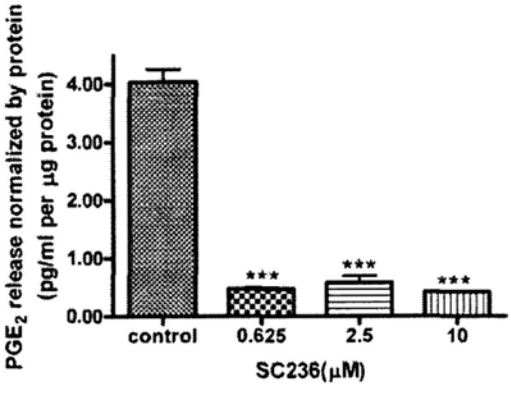
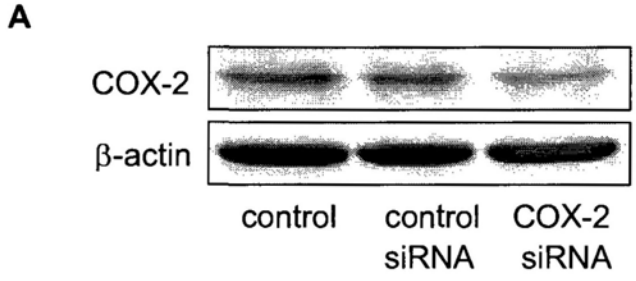
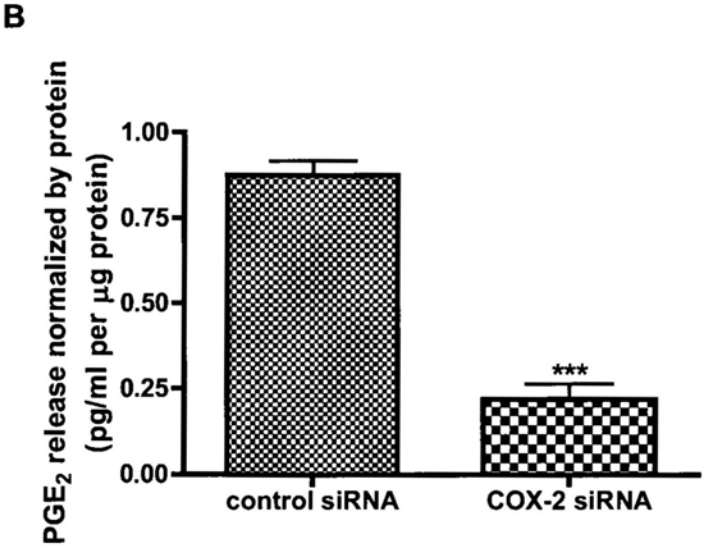

PGE2 release normalized by protein (pg/ml per μg protein)
4.00
3.00
2.00
1.00
0.00
***
control
0.625
2.5
10
SC236(μM)

Figure 3.59 Effects of COX-inhibitors on PGE_2 production in HKESC-1 cells. Cells were treated with indicated COX-inhibitors (20 μM indomethacin, 10 μM SC236, 20 μM SC560, 10 μM nimesulide, or 10 μM NS398) (A), various concentrations of indomethacin (B) or SC236 (C) for 24 h. Supernatants were then collected for PGE_2 measurement. The PGE_2 level was expressed as picogramme per milliliter per microgramme of protein. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. ***, $p<0.001$ compared with control group.


A
COX-2
β-actin
control
control siRNA
COX-2 siRNA


B
PGE2 release normalized by protein (pg/ml per μg protein)
1.00
0.75
0.50
0.25
0.00
***
control siRNA
COX-2 siRNA

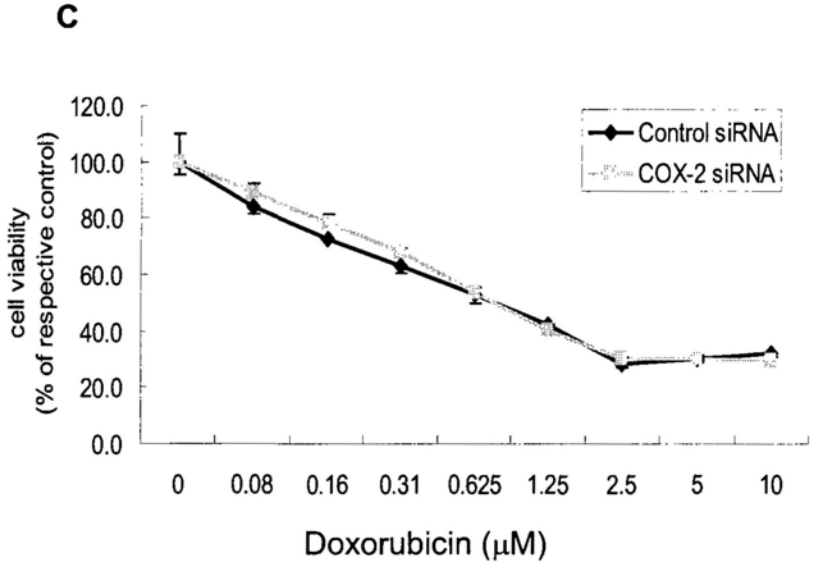

C
cell viability (% of respective control)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Control siRNA
COX-2 siRNA
0
0.08
0.16
0.31
0.625
1.25
2.5
5
10
Doxorubicin (μM)

Figure 3.60 Effects of siRNA-mediated knockdown of COX-2 on doxorubicin-induced cytotoxicity in HKESC-1 cells. (A) The efficacy of COX-2 depletion by COX-2 siRNA was verified by Western blot analysis. Non-targeting siRNA was used as control siRNA. β-actin was used to evaluate protein loading. These results are representative of three independent experiments. (B) After transfection with the control siRNA and COX-2 siRNA, cells were incubated in the growth medium for 24 h. Supernatants were then collected for PGE_2 measurement. The PGE_2 level was expressed as pg/ml per μg protein. Data are presented as mean ±SEM (n=3) of a representative experiment performed in triplicate. ***, $p<0.001$ versus control siRNA. (C) After transfection with the control siRNA or COX-2 siRNA, cells were treated with doxorubicin at indicated concentrations for 24 h before MTT assay. Data are presented as mean±SEM (n=3) of a representative experiment performed in triplicate.

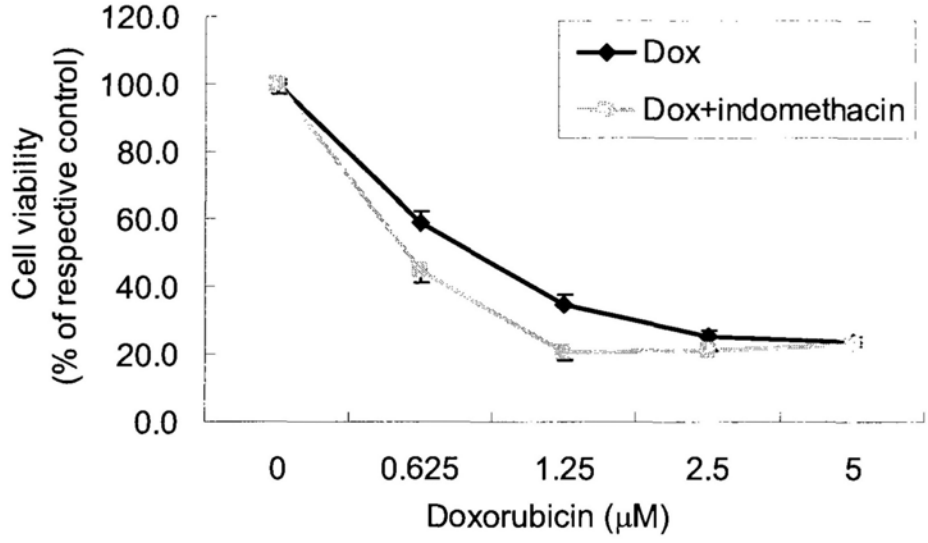


Figure 3.61 Effects of indomethacin on doxorubicin-induced cytotoxicity in TMK-1 cells. Cells were treated for 24 h with doxorubicin (0-10 μM) alone, or in combination with indomethacin (20 μM). Cell viability was then determined by MTT assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate.

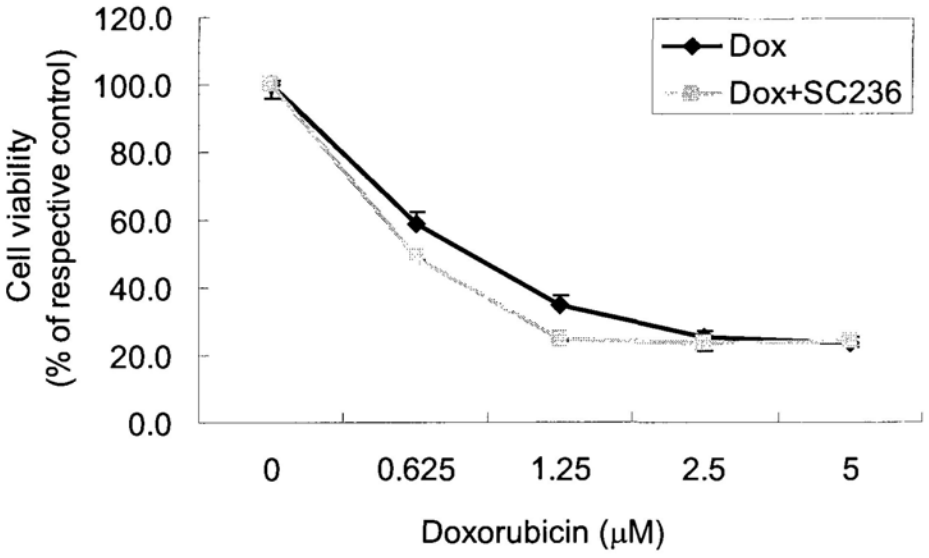


Figure 3.62 Effects of SC236 on doxorubicin-induced cytotoxicity in TMK-1 cells. Cells were treated for 24 h with doxorubicin (0-10 μM) alone, or in combination with SC236 (10 μM). Cell viability was then determined by MTT assay. Data are presented as mean ± SEM (n=3) of a representative experiment performed in triplicate.

3.6 Indomethacin and COX-2 selective inhibitors function as P-gp inhibitors

3.6.1 Indomethacin and SC236 showed no inhibitory effect on NF-κB

As COX-2 inhibitors have been demonstrated to increase intracellular doxorubicin accumulation and subsequently enhance its cytotoxicity in human breast tumor cells through inhibition of NF-κB activity as indicated by the reduced nuclear translocation of the p65 subunit (van Wijingaarden et al., 2007), we examined the effects of indomethacin and SC236 on NF-κB activity in HKESC-1 cells. In an inactive state, NF-κB is sequestered in the cytoplasm as a heterodimer consisting of p50, p65, and IκBα subunits. In response to an activation signal, the IκBα subunit becomes phosphorylated, ubiquitinated, and ultimately degraded through the proteasomal pathway. This process exposes the nuclear localization signals on the p50-p65 heterodimer, facilitating its nuclear entry, binding to specific sequence in DNA and activating transcription of target genes (Dorai and Aggarwal, 2004). In the present study, results showed that neither indomethacin nor SC236 altered the cytosolic or nuclear expressions of p65 subunit (Figure 5.63A). Furthermore, combination of doxorubicin with indomethacin or SC236 also showed no influence on the cytosolic and nuclear p65 staining (Figure 5.63B). These findings indicated that the enhancing effect of indomethacin and SC236 on cytotoxicity is not mediated through inhibition of NF-κB activity.

3.6.2 Indomethacin and SC236 inhibited P-gp ATPase activity

The results presented so far indicated that indomethacin or SC236 enhanced the

cytotoxic effect of doxorubicin is not mediated through inhibition of COX-2 and subsequent PGE_2 production as well as inhibition of NF-κB. Although both indomethacin and SC236 increased intracellular accumulation and retention of doxorubicin which is a substrate for a membrane drug efflux pump P-glycoprotein (P-gp), whether this phenomenon is related to P-gp activity via COX-independent manner has not yet been determined. As shown in Figure 3.64, doxorubicin, indomethacin and SC236 alone or in combination did not alter the expression of P-gp. However, doxorubicin obviously increased P-gp ATPase activity to a comparable extent to that of the positive control verapamil, which functions as a P-gp competitive inhibitor (Figure 3.65). Unlike doxorubicin, indomethacin or SC236 significantly decreased P-gp ATPase activity, indicating both of these two COX-inhibitors functioned as P-gp non-competitive inhibitors (Figure 3.66). These findings suggest that indomethacin or SC236 enhanced cytotoxic action of doxorubicin through direct inhibitory actions on P-gp activity in cancer cells. Moreover, other tested COX-inhibitors (SC560, nimesulide, and NS398) showed no effects on P-gp ATPase activity (Figure 3.67). In addition, indomethacin at concentrations of 5 and 10 μM and SC236 at concentrations of 2.5 and 10 μM significantly reduced doxorubicin-induced P-gp ATPase activity (Figure 3.68 and 3.69), which agreed with the effects of indomethacin and SC236 on intracellular accumulation and retention of doxorubicin. These findings suggest that indomethacin or SC236 enhanced cytotoxic action of doxorubicin through direct inhibitory actions on P-gp activity in cancer cells.

3.6.3 Preliminary discussion and conclusion

Apart from inhibition of COX enzymes, it has been suggested that additional mechanisms are involved in the actions of NSAIDs and COX-2 selective inhibitors. One possible mechanism is that these inhibitors repress NF-κB activity which is increased in response to chemotherapeutic agents like doxorubicin (Nakanishi and Toi, 2005). More noteworthy is that a recent study demonstrated COX-2 selective inhibitors increased the intracellular accumulation of doxorubicin and enhanced doxorubicin-induced cytotoxicity in human breast cancer cells via inhibition of doxorubicin-induced NF-κB activation (van Wijingaarden et al., 2007). Unlike these findings, our studies show that NF-κB activity is not increased in response to doxorubicin treatment. Furthermore, neither indomethacin nor SC236 exerted inhibitory effect on NF-κB activity in the absence or in the presence of doxorubicin (Figure 3.63). These results suggest that NF-κB is not involved in the enhancing effect of indomethacin or SC236 on doxorubicin-induced cytotoxicity. This discrepancy between the findings of our study and others may be due to the intrinsic difference among the cell lines used.

The efficacy of chemotherapeutic drugs is greatly compromised by emergence of multidrug resistance (MDR), which is regarded as a major obstacle to effective cancer chemotherapy. Such resistance pattern is mainly mediated by ATP-binding cassette (ABC) transporters via ATP-dependent drug efflux. Among these ABC transporters, P-gp and multidrug resistance protein 1 (MRP1) are two major transporters which function as pumps to extrude doxorubicin from cancer cells

(Gillet et al., 2007; Mimeault et al., 2007). It has been suggested that COX-inhibitors may sensitize cancer cells to chemotherapeutic drugs like doxorubicin via inhibiting P-gp (Awara et al. 2004; Zatelli et al., 2005; Puhlmann et al., 2005; Zrieki et al., 2008; Zatelli et al., 2007) or MRP1 (Draper et al., 1997; Duffy et al., 1998; Roller et al., 1999; O'Connor et al., 2004). Although COX-inhibitors like indomethacin has been reported to reverse MRP-mediated efflux of doxorubicin via inhibition of MRP1 pumping system (Draper et al., 1997; Duffy et al., 1998; Roller et al., 1999), the involvement of MRP1 in augmentation of doxorubicin toxicity by indomethacin or SC236 is excluded in our study based on the findings that MRP1 protein is undetectable in HKESC-1 and HKESC-2 cells (data not shown). In contrast, P-gp expression is expressed in esophageal squmous cell carcinoma cell lines. However, its expression is quite stable in response to doxorubicin in the absence or in the presence of indomethacin or SC236 (Figure 3.64). We therefore directly measured the activity of P-gp. In this respect, doxorubicin functions as a substrate for P-gp, which is manifested as increased P-gp ATPase activity (Figure 3.65). Conversely, both indomethacin and SC236 act as non-competitive inhibitors for P-gp, decreasing the basal P-gp ATPase activity and doxorubicin-induced P-gp ATPase activity and thereby preventing the transport of doxorubicin out of cells (Figures 3.66, 3.68 and 3.69). These findings suggest a direct inhibitory action of indomethacin or SC236 on P-gp function, which may contribute to the increased intracellular doxorubicin accumulation and retention as well as the subsequent enhancement of cytotoxicity on esophageal cancer cells. The minimal effective concentration (2.5 μM) of

indomethacin used in the present experiments is almost 3-times lower than that in the plasma of the patients treated with indomethacin in the clinical trial (Helleberg, 1981). With respect to SC236, it has been reported that SC236 at the plasma concentration of 5 μg/ml (12.5 μM) which is 5-times higher than the minimal effective concentration (2.5 μM) in our study was employed in an orthotopic xenograft mouse model (Lee et al., 2006). Although both indomethacin and SC236 at their effective concentrations in the present study showed no cytotoxic action on the tested cells *in vitro*, given the concerns regarding the safety of COX-inhibitor, further safety study on the combination of these COX-inhibitors with doxorubicin *in vivo* is warranted.

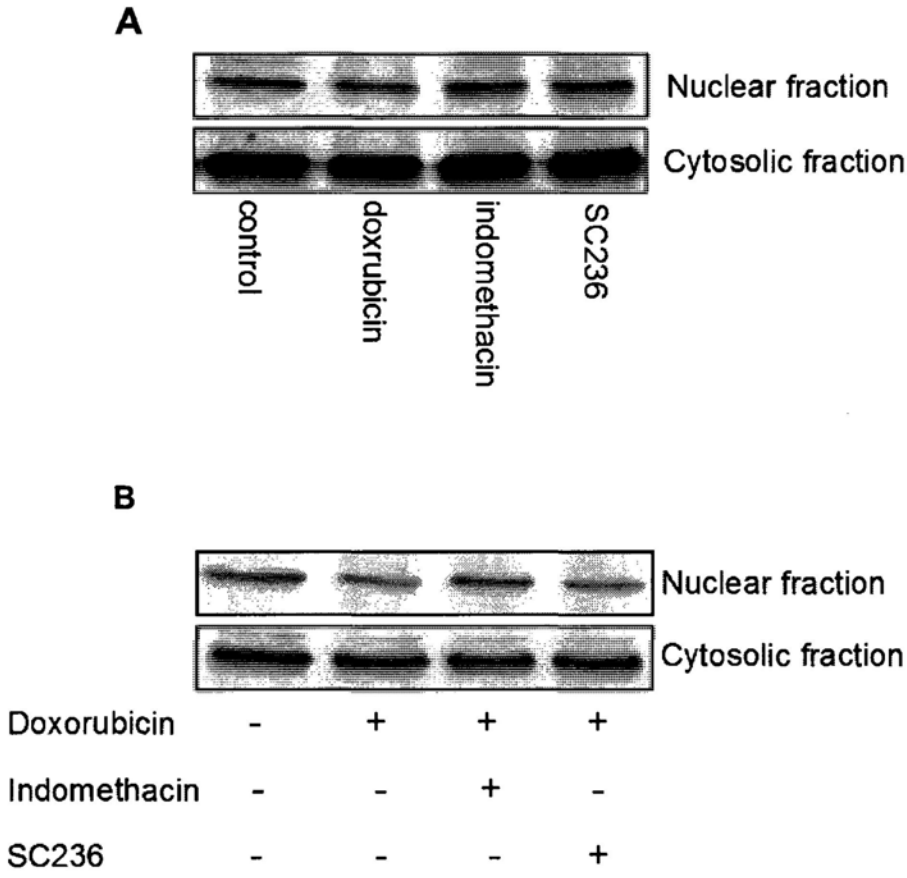


Figure 3.63 Western blot detection of NF-κB subunit p65 in nuclear and cytosolic extracts from HKESC-1 cells. (A) Cells were incubated for 6 h in the absence or the presence of 1.25 μM doxorubicin, 20 μM indomethacin or 10 μM SC236. (B) Cells were incubated for 6 h with 1.25 μM doxorubicin alone, or in combination with 20 μM indomethacin or 10 μM SC236. These results are representatives of three independent experiments.

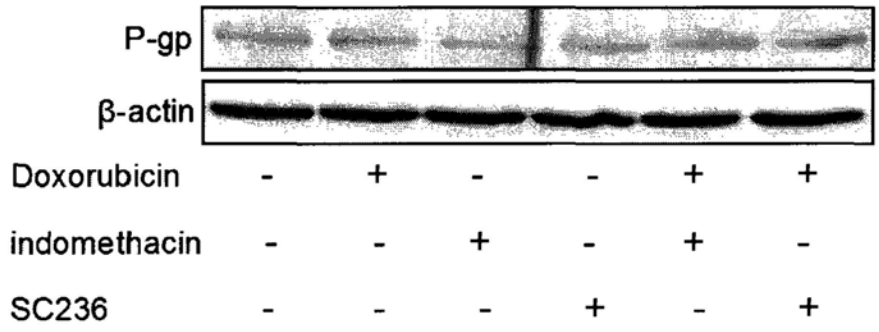


Figure 3.64 Effects of indomethacin and SC236 on P-gp protein expression in HKESC-1 cells. Cells were incubated for 6 h with 1.25 μM doxorubicin alone, or in combination with 20 μM indomethacin or 10 μM SC236. Cells were then collected for determination of P-gp expression by Western blot analysis. Beta-actin was used to evaluate protein loading. These results are representative of three independent experiments.

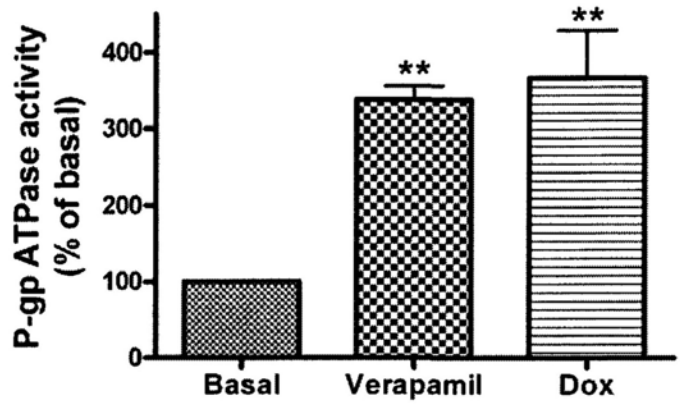


Figure 3.65 Effects of doxorubicin on P-gp ATPase activity. Verapamil functions as the positive control. Results are expressed as mean ± SEM (n=3) of three independent experiments. **, $p<0.01$ compared with the basal level of P-gp ATPase activity.

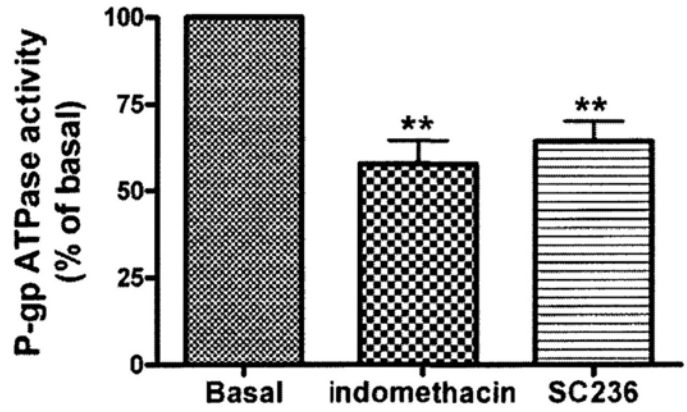


Figure 3.66 Effects of indomethacin and SC236 on P-gp ATPase activity. Results are expressed as mean ± SEM (n=3) of three independent experiments. **, $p<0.01$ compared with the basal level of P-gp ATPase activity.

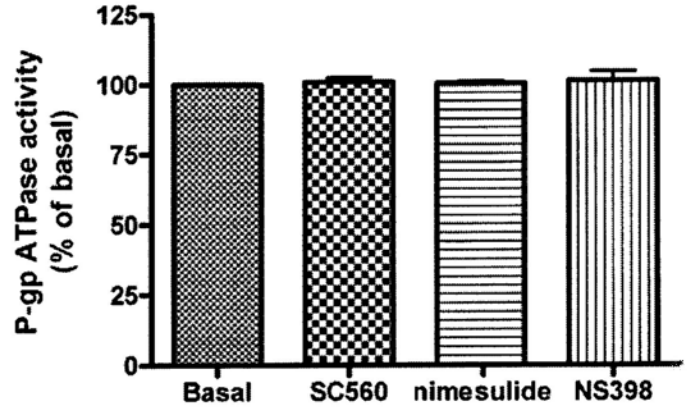


Figure 3.67 Effects of SC560 (20 μM), nimesulide (10 μM) and NS398 (10 μM) on P-gp ATPase activity. Results are expressed as mean ± SEM (n=3) of three independent experiments.

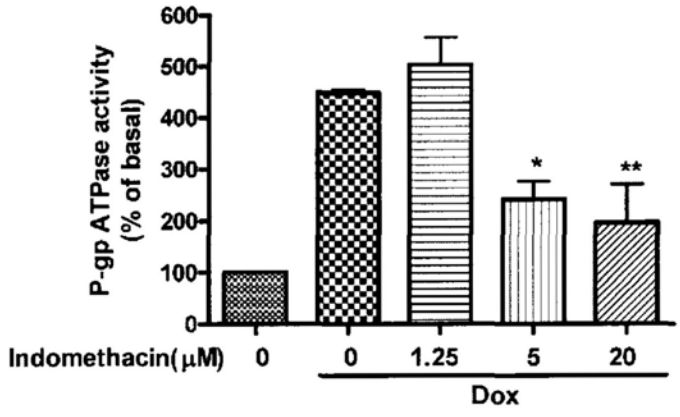


Figure 3.68 Effects of indomethacin on 10 μM doxorubicin-induced P-gp ATPase activity. Results are expressed as mean ± SEM (n=3) of three independent experiments. *, $p<0.05$ and **, $p<0.01$ compared with the doxorubicin-treated group.

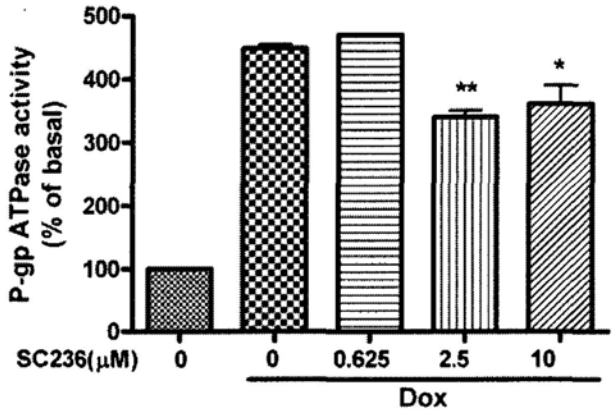


Figure 3.69 Effects of SC236 on 10 μM doxorubicin-induced P-gp ATPase activity. Results are expressed as mean ± SEM (n=3) of three independent experiments. *, $p<0.05$ and **, $p<0.01$ compared with the doxorubicin-treated group.

Chapter 4

Final summary and conclusion

The first part of our research focused on the effect of PGE_2 on cell proliferation of human esophageal squamous cell carcinoma cells (HKESC-1). Our findings demonstrated that PGE_2 at concentrations ranging from 0.1 to 10 μM significantly increased HKESC-1 cell proliferation in a dose-dependent manner. The actions of PGE_2 are mediated via four subtypes of G-protein-coupled receptors, designated EP1, EP2, EP3 and EP4, based on their different pharmacological properties and secondary messenger pathways. In the present study, we show that all four EP receptor subtypes are expressed in a panel of human esophageal squamous cell carcinoma cell lines (HKESC-1, HKESC-2, HKESC-3, EC109, and KYSE150). Further characterization by pharmacological and RNA interference approaches revealed that EP2 receptor mediated the mitogenic action of PGE_2 in HKESC-1 cells. In this regard, EP2 receptor agonist butaprost mimicked the mitogenic effect of PGE_2, whereas knockdown of the EP2 receptor by specific siRNA attenuated the PGE_2-induced cell proliferation. However, activation of other EP receptor subtypes by respective agonists showed no or minimal mitogenic effect.

In relation to the signaling mechanism, PGE_2 and butaprost induced phosphorylation of ERK1/2, whose down-regulation by RNA interference significantly attenuated PGE_2-induced cell proliferation. Moreover, ERK1/2 activation by PGE_2 was completely abolished by PKC inhibitor, Ro-31-8425. The mitogenic action of PGE_2 was also attenuated by Ro-31-8425. The activation of ERK1/2 is an early event after mitogenic stimulation, which subsequently triggers

multiple signaling pathways. In the present study, we demonstrated that activation of AP-1 and c-Myc pathways in the downstream of ERK1/2 contributed to the mitogenic action of PGE_2 in esophageal squamous cell carcinoma cells.

The AP-1 transcription factor is a heterodimetic complex that mainly comprises members of the Fos (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) families. Our study showed that PGE_2 increased c-Fos expression and AP-1 transcriptional activity, both of which can be abolished by the MEK inhibitor U0126, suggesting that activation of ERK1/2 is required for PGE_2-induced c-Fos expression and AP-1 activation. Moreover, AP-1 binding inhibitor curcumin attenuated the mitogenic action of PGE_2, revealing that AP-1 activation is required for PGE_2-induced cell proliferation. In parallel, the EP2 receptor agonist butaprost induced ERK1/2 phosphorylation, c-Fos expression, and AP-1 activity to a similar extent as PGE_2 stimulation, indicating that the EP2 receptor mediates the effects of PGE_2 on these parameters. This conclusion is substantiated by the findings that activation other EP receptor subtypes by respective agonists failed to induce c-Fos expression.

In addition to AP-1, activation of c-Myc pathway following ERK1/2 activation is also demonstrated to contribute to the mitogenic action of PGE_2 in esophageal squamous cell carcinoma cells. In this regard, PGE_2 increased c-Myc expression both at mRNA and protein levels. Although the expression of Max, the c-Myc binding partner, was quite stable in response to PGE_2 stimulation, the association between c-Myc and Max was enhanced, which suggests an increase in functional c-Myc

protein. Moreover, knockdown of c-Myc by specific siRNA decreased the basal and PGE_2-induced cell proliferation. These findings strongly suggest the involvement of c-Myc in the mitogenic action of PGE_2 in esophageal squamous cell carcinoma cells. Considering c-Myc protein is a highly unstable protein, we further determined whether post-transcriptional control of c-Myc protein contributes to PGE_2-induced c-Myc expression. Our study showed that PGE_2 increased the protein stability and nuclear accumulation of c-Myc via phosphorylation on serine 62 in an ERK1/2-dependent manner. Moreover, the effect of PGE_2 on c-Myc expression was mimicked by the EP2 receptor agonist butaprost, but not by other EP receptor agonists. These findings suggest that EP2 receptor mediates the PGE_2-induced c-Myc expression.

Collectively, the above findings suggest that PGE_2 promotes cell proliferation via EP2/PKC/ERK-dependent activation of AP-1 and c-Myc pathways in human esophageal squamous cell carcinoma cells. Figure 4.1 summarizes the possible mechanisms by which PGE_2 exerts mitogenic action on human esophageal squamous cell carinoma cells. Given the recent concerns regarding the safety of NSAIDs and COX-2 selective inhibitors, our findings suggest that, by blocking the specific PGE_2 signaling pathway through the EP2 receptor instead of global prostaglandin synthesis, may represent a promising therapeutic strategy for the treatment of esophageal squamous cell carcinoma. This definitely deserves further clinical investigation in the future.

Considering the deleterious effects of PGE_2 which have been demonstrated

above, as well as the reported chemotherapeutic and chemoprophylactic actions of COX-inhibitors in esophageal squamous cell carcinoma, it is worthwhile to examine the possible benefit of combining COX-inhibitors with conventional anticancer drug during chemotherapy and study further whether PGE_2 if any plays a role in this action. Therefore, the second part of our study was focused on the role of COX-inhibitors in cancer cell drug resistance. To study whether COX-inhibitors could enhance the cytotoxic action of doxorubicin, a non-selective COX-inhibitor (indomethacin), COX-1 selective inhibitor (SC560), and COX-2 selective inhibitors (SC236, nimesulide, and NS398) were employed in the present study. Although all of these tested COX-inhibitors substantially suppressed PGE_2 production to a similar extent, only the non-selective COX inhibitor indomethacin and the COX-2 selective inhibitor SC236 enhanced the cytotoxic effect of doxorubicin on HKESC-1 and HKESC-2 cells, and these effects could not be reversed by the addition of PGE_2. Knockdown of COX-2 by specific siRNA substantially decreased PGE_2 production, also failed to mimic the enhancing effect of indomethacin or SC236 on cytotoxic action of doxorubicin. These findings indicate that indomethacin and SC236 enhance the cytotoxicity of doxorubicin via COX- and PGE_2-independent mechanism. In an attempt to explain the enhancement of doxorubicin cytotoxicity caused by indomethacin and SC236, the effect of these two compounds on the function of P-gp was investigated. To this end, although indomethacin and SC236 showed no effects on P-gp expression, these two COX-inhibitors acted as non-competitive inhibitors of P-gp ATPase, which retarded the efflux of doxorubicin from cancer cells and thus

augmented its cytotoxicity on esophageal squamous cell carcinoma.

Throughout the last two decades, much effort has been made to identify agents that are able to inhibit P-gp as a way to reverse cancer cells drug resistance. A variety of agents with different chemical structures that modulate the function of P-gp have been identified. Pharmacologically, these agents can be categorized into competitive inhibitors and noncompetitive inhibitors. Early agents like verapamil belong to competitive inhibitors and work by competing with the chemotherapeutic drugs for efflux by the P-gp pump. One of the drawbacks of this group of compounds is that high serum concentrations are necessary to produce adequate intracellular concentrations of the chemotherapeutic drugs. Thus, they typically reverse drug resistance at concentrations that result in unacceptable toxicity. In contrast, non-competitive inhibitors act via non-competitive binding to P-gp pump and thereby overcome the limitations of competitive inhibitors, which are more promising for the therapeutic application. It is noteworthy that both indomethacin and SC236 are most likely functioning as non-competitive inhibitors for P-gp, which manifest by the inhibition of P-gp ATPase activity.

In summary, our studies show a new mechanism by which indomethacin and SC236 exert enhancing effect on cytotoxic effect of doxorubicin via direct inhibition of P-gp ATPase activity in human esophageal squmous cell carcinoma cells. Based on these findings, the combination of indomethacin or SC236 with doxorubicin may have potential clinical applications, especially in the circumvention of P-gp-mediated multidrug resistance in cancer cells.

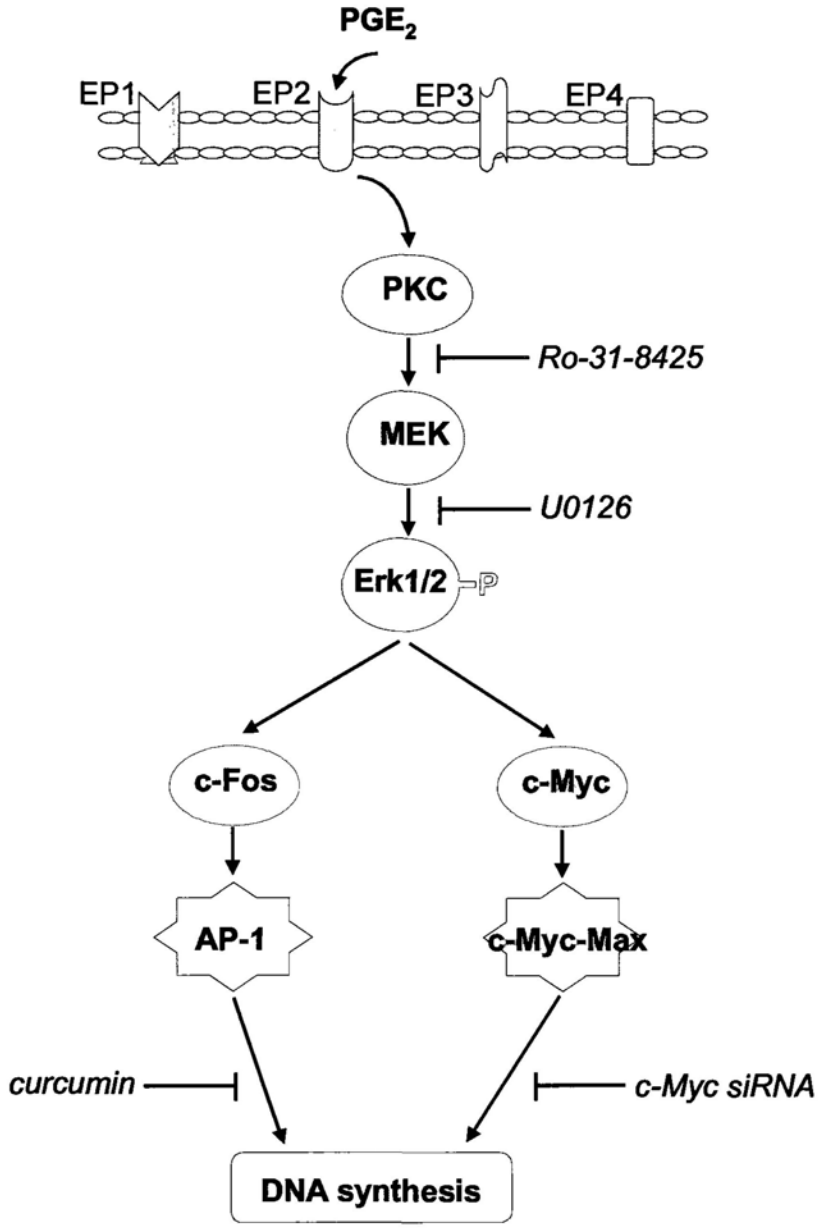


Figure 4.1 Proposed mechanisms for PGE_2-mediated human esophageal squamous cell carcinoma cell proliferation. (PKC: protein kinase C; MEK: mitogen-activated protein kinase kinase; Erk: extracellular signal-regulated kinase; AP-1: activator protein-1; Ro-31-8425: PKC inhibitor; U1026: MEK inhibitor; cucumin: AP-1 binding inhibitor; ⊢ represents blockade of the action and → stands for stimulation of the action).

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