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Synthesis of 3-carbethoxy-4(3'-chloro-4'-hydroxy) phenyl-but-3-en-2-one and its cytotoxicity evaluation against cancer cell carrying mutant p53

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apoptosis,
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ABSTRACT

Overexpression of mutant p53 in cancer cell inactivates the p53 pathways to execute apoptosis and cell cycle arrest. This study aimed to synthesize new kardiena derivative compound, 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one, to reactivate the p53 pathways to execute apoptosis and cell cycle arrest. Characterization of the synthetic compound employing melting point, IR, EI-MS, ^{1}H-NMR, and ^{13}C-NMR spectra revealed 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one was successfully synthesized from 3-chloro-4-hydroxybenzaldehyde and ethyl acetoacetate using dimethylamine as a catalyst. This compound had antiproliferative activity against the WiDr cells which carried mutant p53. Its antiproliferative activity was better than 5'-FU as a reference standard to treat colon cancer. Increasing WiDr cell accumulation in the G2-M phase, the active form of caspase-3, and inducing apoptosis demonstrated the ability of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one to reactivate p53 pathways to execute apoptosis and cell cycle arrest in cancer cells carrying mutant p53.

ABSTRAK

Overekspresi p53 mutan pada sel kanker dapat menginaktivasi jalur p53 dalam proses apoptosis dan penghentian siklus sel. Tujuan penelitian ini adalah mensintesis senyawa baru turunan kardiena untuk mengaktifkan kembali jalur p53 dalam proses apoptosis dan penghentian siklus sel. Karakterisasi dari senyawa hasil sintesis menggunakan titik lebur, spektra IR, EI-MS, ^{1}H-NMR, dan ^{13}C-NMR menunjukkan senyawa 3-karbetoksi-4(4'-hidroksi-3-kloro)fenil-but-3-en-2-on berhasil disintesis dari 3-kloro-4-hidroksibenzaldehida dan etilasetoasetat menggunakan katalis dimetilamina. Senyawa ini mempunyai aktivitas antiproliferasi terhadap sel WiDr yang membawa p53 mutan. Aktivitas antiproliferasi senyawa ini lebih baik dibanding 5'-FU yang digunakan sebagai standard dalam pengobatan kanker kolon. Peningkatan akumulasi sel WiDr pada fase G2-M, bentuk aktif caspase-3 dan induksi apoptosis menunjukkan kemampuan 3-karbetoksi-4(4'-hidroksi-3-kloro)fenil-but-3-en-2-on untuk mengaktifkan kembali jalur p53 dalam melakukan apoptosis dan penghentian siklus sel kanker yang membawa p53 mutan.

INTRODUCTION

Mutant p53 is a missense mutation of the *p53* gene.[1-3] The thermostability and melting point of mutant p53 are lower than the p53 wild type (wt). Mutant p53 has different conformation (misfolding) compared to p53 wt.[4] The binding site of mutant p53 is also located at a different specific sequence in the DNA position than p53 wt.[5] Mutant p53 do not transactivate the tumor suppressor gene to control cell division fidelity. They transactivate a

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new activity which is distinct from p53 and have proto oncogenic properties termed as a gain of function (GOF) p53 mutant.[6-10] Overexpression of mutant p53 in the cancer cell nuclei contributes to the GOF activities such as: increasing malignancies, proliferation, and resistance to conventional chemotherapy.[11-17] Accordingly, the existence of mutant p53 in cancer cells requires a new therapeutic strategy.

Anticancer treatment using a small molecule which is targeted to mutant p53 results in remarkable implications in cancer therapy[18-22] PRIMA-1 and MIRA-1 demonstrate favourable activity against cancer cells carrying mutant p53, as depicted in FIGURE 1.[23-24] PRIMA-1 is a type of prodrug that undergoes metabolism to methylene quinuclidinone (MQ). The α, β-unsaturated carbonyl moieties in MQ and MIRA-1 have an important role to carry out alkylation of the thiol group of mutant p53's cysteine through Michael addition reactions.[23,25] The residues of mutant p53's amino acids that become alkylation targets are Cys-277 and Cys-124.[26,27] The Michael addition reaction between the small molecule-anticancer with cysteine's thiol group of mutant p53 yields a small molecule-mutant p53 complex. This complex conformation closely resembles p53 wt conformation. This small molecule-mutant p53 complex binds in the same specific sequence of DNA gene target as p53 wt. Subsequently, it leads to reactivation of the p53 pathways to execute apoptosis and cell cycle arrest.[19,28,29]

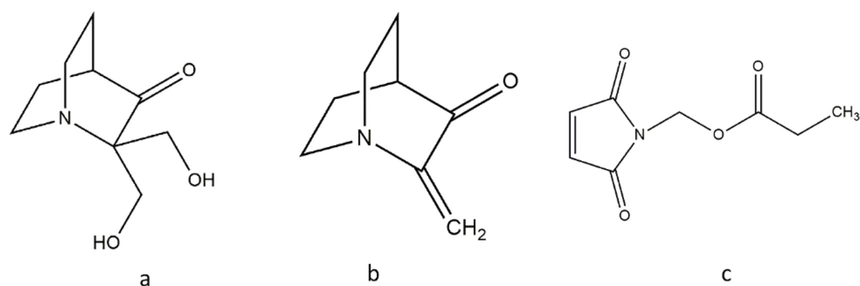

FIGURE 1. Structure of PRIMA-1 (a), MQ (b), and MIRA-1 (c)

This research aimed to synthesize new kardiena derivative compound,3-carbethoxy-4(3'-chloro-4'-hydroxy) phenyl-but-3-en-2-one, targeting Cys-124 amino acid residue in the mutant p53 pockets which are located between the L1 loop and the S3 sheet (L1/S3). Furthermore, in this study, we also evaluated the activity of the synthesized compounds against cancer cells carrying mutant p53 in order to reactivate the p53 pathways. Considering the position of Cys-124 in mutant p53, which is more open and located in a larger pocket size than in p53 wt, it gives some advantage for new compounds to bind selectively.[26,30,31]

The 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one has an α, β-unsaturated carbonylmoiety with an incorporated hydroxyl phenolic group and ester. Incorporation of phenyl and ester forms additional interactions with others amino acid residues in a mutant p53 pocket. The additional interactions increase its ability to reactivate the p53 pathways thus leading to the apoptosis process.[30] The synthesis method of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one was conducted based on the Knoevenagel condensation principle between 3-chloro-4-hydroxybenzaldehyde and ethyl acetoacetate using dimethylamine as the organic base catalyst.

A WiDr cell linewas employed in order to measure the efficacy of 3-carbethoxy-4(3'-chloro-4'-hydroxy) phenyl-but-3-en-2-one in reactivating the p53 pathways. This cell line is a colon

cancer cell having a missense mutation in the amino acid residue Arg-273 of the p53 gene.[32] Several parameters were examined such as antiproliferative activity using MTT assay, cell cycle analysis, caspase-3 activation, and apoptosis using flow cytometry.

MATERIALS AND METHODS

Materials

The starting materials had synthetic grade and were purchased from Sigma-Aldrich (Indonesia). All of the solvents had pro analytic grade and were purchased from Merck (Indonesia). Materials for the cell culture assays, RPMI-1640 medium (Gibco, USA), fetal bovine serum (Gibco, Thermo Fischer USA), MTT reagent (Sigma-Aldrich, Indonesia), penicillin-streptomycin 1% (v/v) (Gibco, USA) were provided. DNA Cycle test (BD Biosciences, USA), Annexin-V-(FITC) and PI (Roche, USA), Caspase-3 FITC (BD Pharmingen, USA), 5-fluorouracil (5'-FU) (Curacil®) in a liquid dosage form for IV injection was obtained from PT Kalbe Farma, Indonesia. The WiDr human colon cancer cell line that carries mutant p53 (Arg-273-His) was obtained from the Laboratory of Parasitology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Instrumentation

The ^{1}H-NMR spectra were recorded in a JEOL 500 MHz Spectrometer. ^{13}C-NMR spectra were recorded in a JEOL 125 MHz Spectrometer. Chemical shifts (δ) were reported in ppm, using TMS (δ = 0) in $CDCl_3$ as an internal standard. ESI-MS spectra were recorded in The Waters Xevo TQD. IR spectra were recorded in Shimadzu Prestige-21. Melting points were obtained using a Mettler Toledo MP70. Instrumentations for cell culture assays, laminar air flow cabinet (Labconco, Purifier Delta Series Class II), ELISA reader (BIO-RAD Benchmark), flow cytometer (BD FACSCalibur), inverted microscope (Olympus).

Synthesis of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one

3-Chloro-4-hydroxybenzaldehyde (0.01 mol, 1.56 g), ethyl acetoacetate (0.01 mol, 1.3 mL), dimethylamine (0.01 mol, 0.6 mL) and ethanol (10 mL) were stirred under nitrogen condition for 1 h. HCl 1N (10 mL) and aquadest (100 mL) were added to make the mixture. This mixture was kept in refrigerator for overnight. The obtained crude product was recrystallized from ethanol: water (1:9).

Cytotoxicity assay

Cytotoxic effects of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one and 5'-FU in WiDr cells were measured by MTT assay.[33] We employed 5'-FU as the positive control.[34] WiDr cells ($5x10^3$ cells/well) 100 μL were seeded in 96 well-plate and incubated for 24h. Cells were treated with various concentrations of 3-carbethoxy-4(3'-chloro-4'-hydroxy) phenyl-but-3-en-2-one, i.e. 200; 100; 50; 25; 12.5; 6.25; 3.12 (μM). As the positive control, the cells were treated with 5'-FU i.e. 3800; 1900; 950; 475; 237.5; 118.75; 59.375 (μM). Both groups of treatment were incubated for 72h. After incubation, the cells were washed with phosphate-buffered saline (PBS) and then added with MTT reagent. Around twoh later, a 10% SDS in 0.01 M HCl was added and incubated for overnight in the dark. The absorbance of each wells was measured by using a microplate reader at λ 570 nm. Cell viability (%) was defined as (absorbance of treated cells – absorbance of blank)/(absorbance of untreated cells – absorbance of blank) x 100%.

Cell cycle analyses

WiDr cells ($1x10^5$ cells/well) were seeded in six well-plate and incubated for 24h. Cells were treated with 37.3 μM (¾ of IC_{50} value) of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one and incubated for 72h. Cell cycle analyses were performed using the DNA Cycle test according to the manufacturer's instructions. Cell cycle distribution was analyzed by flow cytometry using BD

FACSCalibur. The percentage of the cells in every phase was determined by BD CellQuest Pro software.

Caspase-3 activation assay

Activation of caspase-3 due to 3-carbethoxy-4(3'-chloro-4'-hydroxy) phenyl-but-3-en-2-one treatment with concentration 37.3 μM (¾ of IC_{50} value) was analyzed by flow cytometry using BD FACSCalibur. Caspase-3 FITC was used as a caspase-3 antibody according to the manufacturer's instructions. Preparation and treatment of WiDr cells were similar to cell cycle analyses. The percentage of activating caspase-3 was determined by BD CellQuest Pro software.

Cell apoptosis assay

Apoptosis effect of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one with concentration of 37.3 μM (¾ of IC_{50} value) was detected using Annexin-V-(FITC) and PI as a staining agent according to the manufacturer's instructions. Preparation and treatment of WiDr cells were similar to cell cycle analyses. The apoptotic cells were counted by flow cytometry using BD FACSCalibur. The percentage of the apoptotic cells was determined by BD CellQuest Pro software.

RESULTS

Characterization of synthetic compounds

The synthetic compound was a pale yellow solid. Its yield was 53% (0.224 g), Mp 127.1-129.1°C andpurity: 90%. This compound had IR (pellet): υ_{max} 3261, 1734, 1698, 1667, 1596, 1260, 1050. ^{1}H-NMR (500 MHz, $CDCl_3$): 1.33 (*t*, *J* = 7 Hz, 3H, $-O-CH_2-CH_3$), 2.42 (*s*, 3H, $-(C=O)-CH_3$), 4.37 (*q*, *J* = 7 Hz, 2H, $-O-CH_2-CH_3$), 7.16 (*d*, J_{5-6}= 8 Hz, 1H, aromatic proton), 7.31 (*dd*, J_{5-6}= 8 Hz, J_{2-6} = 2 Hz, 1H, aromatic proton), 7.44 (*s*, 1H, -CH=C-), 7.48 (*d*, J_{2-6}= 2 Hz, 1H, aromatic proton), and 9.84 (*s*, 1H, C_6H_3-OH). ^{13}C-NMR (125 MHz, $CDCl_3$): 13.93 ($-O-CH_2-CH_3$), 26.55 ($-(C=O)-CH_3$), 62.00 ($-O-CH_2-CH_3$), 116.74, 121.13, 126.33, 130.83, 153.62, and 156.71 (aromatic carbons), 133.33 (CH=C-), 139.75 (-CH=C-), 167.92 (-C=O, α, β-unsaturated ester), and 194.85 (-C=O, β-keto ester in enol form). ESI MS (*m/z*): 269.171 $(M+H)^+$ (calcd. for $C_{13}H_{14}O_4Cl$, 269.201).Based on the structure elucidation performed by IR, ESI/EI-MS, ^{1}H-NMR, and ^{13}C-NMR, we have successfully synthesized the novel 3-carbethoxy-4(3'-chloro-4'-hydroxy) phenyl-but-3-en-2-one compound.

The m/z ratio of ESI-MS informed us about the molecular weight of this compound. The presence of a peak at wave number 1734 cm^{-1} in IR spectra indicated conjugated-carbonyl ester. The peak of ester shifted to the right due to electron delocalization reducing the double bond character of the carbonyl. The peak at a chemical shift of 167.92 ppm in ^{13}C-NMR represented the carbon-carbonyl peak of conjugated-ester. A conjugation in ester caused its carbon-carbonyl peak to be more shielded due to electron delocalization. The appearance of conjugated-ester peak (α, β-unsaturated ester) was important evidence of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one yield.

Antiproliferative activity

We examined the antiproliferative activity of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one against WiDr cell using MTT assay and 5'-FU as a reference compound. 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one demonstrated antiproliferative activity with IC_{50} value 47.5 μM. This compound had better antiproliferative activity than 5'-FU (IC_{50} = 71.84μM). Antiproliferative activity of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one related to existence of α, β-unsaturated group. This functional group was required in antiproliferative activity against cancer cells carrying mutant p53.

Cell cycle analysis

Cell cycle arrest in the G1-S or G2-M phase is one of the downstream events

of p53.[35] The result of cell cycle analysis demonstrated a G2-M arrest after treatment with 47.5 μM of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one. This compound accumulated 22.44% of cells while WiDr cells without treatment accumulated 8.10% of cells in the G2-M phase as shown in FIGURE 2. It indicated that 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one successfully reactivated the p53 pathway.

Caspase-3 activation

Caspase-3 activation is an important step prior to executing apoptosis.[36] The caspase-3 activation was examined by using flow cytometry. It was revealed 4.28% of WiDr cells contained an active form of caspase-3 after treatment with 47.5 μM of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one as shown in FIGURE 3. A treatment with 3-carbethoxy-4(3'-chloro-4'-hydroxy) phenyl-but-3-en-2-one increased the active form of caspase-3 in WiDr cells significantly more than the amount of active form of caspase-3 without treatment which was only 2.24%. Activated caspase-3 executed apoptosis in the WiDr cells which treated with 3-carbethoxy-4(3'-chloro-4'-hydroxy) phenyl-but-3-en-2-one.

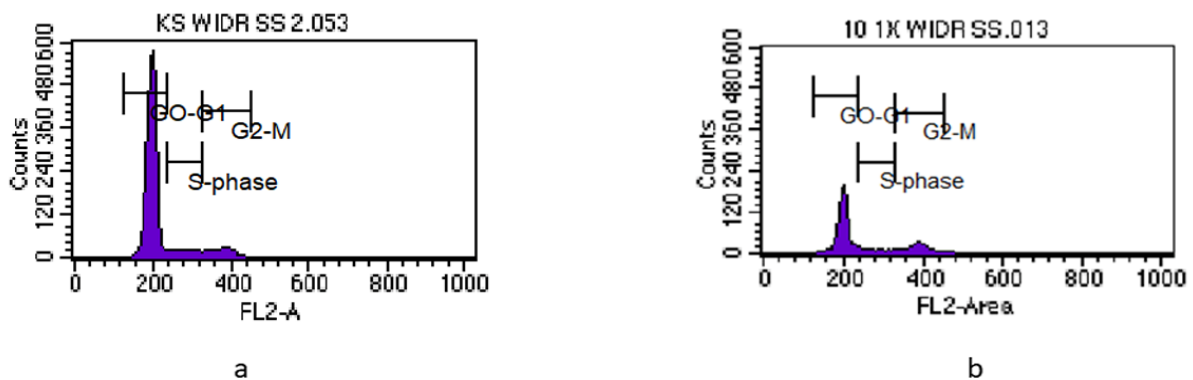

FIGURE 2. Cell cycle analysis of WiDr cell lines using flow cytometry. WiDr as control cell accumulated in 83.2% (G0-G1), 8.92% (S-phase), 8.1% (G2-M) (a). 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one treatment to WiDr cell changed its accumulation phase. WiDr cell accumulated in 67.47% (G0-G1), 10.27% (S-phase), 22.44% (G2-M) (b).

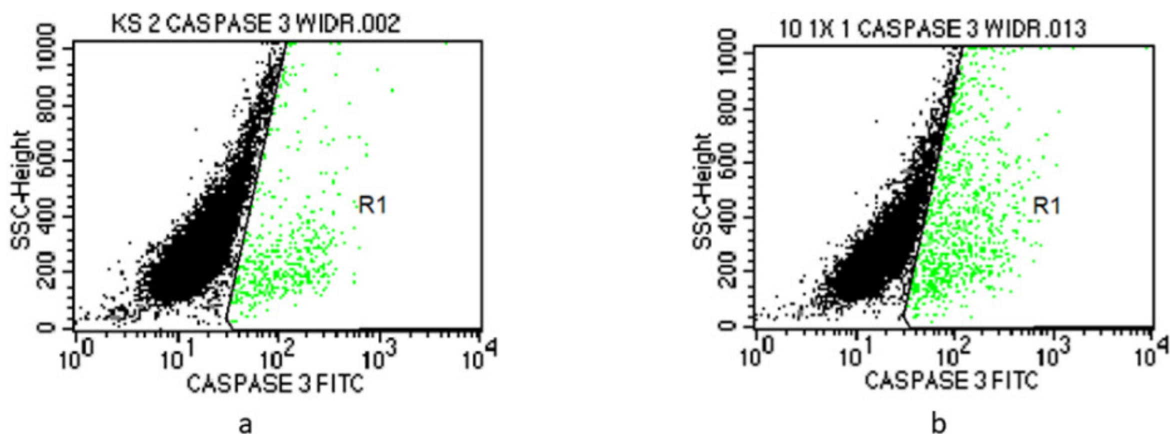

FIGURE 3. Analysis of active form of caspase-3 in WiDr cell lines using flow cytometry. WiDr cells without treatment had 2.24 % active form of caspase-3 (a). 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one treatment to WiDr cell increased active form of caspase-3 become 4.28% (b).

Cell apoptosis

The examination of WiDr cells which experienced apoptosis using flow cytometry after staining with annexin V FITC and PI are shown in FIGURE 4. Treatment of 47.5 μM of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-oneaffected the apoptosis of WiDr cells by 19.19%. The result revealed a significantly increasing amount of apoptosis occurrence in WiDr cells after treatment with 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one than without treatment which was only 4.54%. WiDr cells that experienced apoptosis after 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one treatment was proof of the successful reactivation of the p53 pathways in cancer cells carrying mutant p53. The apoptosis level of the WiDr cell line was higher than its cell cycle arrest after treatment with 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one. It indicated that impairment of DNA in the WIDr cell line preferred to induce apoptosis through the p53 pathway.

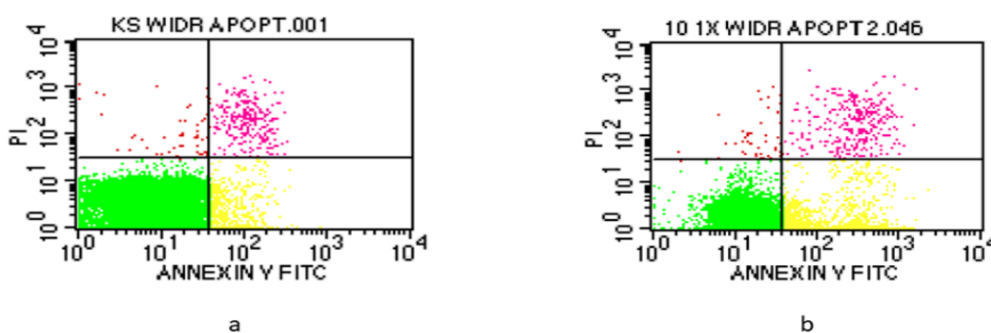

FIGURE 4. Apoptosis analysis with staining Annexin V FITC/PI in WiDr cell lines using flow cytometry. In WiDr cells as control sel, 4.54% WiDr cell experienced apoptosis (a). 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one treatment to WiDr cell causing 19.19% of WiDr cell experienced apoptosis (b).

DISCUSSION

3-Carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one was synthesized using the Knoevenagel condensation principle. The synthesis process was initiated by generating enolate ions of ethyl acetoacetate using dimethylamine. Enolate ions have higher nucleophilicity thus making it easier to react with C of carbonyl of 3-chloro-4-hydroxybenzaldehyde. This reaction yielded a β-hydroxy carbonyl product which was an intermediate compound. β-hydroxy carbonyl underwent dehydration reaction to yield α, β-unsaturated carbonyl product simultaneously. The presence of α, β-unsaturated carbonyl indicated success of the 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one synthesis process, as shown in FIGURE 5.

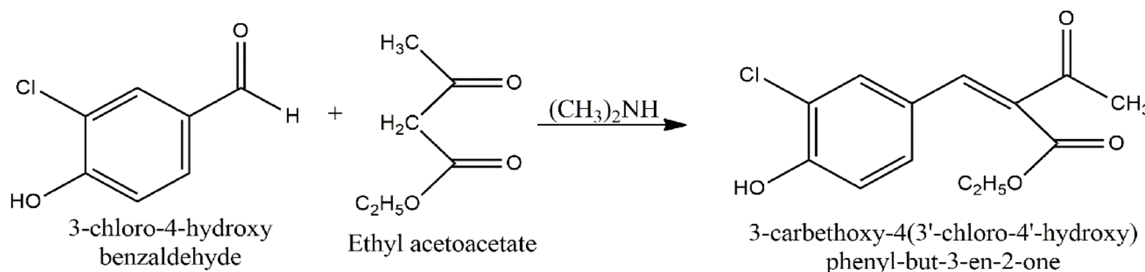

FIGURE5. Synthesis of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one.

The presence of α, β-unsaturated carbonyl in 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one was responsible as antiproliferative effect. This functional group as target of Cys-124 of mutant p53 through Michael addition reaction. The result of this reaction was acomplex3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one-mutant p53. This complex had a resemble conformation with p53 *wt*. Therefore, the complex was able to bind like p53 *wt*at the specific sequence of DNA target. The bind formed successfully reactivated the p53 pathway in WiDr cell line.

The reactivating p53 pathway arrested the cell cycle at G2-M phase and induced apoptosis. We suggested that the complex of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one-mutant p53 transactivated 14-3-3δ and reprimo. 14-3-3δ protein sequestered cyclin B-CDC2 complex to the cytoplasm. This complex sequestration prevented it from entering the nucleus so that inhibit the mitotic process.

Based on correlation between increasing amount of actived form of caspase-3 and experienced apoptosis in WiDr cell line, we suggested that apoptosis mechanism through BAX transactivation. BAX attachment to mitochondria caused mitochondria's channel opening. Cytochrome C which released from mitochondria aggregated with Apaf-1 to form apoptosome. The apoptosome converted procaspase 9 into active caspase 9. The active form of caspase9 cleaved procaspase 3 into active caspase 3, which in turn executed apoptosis.

CONCLUSION

Based on our findings, 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one was successfully synthesized from 3-chloro-4-hydroxybenzaldehyde and ethyl acetoacetate using dimethylamine as the catalyst. This compound demonstrated antiproliferative activity against the WiDr cells, even more potent than 5'-FU. Furthermore, efficacy examination of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one exhibited its ability to increase the accumulation of WiDr cells in the G2-M phase, increasethe active form of caspase-3, and increase the apoptosis. These results demonstrated the anticancer activity of 3-carbethoxy-4(3'-chloro-4'-hydroxy)phenyl-but-3-en-2-one against cancer cells carrying mutant p53 through the p53 pathway reactivation.

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