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Protein tyrosine kinase pathway-derived ROS/NO productions contribute to G2/M cell cycle arrest in evodiamine-treated human cervix carcinoma HeLa cells

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Abstract

A previous study indicated that reactive oxygen species (ROS) and nitric oxide (NO) played pivotal roles in mediating cytotoxicity of evodiamine in human cervix carcinoma HeLa cells. This study suggested that G2/M cell cycle arrest was triggered by ROS/NO productions with regulations of p53, p21, cell division cycle 25C (Cdc25C), Cdc2 and cyclin B1, which were able to be prevented by protein tyrosine kinase (PTK) activity inhibitor genistein or JNK inhibitor SP600125. The decreased JNK phosphorylation by addition of Ras or Raf inhibitor, as well as the increased cell viability by addition of insulin-like growth factor-1 receptor (IGF-1R), Ras, Raf or c-Jun N-terminal kinase (JNK) inhibitor, further demonstrated that the Ras-Raf-JNK pathway was responsible for this PTK-mediated signalling. These observations provide a distinct look at PTK pathway for its suppressive effect on G2/M transition by inductions of ROS/NO generations.

Keywords: *Reactive oxygen species (ROS), nitric oxide (NO), cell cycle arrest, protein tyrosine kinase (PTK), evodiamine, genistein*

Introduction

Reactive oxygen species (ROS) and nitric oxide (NO) are two major forms of free radicals. ROS are small, highly reactive, oxygen-containing molecules that are naturally generated endogenously as by-products of cellular metabolism [1]. NO is a highly reactive gaseous free radical generated by a heme enzyme NO synthase (NOS)-mediated oxidation of L-arginine [2]. Under physiological conditions, free radicals are part of the normal cellular redox state, the balance of which is tightly controlled by a defence system of antioxidants including enzymes such as glutathione peroxidase, catalase and superoxide dismutase. Disturbed balance between pro-oxidants and antioxidants is considered as oxidative stress, which is one major source of the intrinsic DNA damage [3]. Cell cycle checkpoints can be activated by oxidative stress to arrest cell cycle progression to allow repair DNA in response to DNA damage. There are at least two checkpoints to monitor DNA damage: one at the G1/S transition and the other at the G2/M transition. The G1/S checkpoint prevents replication of damaged DNA; whereas the G2/M checkpoint inhibits the segregation of damaged chromosomes [4]. Two key families of proteins, protein kinase sub-units called cyclin-dependent protein kinases (Cdk) and activating proteins called cyclins, can bind together and activate Cdk kinases activities through phosphorylations to control cell cycle progression [5]. These control mechanisms determine a temporary arrest at a specific stage of cell cycle to allow the cell to correct possible defects.

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It is well known that protein tyrosine kinases (PTKs) recruit a large network of protein (Ser/Thr) kinases including the Ras-Raf-mitogen-activated protein kinase (MAPK) cascade to execute their cellular programmes [6]. PTK activity can be suppressed by genistein, which can bind with a common, highly conservative sequence at, or near to, the ATP-binding domain of PTK, parallelled with its enzymatic activity [7]. Extracellular signal-regulated kinases (ERK1/2), p38 MAPK and c-Jun N-terminal kinase (JNK) are the major mammalian MAPKs. In general, ERK1/2 is a major growth-signalling kinase; whereas JNK and p38 MAPK influence cell survival, apoptosis, differentiation and inflammation [8].

Evodiamine is a bioactive quinozole alkaloid extract from the dried, unripe fruit of *Evodia rutaecarpa Bentham* (*Rutaceae*) and has been found to present anti-tumour growth, anti-obesity, anti-anoxic, anti-nociceptive and vasorelaxant effects [9–13]. In our previous study, we have demonstrated that evodiamine can induce human melanoma A375-S2 cell and human cervix carcinoma HeLa cell apoptosis [14–16]. In this study, we provided a detailed look at the effect of evodiamine treatment on HeLa cells. Interesting finding was disclosed after co-incubation of HeLa cells with PTK pathway inhibitors and evodiamine, with the preventive effect of PTK, Ras, Raf or JNK inhibitor on evodiamine-induced G2/M cell cycle arrest and ROS/NO productions. This potential PTK pathway on regulating the redox status in evodiamine-treated HeLa cells attracted us to further explore the possible mechanisms to be involved in.

Materials and methods

Reagents

Evodiamine was obtained from Beijing Institute of Biological Products (Beijing, China); and its purity was determined to be ~ 98% by HPLC measurement. Evodiamine was dissolved in dimethyl sulphoxide (DMSO) to make a stock solution and diluted by RPMI-1640 (Gibco, Grand Island, NY) before the experiments. DMSO concentration in all cell cultures was kept below 0.001%, which had no detectable effect on cell growth or death. 3-(4,5-dimetrylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3,3-diaminobenzidine tetrahydrochloride (DAB), propidium iodide (PI), genistein, SP600125, AG1024, AG1478, manumycin A, GW5074, PD98059, SB203580, *N*-acetyl-cysteine (NAC), N^G-nitro- L-arginine methyl ester (L-NAME), 2′,7′-dichlorofluorescein diacetate (DCF-DA), 4,5-diaminofluorescein diacetate (DAF-2DA), rhodamine-123, PMSF, aprotinin and leupeptin were purchased from Sigma Chemical (St. Louis, MO). Polyclonal antibodies against JNK, phospho-JNK, p53, phospho-p53, p21, phospho-Cdc25C, phospho-Cdc2, cyclin B1, β-actin and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

HeLa, human cervical carcinoma cells, were obtained from American Type Culture Collection (ATCC, #CCL-2, Manassas, VA) and were cultured in RPMI-1640 medium supplemented with 10% heat inactivated (56°C, 30 min) foetal calf serum (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China), 2 mM L-glutamine (Gibco, Grand Island, NY), 100 kU/L penicillin and 100 mg/L streptomycin (Gibco) at 37°C in 5% CO_2. Cells in the exponential phase of growth were used in the experiments.

Flow cytometric analysis using PI

After treatment with drugs, 1×10^6 cells were harvested, washed with PBS, then fixed in 70% methanol and, finally, maintained at 4°C for at least 12 h. Then the cell pellets were stained with the fluorescent probe solution containing PBS, 50 μg/ml PI and 1 mg/ml DNase-free RNaseA for 30 min on ice in the dark. DNA fluorescence of PI-stained cells was evaluated by a FACScan flow cytometer. A minimum of 10 000 cells were analysed per sample and the DNA histograms were gated and analysed further using Modfit software on a Mac workstation to estimate the percentage of cells in various phases of the cell cycle [17].

Measurement of intracellular ROS generation

After drug treatments, the cells were incubated with 10 μM DCF-DA at 37°C for 15 min to assess ROS-mediated oxidation of DCF-DA to the fluorescent compound 2′,7′-dichlorofluorescein (DCF). Then the cells were harvested and the pellets were suspended in 1 ml PBS. Samples were analysed at an excitation wavelength of 480 nm and an emission wavelength of 525 nm by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) [18].

Measurement of intracellular NO generation

The intracellular NO was detected using DAF-2DA as described [19] with some modifications. DAF-2DA, a nitric oxide fluorescent probe, can react with NO within viable cells to produce a fluorescent compound DAF-2T. After drug treatments, the cells were collected and resuspended in PBS and then incubated with 10 μM DAF-2DA at 37°C for 45 min. Samples were then analysed at an excitation wavelength of 485 nm and an emission wavelength of 515 nm by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Measurement of ΔΨm

ΔΨm was measured by the incorporation of a cationic fluorescent dye rhodamine 123 as described [20]. After incubation with 21 μM evodiamine for 24 h with or without 80 μM genistein or 40 μM SP600125, the cells were stained with 1 μg/ml rhodamine 123 and incubated at 37°C for 15 min. The fluorescence intensity of cells *in situ* was observed under fluorescence microscopy. Quantitative assay was performed by a similar staining procedure as above. After treatment with evodiamine, the cells were instead collected and suspended in 1 ml PBS containing 1 μg/ml rhodamine 123 and incubated at 37°C for 15 min. The fluorescence intensity of cells was analysed within 15 min by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Assessment of cell viability

HeLa cells were dispensed in 96-well flat bottom microtiter plates (NUNC, Roskilde, Denmark) at a density of 1×10^5 cells/ml. After 12 h incubation, the cells were treated with or without genistein, SP600125, AG1024, AG1478, manumycin A, GW5074, PD98059, SB203580, NAC or L-NAME at given concentrations 1 h prior to the administration of 21 μM evodiamine for the indicated time periods. Cell viability was measured using the MTT assay as described elsewhere [21] with a plate reader (Bio-Rad, Hercules, CA).

The percentage of cell viability was calculated as follows:

Cell viability (%) = (A490,sample – A490,blank)/(A490,control – A490,blank)] × 100

Western blot analysis

HeLa cells were treated with 21 μM evodiamine for 0, 12 or 24 h or co-incubated with the given inhibitors for 24 h. Both adherent and floating cells were collected and then Western blot analysis was carried out as previously described [22] with some modification. Briefly, the cell pellets were resuspended in lysis buffer, including 50 mM Hepes (pH 7.4), 1% Triton–X 100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM edetic acid, 1 mM PMSF (Sigma), 10 μg/mL aprotinin, 10 μg/mL leupeptin and lysed on ice for 60 min. After centrifugation of the cell suspension at 13 000×g for 15 min, the protein content of supernatant was determined by Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). The protein lysates were separated by electrophoresis in 12% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Proteins were detected using polyclonal antibody and visualized using anti-rabbit, anti-mouse or anti-goat IgG conjugated with horseradish peroxidase (HRP) and 3,3-diaminobenzidine tetrahydrochloride (DAB) as the substrate of HRP.

Statistical analysis

The results are presented as Mean ± SD. Comparisons between groups were made using Student's *t*-test. A *p*-value less than 0.05 was considered to represent a statistically significant difference.

Results

ROS and NO generations contributed to the evodiamine-stimulated G2/M cell cycle arrest

To obtain a more comprehensive view of the effects of ROS and NO productions on evodiamine-triggered HeLa cell damage, flow cytometry was introduced to analyse cell cycle progression by PI staining. The peak of DNA fragments in cell cycle analytic diagrams locates at the left of the normal G0/G1 phase peak, which is defined as SubG0/G1 peak [23]. As shown in Figure 1, G2/M percentage was dramatically decreased from 43.1% by evodiamine alone to 6.6% ($p < 0.01$) or 22.2% ($p < 0.01$) in the presence of NAC or L-NAME, as well as the markedly reduced SubG0/G1 percentage from 28.6% by evodiamine alone to 8.4% ($p < 0.01$) or 15.2% ($p < 0.01$) in the presence of NAC or L-NAME. Therefore, the productions of ROS and NO played pivotal roles in regulating evodiamine-induced apoptosis and G2/M cell cycle arrest.

Genistein and SP600125 prevented the evodiamine-triggered ROS generation

Genistein was shown to be a specific and potent inhibitor of PTK activity, as well as an effective antioxidant. SP600125 is a reversible ATP-competitive inhibitor specific for the three isoforms of JNK [24]. To detect the relationship between PTK signalling and ROS generations, intracellular ROS level was determined by using ROS-detecting fluorescent dye DCF-DA after pre-treatment with genistein or SP600125 in evodiamine-administered cells. As shown in Figure 2, the ratio of DCF positive cells dropped remarkably from 67.4% by evodiamine alone to 10.7% ($p < 0.01$) or 20.7% ($p < 0.01$) in the presence of genistein or SP600125 at 24 h; while pre-incubation with NAC abolished ROS generation to 7.1% ($p < 0.01$), which served as the positive control group. This result revealed that the intracellular ROS productions were probably regulated by PTK and JNK.

Genistein and SP600125 decreased the evodiamine-induced NO generation

Similar investigation was performed to examine the relationship between PTK signalling and NO

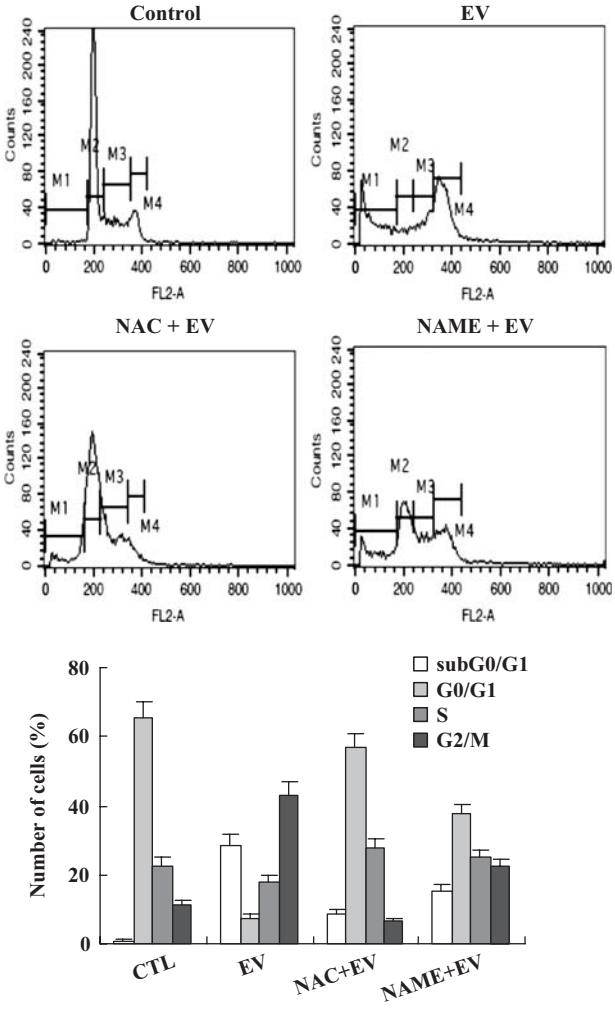

Figure 1. Suppressive effect of NAC or L-NAME on evodiamine-induced G2/M cell cycle arrest. The cells were pre-treated with 10 mM NAC or 20 mM L-NAME (NAME) for 1 h, followed by an additional 21 μM evodiamine for 24 h. The DNA content was analysed by flow cytometry after PI staining. The corresponding linear diagram of the FACScan histograms is expressed at the bottom. Data from a representative experiment ($n = 3$) are shown.

production. By staining the cells with DAF-2DA, a membrane-permeable derivative of the NO sensitive fluorophore DAF-2, the burst of DAF-2T fluorescence in evodiamine-treated cells at 24 h was largely reduced from 45.3% to 20.21% ($p < 0.01$) or 25.06% ($p < 0.01$) by pre-incubation with genistein or SP600125; while pre-treatment with NOS inhibitor L-NAME, a methyl ester derivate of the NOS substrate L-arginine, attenuated NO generation to 17.48% ($p < 0.01$) (Figure 3). Thus, NO production was closely related to PTK signalling.

Genistein and SP600125 prevented evodiamine-induced $\Delta\Psi$m dissipation

To test whether mitochondrial damage was also affected by genistein or SP600125, the change in $\Delta\Psi$m was determined by rhodamine 123 staining. FACScan analysis showed that exposure of HeLa cells to evodiamine decreased the fluorescent intensity of rhodamine 123 staining from 95.4% in untreated cells to 50.5%; whereas pre-treatment with genistein or SP600125 rescued evodiamine induced the loss of $\Delta\Psi$m from 50.5% to 83.5% ($p < 0.001$) or 73.9% ($p < 0.001$) (Table I), respectively. These data suggested that notable deprivation of $\Delta\Psi$m was triggered by PTK and JNK signalling in evodiamine-treated HeLa cells.

Genistein and SP600125 suppressed evodiamine-triggered G2/M cell cycle arrest

Since genistein and SP600125 are both indicated here to be closely related to the stimulation of ROS and NO burst from our results, we tested whether apoptosis and G2/M cell cycle could be prevented by administration of genistein or SP600125. After treatment with evodiamine for 24 h, FACScan analysis of DNA content by PI staining showed that the percentage of SubG0/G1 or G2/M was obviously increased to 28.6% or 43.1%, as compared with 1.0% ($p < 0.001$) or 11.1% ($p < 0.001$) in the untreated group, indicating that evodiamine induced significant apoptosis and G2/M arrest in HeLa cells. When co-incubated with genistein or SP600125, the percentage of SubG0/G1 or G2/M was remarkably reduced from 28.6% or 43.1% for evodiamine alone to 5.5% ($p < 0.01$) or 18.8% ($p < 0.01$) in the presence of genistein, as well as to 11.2% ($p < 0.01$) or 21.1% ($p < 0.01$) in the presence of SP600125 (Figure 4). Therefore, genistein and SP600126 contributed their protective roles in promoting cell cycle progression in evodiamine-incubated HeLa cells.

Evaluate critical factors in mediating evodiamine-induced cell growth inhibition

MTT assay displayed that cell viability in evodiamine-treated cells was markedly elevated from 49.7% to 70.9% ($p < 0.001$) by 40 μM genistein, 84.9% ($p < 0.001$) by 80 μM genistein, 60.4% ($p < 0.01$) by 20 μM SP600125 or 73.7% ($p < 0.001$) by 40 μM SP600125 (Figure 5A), respectively. This result further illustrated the possible role of PTK and JNK in cell growth inhibition.

Increasing evidence suggests that the mechanism underlying the transactivation of PTK, such as epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 receptor (IGF-1R), involves the signalling pathway of Ras-Raf-MEK-MAPK [25,26]. Since genistein was found here to present its remarkable protective effect on evodiamine-treated HeLa cells, we further tested whether PTK pathway molecules were stimulated and exerted their roles in cell growth inhibition. Thus, IGF-1R inhibitor AG1024, EGFR inhibitor AG1478, Ras inhibitor manumycin A,

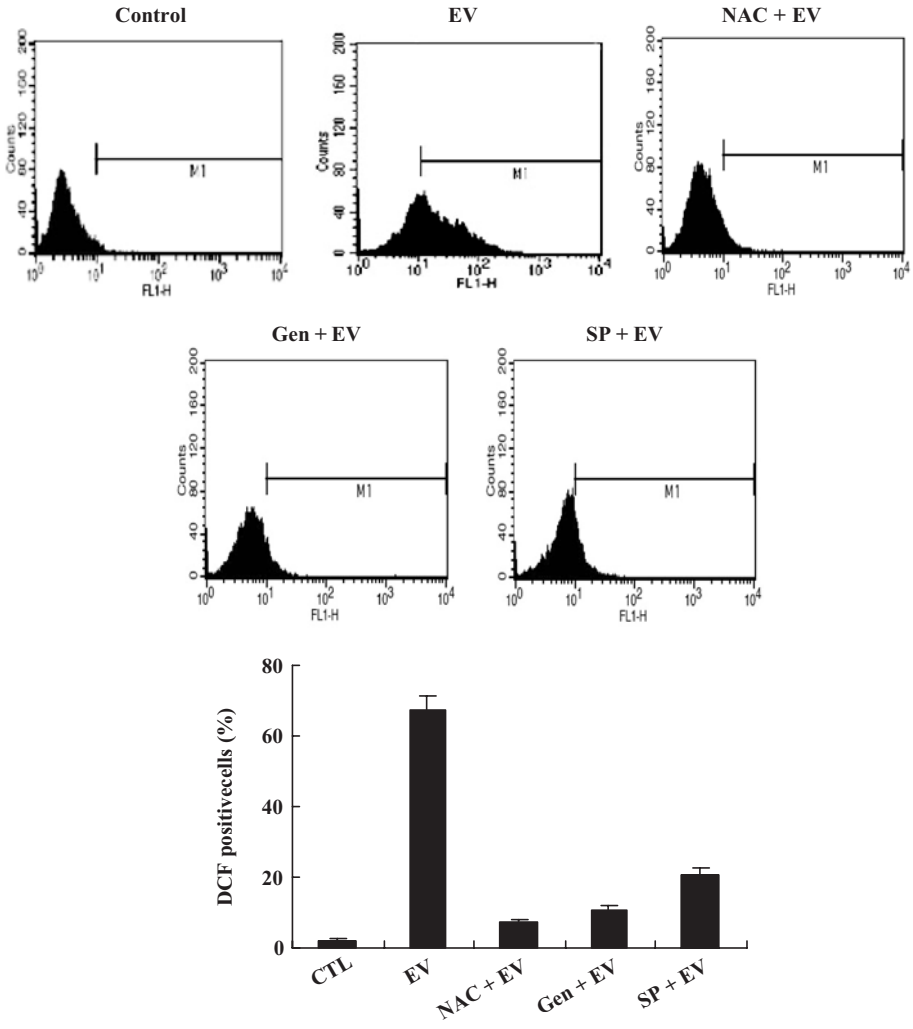

Figure 2. Suppressive effect of NAC, genistein or SP600125 on evodiamine-induced ROS production. The cells were cultured in the presence or absence of 10 mM NAC, 80 μM genistein (Gen), 40 μM SP600125 (SP) for 1 h prior to the addition of 21 μM evodiamine and then incubated for 24 h. Then the cells were loaded with DCF-DA and examined by flow cytometry. The corresponding linear diagram of the FACScan histograms is expressed at the bottom. Data from a representative experiment ($n = 3$) are shown.

Raf inhibitor GW5074, ERK inhibitor PD98059 and p38 inhibitor SB203580 were introduced in the MTT assay. As shown in Figure 5B, after pre-incubation with increasing concentrations of AG1024, manumycin A or GW5074, a significant protective effect of each inhibitor on cell viability was found in a dose-dependent manner, as compared with the evodiamine-treated group, indicating that IGF-1R, Ras and Raf all played cytotoxic roles in evodiamine-treated cells. However, AG1478, PD98059 or SB203580 was not shown significant protective effect on evodiamine-treated cells by MTT assay, suggesting that EGFR, ERK and p38 MAPK were not involved in mediating evodiamine-induced cytotoxicity. Additionally, by introducing ROS non-enzymatic scavenger NAC and NOS inhibitor L-NAME, the result from MTT assay was observed to be consistent with the result obtained in Figure 1 that ROS and NO contributed their potential roles in mediating cell growth inhibition induced by evodiamine.

Since SP600125 was found to be able to reverse evodiamine-triggered growth inhibition, JNK was believed to be an essential downstream effector of evodiamine. Western Blot analysis showed us that JNK was triggered by evodiamine through phosphorylation with the intact total JNK; while the phosphorylation of JNK was markedly attenuated by addition of genistein, NAC or L-NAME and was moderately decreased by addition of manumycin A or GW5074 (Figure 5C). Taken together, these data suggested PTK signalling cascade PTK-Ras-Raf as well as generations of ROS and NO both acted as critical factors in regulating JNK activation to facilitate evodiamine-induced cell growth inhibition.

Evaluate important downstream effectors in mediating G2/M cell cycle arrest

p53 is acknowledged as the mediator of either apoptosis or cell cycle arrest in response to DNA

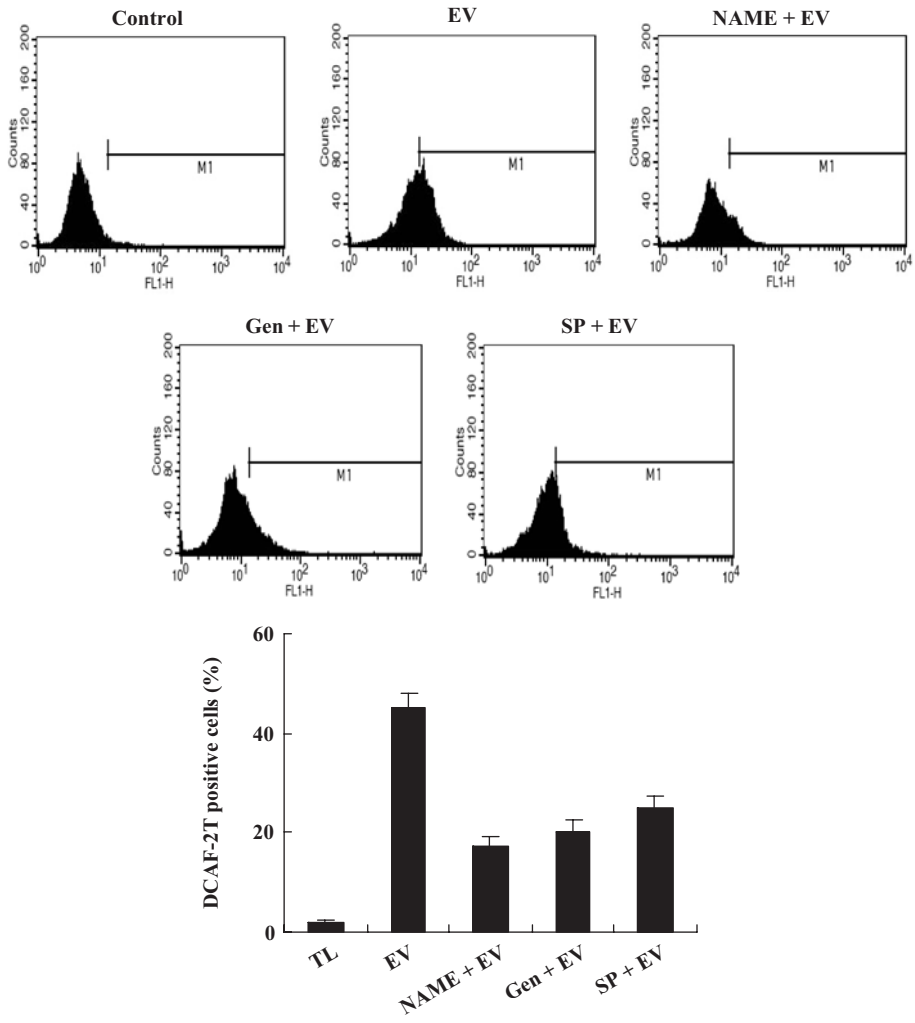

Figure 3. Inhibitory effect of L-NAME, genistein or SP600125 on evodiamine-induced NO production. The cells were cultured in the presence or absence of 20 mM L-NAME (NAME), 80 μM genistein (Gen) or 40 μM SP600125 (SP) for 1 h prior to the addition of 21 μM evodiamine and then incubated for 24 h. DAF-2T, the fluorescent dye product of DAF-2 in reaction with NO, was measured fluorometrically at 1 h post-treatment. The corresponding linear diagram of the FACScan histograms is expressed at the bottom. Data from a representative experiment ($n = 3$) are shown.

damage, thus acting as a molecular 'guardian of the genome' [27]. p21, an inhibitor of most of the cyclin-dependent kinases (CDKs) which help to regulate the cell cycle, is an important target of p53 [28]. Detections of expressions of p53 and phospho-p53 by Western blot analysis revealed that phospho-p53, one active form of p53, and p21 were both notably upregulated by evodiamine in a time-dependent manner, without any changes in p53 expression. However, co-incubation with genistein, SP600125, NAC or L-NAME for 24 h apparently prevented these enhancements (Figure 6A), suggesting that the productions of ROS/NO and the stimulation of PTK signalling lead to the inductions of p53 and p21.

Table I. FACScan analysis of the protective effect of genistein or SP600125 on evodiamine-triggered ΔΨm dissipation.

Group	M1 (%)
Control	95.4 ± 2.4
EV	50.5 ± 3.6
Gen + EV	83.5 ± 2.7#
SP + EV	73.9 ± 3.0#

The cells were incubated with 21 μM evodiamine or coincubated with 21 μM evodiamine and 80 μM Genistein (Gen) or 40 μM SP600125 (SP) for 24 h. After loading with rhodamine-123 1 μg/ml, cells were measured for $\Delta\Psi$m b y a FACScan flowcytometery. The cells in M1 zone represent the rhodamine positive cells. Data from a representative experiment ($n = 3$) are shown. Values are expressed as mean ± SD. #$p < 0.001$ vs evodiamine-treated group.

The final effector in the G2/M checkpoint is the Cdc2/cyclin B1 complex as being essential for the transition from G2 into mitosis [29]. Upon DNA damage, the cell division cycle 25C (Cdc25C) phosphatase can no longer remove inhibitory phosphates from Cdc2, thus preventing the Cdc2/cyclin B1 complex from breaking down the nuclear envelope, condensing chromosomes and other events that

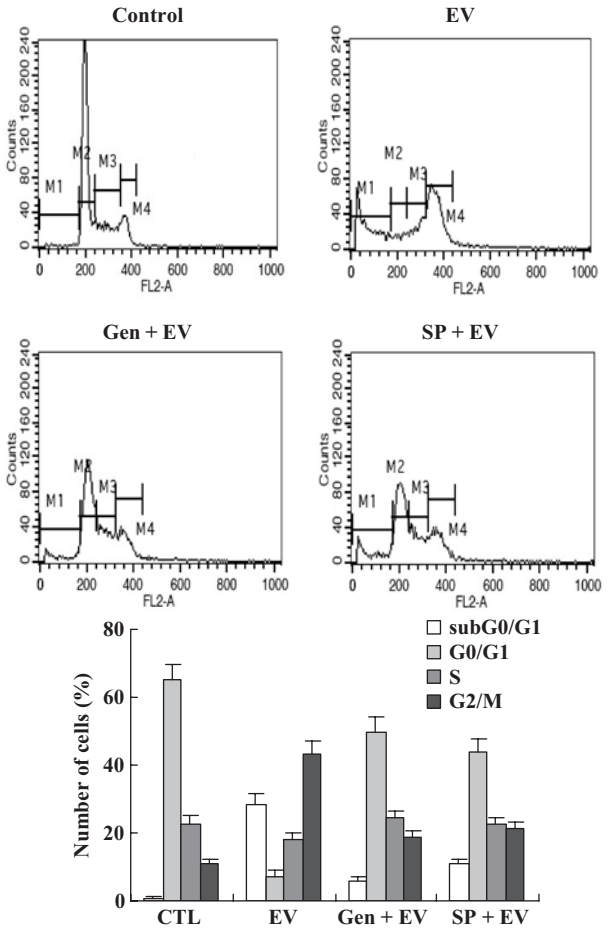

Figure 4. Preventive effect of genistein or SP600125 on evodiamine-induced G2/M cell cycle arrest. The cells were pre-incubated with 80 μM genistein (Gen) or 40 μM SP600125 (SP) for 1 h, followed by an additional 21 μM evodiamine for 24 h. The DNA content was analysed by flow cytometry after PI staining. The corresponding linear diagram of the FACScan histograms is expressed at the bottom. Data from a representative experiment (n = 3) are shown.

occur in early mitosis [30]. To further delineate the role of PTK signalling and ROS/NO in G2/M cell cycle arrest, crucial G2/M regulators Cdc25C, Cdc2 and cyclin B1 were examined in the presence of these inhibitors. As shown in Figure 6B, the inactive form of Cdc25C, phospho-Cdc25C, increased with time obviously after evodiamine treatment; while the active form Cdc25C decreased simultaneously. In addition, phosphorylation of Cdc2 at Tyr15 was also found augmented time-dependently after incubation with evodiamine, with the detection of a slight decrease in cyclin B1 expression. However, pre-treatment with genistein, SP600125, NAC or L-NAME efficiently prevented the phosphorylations of Cdc25C and Cdc2, as well as the detectable increase in cyclin B1. Taken together, these results indicated that ROS/NO generation and PTK signalling both acted as upstream regulators of G2/M cell cycle arrest by targeting various G2/M cell cycle checkpoints.

Discussion

PTKs are a large family of enzymes that transfer γ–phosphate of ATP to Tyr hydroxyl group in proteins, which has been proposed to play a major role in regulating cell proliferation and differentiation, promoting cell migration and survival and modulating cellular metabolism [31]. In our study, it was shown that PTK signalling molecules Ras and Raf were triggered in mediating evodiamine-induced HeLa cell growth inhibition, in the process of which JNK, rather than ERK and p38 MARK, acted as an central downstream effector (Figure 7). This interesting finding gives us an important clue that the PTK pathway can not only play a known proliferative role, but also an anti-proliferative role in cancer cell growth by using the same signalling cascade Ras-Raf-MAPK, which was then found to be closely related to the burst of ROS/NO productions.

Recently, the activation of Ras-Raf-MAPK pathway has been shown to cause growth arrest in several cell types. The Ras-Raf-ERK pathway was reported to be involved in naringin-induced G1-cell cycle arrest via p21 expression in 5637 cancer cells [32]. Similarly, Ras-induced G1 arrest and cell senescence in primary human or rodent cells was reported to be associated with the accumulation of p53 and p16 [33]. Thus, the Ras-Raf-MARK pathway can promote growth arrest by inducing several CDK inhibitors, including p21, p53 and p16, in a cell-specific manner. Consistent with these findings, the potent inhibitory effects of both genistein and SP600125 on p53 and p21 activations observed in this study further illustrated that Ras-Raf-MARK pathway could trigger cell cycle arrest by inducing several CDK inhibitors. Moreover, the present study revealed that among PTKs, the RTK member IGF-1R, a membrane-associated tyrosine kinase receptor that was believed to play an important role in cell growth, transformation and tumourigenesis [34], was found to be one potential upstream regulator of this cascade to inhibit cell growth. The contribution of IGF-1R signalling to cell death has been shown to be related to the C terminus of the IGF-1R β sub-unit [35]. Additionally, IGF-1R signalling was reported to be participated in p53-mediated apoptosis through translational modulation of the p53-murine double minute 2 (Mdm2) feedback loop [36]. Thus, these reports together with our finding provide a fresh look at the function of IGF-1R, indicating that cancer intervention by disruption of IGF-1R signalling should be carefully operated.

Oxidative stress can damage cellular macromolecules such as lipids, protein and DNA and is known to be involved in triggering apoptosis induced by exterior factors such as environmental pollutants, ionized radiation and UV irradiation [37]. Numerous anticancer drugs exert their cytotoxicity via oxidative stress-mediated mechanisms. It was suggested that

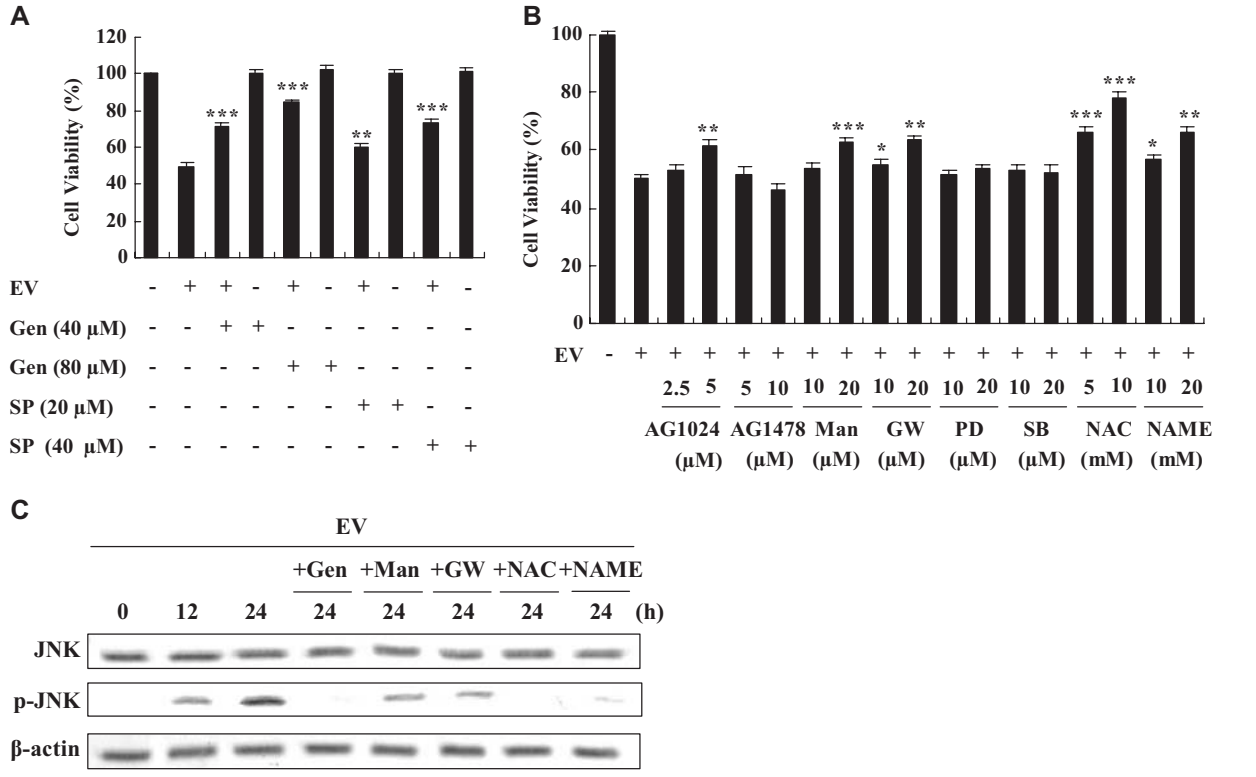

Figure 5. Assessment of the possible signalling pathway involved in evodiamine-induced cell growth inhibition. (A) The cells were pretreated with genistein (Gen) or SP600125 (SP) at the indicated concentration for 1 h and then incubated with 21 μM evodiamine (EV) for 24 h. The viability of cell was measured by MTT assay. $^{***}p < 0.001$, $^{**}p < 0.01$, as compared with the viability in group treated with evodiamine alone. Data are presented as mean ± SD ($n = 3$). (B) The cells were cultured in the presence or absence of AG1024, AG1478, manumycin A (Man), GW5074 (GW), PD98059 (PD), SB203580 (SB), NAC or L-NAME (NAME) at indicated concentrations for 1 h prior to the addition of 21 μM evodiamine and then incubated for 24 h. The viability of cells was determined by MTT assay ($n = 3$). Values are expressed as mean ± SD. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs the viability in group treated with evodiamine alone. (C) The cells were treated with 21 μM evodiamine for the indicated time periods in the presence or absence of 80 μM genistein (Gen), 20 μM manumycin A (Man), 20 μM GW5074 (GW), 10 mM NAC or 20 mM L-NAME (NAME), followed by Western blot analysis for detections of JNK and p-JNK expressions. β-actin was used as an equal loading control.

mitochondria and NADPH oxidase are two major sources of ROS induction. Mitochondria generate ROS as byproducts of respiration; while NADPH oxidase is a membrane enzyme that is responsible for the oxidative burst [38]. Recent work has suggested that ligand-induced activation of RTKs is reported to lead to the phosphatidylinositol 3-kinase (PI3K)-mediated activation of Rac, which switches on the production of ROS from NADPH oxidase. The intracellular ROS then catalytically inactivated protein tyrosine phosphatases (PTPs) through oxidation of active-site cysteine residues, which negatively regulated RTK activity and downstream signalling, hence allowing the sustained RTK phosphorylation and activation [39]. Our present finding that $\Delta\Psi$m dissipation can be prevented by PTK or JNK inhibitor also suggested that the intact mitochondria, which are crucial for maintaining the normal redox environment in cell, can be damaged by PTK signalling. Therefore, the PTK pathway might interfere with both two major sources of ROS to execute cell damage induced by oxidative stress. Moreover, we found that there was a mutual cross-talk between JNK activation and ROS/NO productions, as shown by the suppressive effect of JNK inhibitor SP600125 on ROS/NO generations and the preventive role of ROS scavenger or NOS inhibitor in JNK phosphorylation. Several studies have provided evidence that JNK can be activated by hydrogen peroxide though the activation of MAPK kinase kinases (MAPKKKs) or suppression of phosphatases involved in JNK inactivation by oxidizing cysteine residues critical for their phosphatase activities and then affect the cellular redox status in turn [40]. Thus, PTK signalling might target at multiple levels to enlarge the loop to induce ROS productions.

It has been well accepted that many of the G2/M regulators appear to ultimately target Cdc2, the activation of which requires its association with cyclin B1 and phosphorylation at Thr161 and dephosphorylation at Thr14/Tyr15, thus controlling the G2/M phase transition in eukaryotes [41]. Cdc25C is a protein phosphatase responsible for dephosphorylating the two conserved inhibitory Thr and Tyr residues on Cdc2 in order to promote its activation. When phosphorylated at Ser216, Cdc25C binds to members of the 14-3-3 family of proteins, preventing its activation

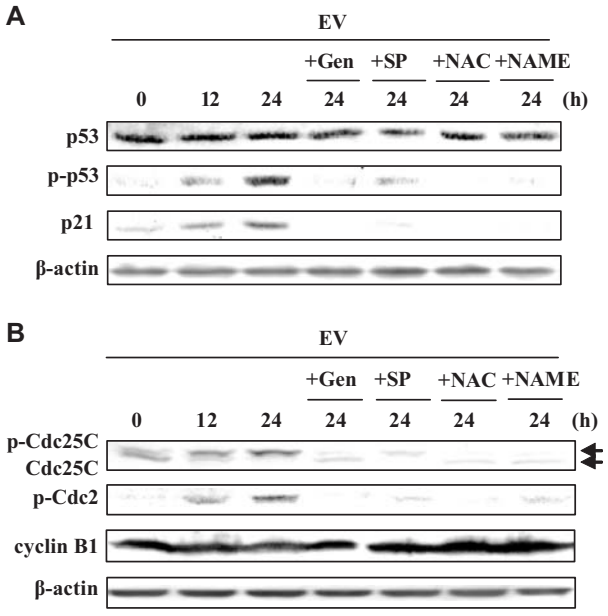

Figure 6. Impairment of genistein, SP600125, NAC or L-NAME on the increased expressions of p-p53, p21, p-Cdc25C, p-Cdc2 and cyclin B1 induced by evodiamine. The cells were treated with 21 μM evodiamine for the indicated time periods in the presence or absence of 80 μM genistein (Gen), 40 μM SP600125 (SP), 10 mM NAC or 20 mM L-NAME (NAME), followed by Western blot analysis for detections of p53, p-p53, p21 (A), Cdc25C, p-Cdc25C, p-Cdc2 and cyclin B1 (B) expressions. *β*-actin was used as an equal loading control.

of Cdc2 [42]. The data from our study revealed that the G2/M checkpoints were deregulated by evodiamine through phosphorylating Cdc25C at Ser216 and Cdc2 at Tyr15 as well as by decreasing the expression of cyclin B1 (Figure 7). It has been shown that DNA damage was able to suppress Cdc2 activity by inhibiting the accumulation of cyclin B1 mRNA and protein [43]. A similar report demonstrated that decreased Cdc2 kinase activity after radiation was mediated by reduced expression of cyclin B1 protein due to shortened cyclin B1 mRNA half-life in HeLa cells [44]. Thus, the reductions of cyclin B1 and the inability of Cdc25C were two contributors to the inactivation of Cdc2 in this study. Moreover, it was shown that ROS generations were associated with diallyl trisulphide-induced G2/M phase cell cycle arrest in PC-3 and DU145 human prostate cancer cells, which caused rapid degradation and phosphorylation of Cdc25C at Ser216, leading to accumulation of Tyr15-phosphorylated Cdc2 [45]. JNK was demonstrated to regulate the Cdc2/cyclin B1 complex following stress events by phosphorylation of Cdc25C [46]. Together with our finding, ROS/NO generations as well as JNK signalling were considered as two key regulators of Cdc25C, Cdc2 and cyclin B1 for controlling the G2/M transition.

ROS are also considered to be potent activators of p53 function and are key factors in the activation of p53 by many chemotherapeutic agents [47]. 14-3-3 is one of the p53 downstream genes and p53 locates upstream of Cdc25C by promoting the formation and maintenance of the Cdc25C/14-3-3 complex, hence inhibiting the formation of the Cdc2/cyclin B1 complex [48]. ROS contribute to p53 activation in

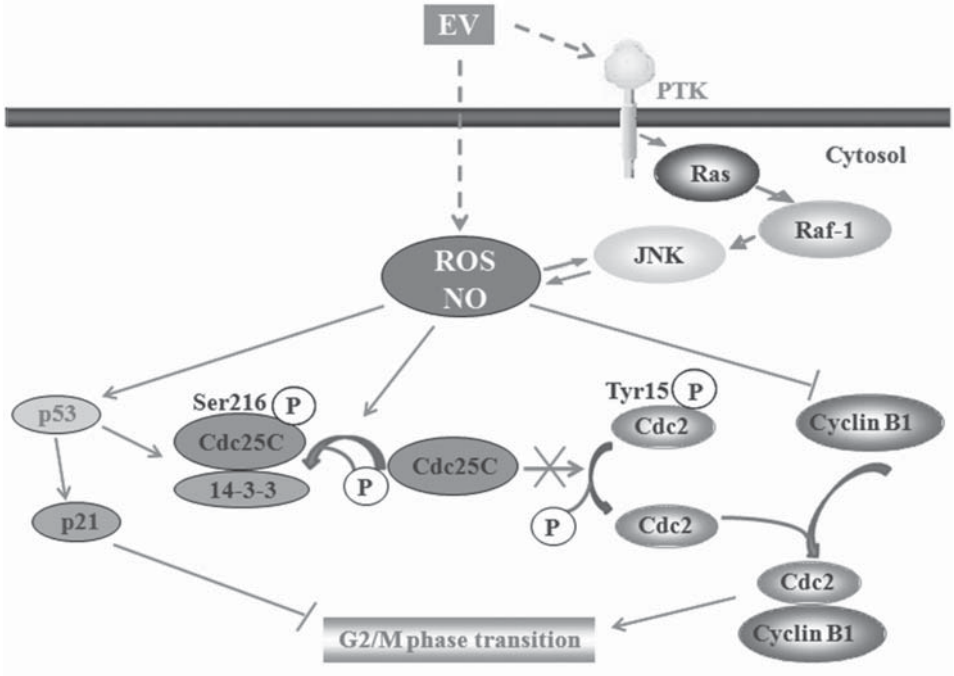

Figure 7. Schematic diagram of the possible mechanism for evodiamine-induced G2/M cell cycle arrest in HeLa cells. Evodiamine (EV) may act through PTK signalling pathway, Ras-Raf-JNK, resulting in the increased enrichment of ROS and NO productions through a positive feedback loop. ROS/NO then disrupt the normal transition of G2 to M phase by inducing p53 and p21 activation, inactivating Cdc25C, which is vital for Cdc2 phosphorylation at Tyr15, by promoting Cdc25C phosphorylation at Ser216 and suppressing the expression of cyclin B1, hence preventing the formation of Cdc2/cyclin B1 complex that regulates G2 to M phase transition.

many ways, such as through direct damage to DNA, via NF-κB dependent upregulation of p53 and by modulating the redox status of a critical cysteine in the DNA-binding domain of p53 which affects its DNA binding activity [49–51]. ROS were shown to induce p21 gene expression by an unknown mechanism that may involve p53 [52]. JNK are also capable of phosphorylating p53 and have been implicated in regulating p53 expression levels through stabilization of the p53 protein under oxidative stress [53]. In view of these reports, our study suggested that p53 activation was stimulated by ROS/NO generations and JNK signalling, which might participate in evodiamine-induced G2/M cell cycle arrest by controlling Cdc25C activity.

In conclusion, our study interpreted a distinct mechanism for ROS/NO generations by induction of IGF-1R-involved Ras-Raf-JNK signalling pathway in evodiamine-treated human cervix carcinoma HeLa cells (Figure 7). A positive feedback loop was found to be enlarged between JNK and ROS/NO bursts, mediating the cytotoxicity of evodiamine through deregulations of p53 and p21 and leading to G2/M cell cycle arrest by crippling critical G2/M checkpoints Cdc25C, Cdc2 and cyclin B1. This finding gives us a novel look at the correlations between PTK signalling and ROS/NO productions, therefore providing a different view for anti-cancer drug development.

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