Exploring the effectiveness of existing anticancer drugs and presenting an alternative solution for cancer treatment

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Abstract

Today, although numerous anticancer drugs and treatments were developed and used, cancer is the second leading cause of death in the world. Through this study, I explored potential reasons for the substantial number of cancer deaths despite numerous anticancer drugs out there. Here I show the effectiveness of the eight anticancer drugs available at the moment, which are known to have significant impact on cancer cells. Four out of the eight anticancer drugs show a dramatic reduction of cancer cell viability. These drugs induce translational expression of p53 and trigger p53-dependent mechanism to induce Bax, a target gene of p53 responsible for apoptosis. Unfortunately, despite the notable effects of these anticancer drugs, those described above cannot reduce cancer mortality completely. In this study, I suggest possible reasons why mutations in p53 in cancer cells make p53-dependent anticancer treatments ineffective and that early detection may be a more efficacious way to decrease cancer mortality. Consequently, I would like to examine various methods that would help accelerate the diagnosis of cancer for everyone.

Keywords

TP53, Cancer, Anti-cancer drug, Early cancer diagnosis

Introduction

Cancer is one of the major causes of death worldwide. According to World Health Organization, the estimated number of cancer deaths in 2020 was around 10 million.[1] The number of new cancer cases is expected to rise in the following decades, due to increasing life expectancy. Incidence rate of cancer has a strong direct correlation with life expectancy.[2,3] Cancer is caused by accumulations of gene mutations over aging. At an older age, more DNA mutations would be deposited in the body, posing more cancer risks than at a younger age.[4,5]

These mutations mainly occur in oncogenes and tumor suppressor genes in cancer and lead to an activation of oncogene and inactivation of tumor suppressor genes. The most common oncogenes are Ras and MYC, which promote cell proliferation through avoiding the function of tumor suppressor genes such as cell cycle arrest and apoptosis. Retinoblastoma (Rb), p16, and p53 are representative tumor suppressor genes. Rb is a transcriptional regulator that gathers transcriptional co-repressors or blocks transcriptional co-activators.[6,7] p16, another tumor suppressor gene, activates an inhibitor of cyclin-dependent kinases (CDK) that drives the cell cycle.[8,9] Lastly, p53 is a tumor suppressor gene that is known to have most mutations in cancer. p53 inactivation is common in cancer development.[10,11] Thus, anticancer drugs were developed to inhibit oncogene and activate tumor suppressor or its related signals.

Three major types of cancer treatment are surgery, chemotherapy, and radiation therapy. Unlike surgery and radiation therapy, chemotherapy is appropriate for widespread cancers. Surgery and radiation therapy can only treat cancers in certain areas, while chemotherapy can be used for cancers that have spread throughout the body.[12] Major

categories of anticancer drugs include alkylating agents, Cytotoxic antibiotics, antimetabolites, anti-microtubule agents, topoisomerase inhibitors, and kinase inhibitors.[13] For each cancer type and stage, different types of anticancer drugs are used in combination.[14] In the early stage of breast cancer, for example, Doxorubicin, one of the cytotoxic antibiotics, is mainly used with other drugs.[15] For lung cancer, common treatment consists of Cisplatin or Carboplatin, members of alkylating agents.[16] In colorectal cancer treatment, Fluorouracil or Capecitabine, members of antimetabolites, are often used.[17]

While exploring about the lifespan of human, I discovered that heart disease and cancer were major causes of death. I wondered why cancer, not other diseases, is the leading cause of death until now despite the development of numerous anticancer drugs. During this experiment, I was trying to learn more about cancer in general, and how specific genes take crucial role in tumor. Moreover, I was able to learn about how anticancer drugs work in general.

Results

To investigate the effectiveness of anticancer drugs, I treated with eight different anticancer drugs used in clinic on colon cancer cell line (HCT116). Out of these anticancer drugs, Doxorubicin is a cytotoxic antibiotic, Etoposide is a topoisomerase inhibitor, Paclitaxel is a member of microtubule disruptors, and Imatinib, Gefitinib, Erlotinib, and Vactosertib are kinase inhibitors. MTT assay was conducted to test the effects of anticancer drugs in relative cell viability. The cell viability decreased significantly after the treatment of Doxorubicin, Etoposide, Nutlin, and Paclitaxel, but kinase inhibitors did not show significant changes in cell viability (Figure 1A).

To examine about working modes of drugs that showed significantly reduced cell viability in more detail (Figure 1A), I observed the change in cell morphology after treatment of each drug using microscope. Microscopic images showed that cells treated with Doxorubicin, Etoposide, and Nutlin exhibit shrunken morphologies. However, cells treated with Paclitaxel did not show shrunken morphology, but cell size was increased, and nucleus was fused (Figure 1B). Cell shrinkage is a characteristic of apoptosis, suggesting that Doxorubicin, Etoposide and Nutlin induced apoptosis in cells. Paclitaxel, on the other hand, did not have an apoptotic morphology, which indicates an activation of other activities such as cell cycle arrest.

Since anticancer drugs that showed a strong decrease in cell viability are known to have DNA damage mechanisms and cell shrinkage related to apoptosis, I hypothesized that treatment of these anticancer drugs would increase the level of p53 to induce apoptosis or cell cycle arrest. p53 has been reported as the most important regulator on cell cycle arrest or apoptosis, which are the responses to DNA damage. To test my assumption, I

performed western blot analysis to validate induction of the protein level of p53. The result showed an increase in p53 expression level in cells treated with Doxorubicin, Etoposide, and Nutlin. There was no change, however, in the p53 level in cells treated with Paclitaxel (Figure 1C). These results indicate that p53 might have induced cell death in cells treated with anticancer drugs.

Such roles of p53 are successfully achieved by encoding p53 transcriptional target genes, such as p21 and Bax. To find out whether p53 acted as a transcription factor of p21 and Bax, I conducted RT-PCR to compare the mRNA levels of p21 and Bax in cells treated with chemicals. The mRNA level of p21 only increased in doxorubicin. Still, the level of mRNA of Bax increased in all four chemicals (Figure 1D). These results suggest that apoptosis occurred in all anticancer drugs, and cell cycle arrest only occurred in doxorubicin.

In order to verify that these changes in actions are dependent on p53, the same experiments were conducted in HCT116 p53 knockout cells (HCT116 p53 -/-). Unlike HCT116 cells which have intact p53, results from MTT assay did not show significant decrease in cell viability (Figure 2A). Moreover, PCR results did not show an increase in mRNA level of p21 and Bax (Figure 2B). These results indicate that changes that implied cell death mechanism in cells treated with Doxorubicin, Etoposide, and Nutlin were dependent on p53.

Discussion

The purpose of this study was to find possible reasons for the extremely large number of cancer death even with the development of cancer treatments. Thus, through above experiments, I reviewed the effectiveness of anti-cancer drugs (Doxorubicin, Nutlin-3, Etoposide, Paclitaxel, Imatinib, Gefitinib, Erlotinib, and Vactosertib) used in clinic.

Our data showed that Doxorubicin, Nutlin-3, Etoposide, and Paclitaxel had a significant effect in cancer cells, while Imatinib, Gefitinib, Erlotinib, and Vactosertib did not (Figure 1A). These four chemicals (Doxorubicin, Nutlin-3, Etoposide, and Paclitaxel), unlike four kinase inhibitors (Imatinib, Gefitinib, Erlotinib, and Vactosertib), are relevant with p53 activation. Doxorubicin, a strong DNA double strand break inducer, intercalates with DNA base pairs and causes DNA damage[18]. Etoposide stabilizes the cleavage complex, which also leads to a break in DNA double strand[19]. Doxorubicin and Etoposide induce DNA damage which allows an activation of p53, leading to several pathways for apoptosis[20,21]. Nutlin-3 prevents interaction between MDM2 and p53, leading to a stabilization of p53 protein[22]. Therefore, Nutlin-3 can also activate p53 and apoptosis. On the other hand, Paclitaxel stabilizes microtubules and prevents their dissociation. During the mitosis, cells become abnormal and contain multiple spindle poles, inhibiting the growth of cells, which is also related to general p53 function[23,24] . Results in Figure 1A to C indicates that Doxorubicin, Nutlin-3, and Etoposide induced apoptosis of cancer cells, which is managed by p53 and the level of p53 increased in cells that show apoptotic morphology.

The major role of p53 is to function as a transcriptional factor of p21 and BAX[25]. p53-induced p21 mRNA is translated to p21 protein and binds to Cdk4/cyclin D and Cdk2/cyclin E complexes to inhibit their kinase activities, resulting in G1 cell cycle arrest

[26]. BAX is one of the proapoptotic transcriptional targets of p53[27]. Thus, p53, as a transcription factor that regulates signaling pathways in order to induce cell cycle arrest or apoptosis, p53-induced cell cycle arrest, and apoptosis play significant roles in tumor suppression[28,29]. In our results of Figure 1D, the mRNA levels of both p21 and Bax were increased by Doxorubicin treatment. Other anticancer drugs did not increase p21, but increased Bax (Figure 1D). Upregulation of BAX by p53 leads to mitochondrial outer membrane permeabilization (MOMP), which is one of the two ways of apoptosis. These results suggest that Doxorubicin induces both apoptosis and cell cycle arrest simultaneously and the other drugs (Etoposide, Nutlin, and Paclitaxel) induce only apoptosis.

Apoptosis, also called programmed cell death or cellular suicide, is one of the responses of p53 to DNA damage. Its representative morphological features include cell shrinkage, nuclear fragmentation, and cell membrane blebbing[30]. Observing the microscopic images of cancer cells treated with Doxorubicin, Nutlin-3, and Etoposide, they showed cell shrinkage, which corresponds to an apoptotic morphology (Figure 1B).

On the other hand, cells treated with Paclitaxel did not show any apoptotic morphology (Figure 1B). This might imply that Paclitaxel inhibited cell proliferation by cell cycle arrest or other p53 independent activity, rather than apoptosis. Cells treated with Paclitaxel show a flattened and enlarged morphology, which is similar to a morphological feature of cellular senescence. In addition, in HCT116 p53 knockout cells, Paclitaxel showed the strongest effect out of four anticancer drugs. This suggests that Paclitaxel has p53 independent mechanism that induces cell death. Several studies also verify this data, suggesting that Paclitaxel can induce p53-independent mechanism[31].

Based on my data, out of anticancer drugs used, p53 targeting drugs had the most significant effect in cancer cell viability (Figure 1). Even though these anticancer drugs showed sufficient effectiveness, none of these chemotherapies cure cancer perfectly in clinic. One possible reason might be related to the mutation frequency of various genes in cancer. In cancer cells, p53 is known to have the greatest mutation frequency (Figure 3). With mutations in p53, these anticancer drugs treatments alone could not have much impact.

Thus, I could suggest two ways to figure it out. First, to find a better treatment for cancer, other effective anticancer drugs not targeting p53 should be developed to be used together. Actually, combination therapy of two or more therapeutic reagents is used for cancer treatment and has been shown to be more efficient than monotherapy[32]. Another potential way to decrease cancer mortality is increasing the rate of early detection. A study showed that average five-year survival at an early stage of cancer is 91 percent, while average five-year survival at late stage is 26 percent[33]. Hence, detecting cancers at the earliest stage possible is significant for decreasing the number of cancer death. Practically, cancer screening method decreased breast cancer mortality in Korea[34] and several detection method and early diagnosis marker are being developed.

Throughout this study, I was able to learn about the effects and limitations of existing anticancer drugs. Especially, I realized that monotherapy of anticancer drugs showed limits in cancer treatment as it only activates one tumor suppressor gene and its downstream signal pathway. Hence, I recognized the need for diverse and continuous development of a new anticancer drugs for combination therapy. Most of all, I found the

potential of early diagnostic markers of cancer, which could overcome the limitations of anticancer drugs. Therefore, I would like to learn in-depth about these early diagnosis methods to suggest a way to decrease cancer mortality and contribute to conquer cancer.

Competing interests

The author declares no competing interests.

Material & Methods

Cells. HCT116 cell lines were kindly provided by Dr. Jinwoo Choi (Kyung Hee University, Seoul, Korea)). Cell lines were cultured in RPMI 1640 (Thermo Scientific, Rockford, IL) with 10% FBS (Thermo Scientific) and 1% antibiotics (Thermo Scientific). All cell lines were maintained in humidified incubator at 37°C with 5% CO2.

Antibodies. Antibodies against P53 (1:1000 for immunoblotting, sc-126) and β-Actin (1:10000 for immunoblotting, sc-47778) was purchased from Santa Cruz Biotechnology.

Western blot analysis. Cells, treated with chemicals, were lysed with RIPA (50 mM TrisCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 10% sodium deoxycholate). Protein concentrations in samples were measured using a SPECTROstar Nano (BMG LABTECH, Baden-Württemberg, Germany) protein assay kit and a bovine serum albumin standard.20 μg of cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). Blots were blocked for 1 hour at room temperature in TBS-T buffer (Tris-HCl based buffer containing 20 mM Tris pH 7.6, 150 mM NaCl and 0.05 % Tween 20) with 3% nonfat dry milk for 1 hour at room temperature. The membrane was incubated for 1 hour to overnight at 4°C with an appropriate primary antibody, followed by a reaction with a secondary monoclonal Horseradish peroxidase (HRP)-conjugated goat anti-mouse (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL, USA) in TBS-T for 2 hours at room temperature. The proteins were visualized using TOPview^{TM} ECL Pico Plus (Enzynomics, Inc. Daejeon, Korea) as recommended by the manufacturer.

Reagents. Doxorubicin, Etoposide, Paclitaxel, Nutlin, Erlotinib, Gefitinib and Imatinib were purchased from Sigma Aldrich (St, Louis, Mo, USA).

RT-PCR. For mRNA analysis, total RNA was Isolated using an EZ^{TM} Total RNA miniprep Kit (EP301-50N; Enzynomics, Inc. Daejeon, Korea), and 1 μg of RNA was converted into cDNA using SuperiorScript III cDNA Synthesis Kit (EZ405S; Enzynomics, Inc. Daejeon, Korea) and random hexamer. After dilution, the cDNA was generated by polymerase chain reactions (PCRs) using 2X TOPsimple^{TM} DyeMIX-*n*Taqi-start Taq (P561T; Enzynomics, Inc. Daejeon, Korea). The PCR conditions contained a denaturing step at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 1 minute, and elongation at 72°C for 32 seconds, and one elongation step at 72°C for 5 minutes. Reverse transcription (RT)–PCR was performed with specific primers of target genes. The primers used in this study were as follows:

Human TP53 (Forward) 5'- CCTCAGCATCTTATCCGAGTGG -3'

Human TP53 (Reverse) 5'- TGGATGGTGGTACAGTCAGAGC -3'

Human p21 (CDKN1A) (Forward) 5'- AGGTGGACCTGGAGACTCTCAG -3'

Human p21 (CDKN1A) (Reverse) 5'- TCCTCTTGGAGAAGATCAGCCG -3'

Human BAX (Forward) 5'- TCAGGATGCGTCCACCAAGAAG -3'

Human BAX (Reverse) 5'- TGTGTCCACGGCGGCAATCATC -3'

Human GAPDH (Forward) 5'- GTCTCCTCTGACTTCAACAGCG -3'

Human GAPDH (Reverse) 5'- ACCACCCTGTTGCTGTAGCCAA -3'

MTT assay. Cell viabilities were determined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded in 24-well plates and treated

with indicated chemical for indicated time. After removing the medium, and 500 µl (0.5 mg/ml) MTT solution in PBS was added to each well. Plates were then incubated at 37°C for 4 hr, MTT solution was removed, and cells were lysed using a solubilization solution (1:1 DMSO:ethanol). Amounts of formazan dye product produced were quantified by measuring absorbance using a microplate reader (SPECTROstar Nano; BMG LABTECH, Baden-Württemberg, Germany) at 560 nm.

Oncogenomic datasets and portals. Analysis of mutation on cancer (Figure. 3) was performed by examination of cancer genomic datasets and tools available from cBioPortal.

Quantification and statistical analysis. Data are expressed as the mean +/- SEM of the values from the independent experiments performed, as indicated in the corresponding figures legends. Statistical analysis was performed with GraphPad Prism v7.00. Two-tailed Student's *t* tests were used for single comparison, and analysis of variance (ANOVA) with Tukey's multiple comparisons tests was used for multiple comparisons unless otherwise specified. p values below 0.05 were considered statistically significant.

Figure legends

Figure. 1 Effectiveness of anticancer drugs in HCT116 cells. A, HCT116 cells were incubated with the indicated chemicals for 48 hr (0, 0.5, 1, and 2 μM) and measured cell viability with MTT assay. Results are presented as means ± standard errors (n = 3; *p<0.05 vs. DMSO controls). Asterisk indicates statistical significance and ns indicates non significantly different. **B,** HCT116 cells were incubated with the indicated chemicals for 48 hr (1 μM) and fixed with paraformaldehyde. Cell morphology was captured by microscope. **C,** HCT116 cells were incubated with the indicated chemical (1 μM) and immunoblotted with indicated antibody. Actin was used as a loading control. **D,** The incubated HCT116 cells with the indicated chemicals were subjected to RT-PCR. B.L.(Blank) indicates sample that was performed RT-PCR using water instead of specific primers. GAPDH was used as a loading control.

Figure. 2 The effect of anticancer drugs is dependent on p53 A, HCT116 p53 -/- cells were incubated with the indicated chemicals for 48 hr (0, 0.5, 1, and 2 μM) and measured cell viability with MTT assay. Results are presented as means ± standard errors (n = 3; *p<0.05 vs. DMSO controls). Asterisk indicates statistical significance and ns indicates non significantly different. **B,** The incubated HCT116 p53 -/- cells with the indicated chemicals were subjected to RT-PCR. B.L.(Blank) indicates sample that was performed RT-PCR using water instead of specific primers. GAPDH was used as a loading control.

Figure. 3 Gene mutation rate in cancer Analysis of mutation on cancer using cancer genomic data sets and tools available from cBioPortal (see “Methods”). The frequency of mutation is shown as a percentage.

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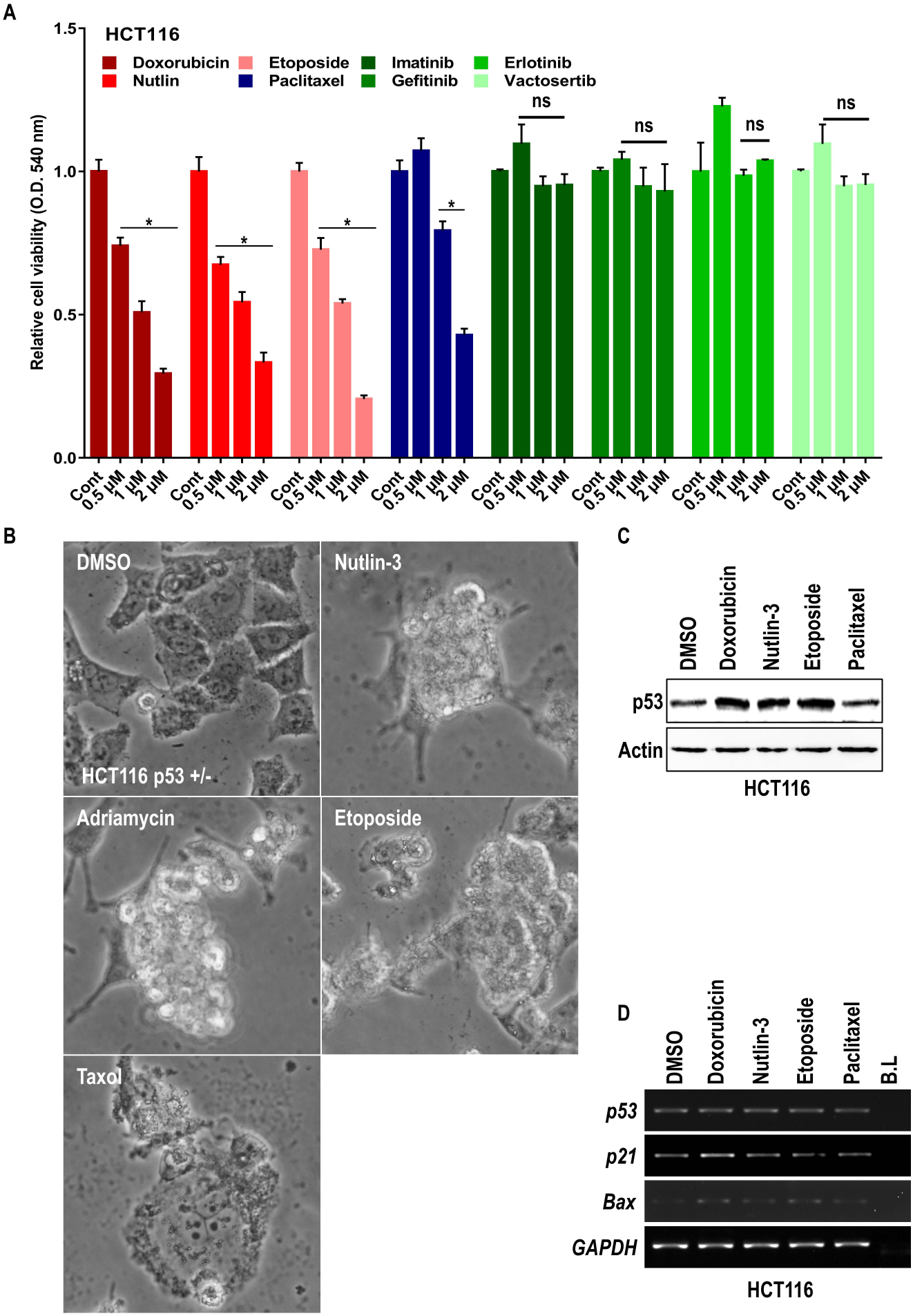
Figure. 1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HCT116
Doxorubicin
Nutlin
Etoposide
Paclitaxel
Imatinib
Gefitinib
Erlotinib
Vactosertib
Relative cell viability (O.D. 540 nm)
1.5
1.0
0.5
0.0
ns
*
Cont
0.5 μM
1 μM
2 μM
B
DMSO
Nutlin-3
HCT116 p53 +/-
Adriamycin
Etoposide
Taxol
C
DMSO
Doxorubicin
Nutlin-3
Etoposide
Paclitaxel
p53
Actin
HCT116
D
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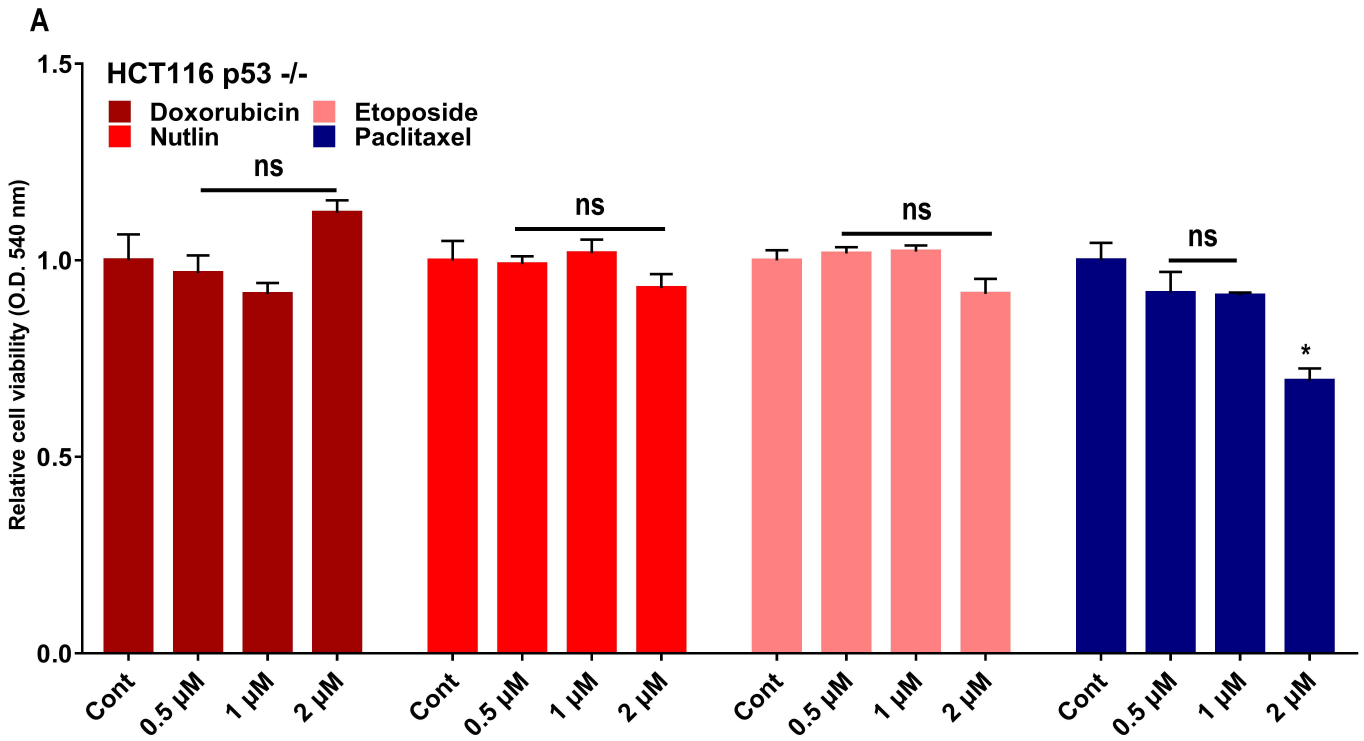
Nutlin-3
Etoposide
Paclitaxel
B.L
p53
p21
Bax
GAPDH
HCT116

Figure. 2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HCT116 p53 -/-
Doxorubicin
Nutlin
Etoposide
Paclitaxel
Relative cell viability (O.D. 540 nm)
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1.0
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ns
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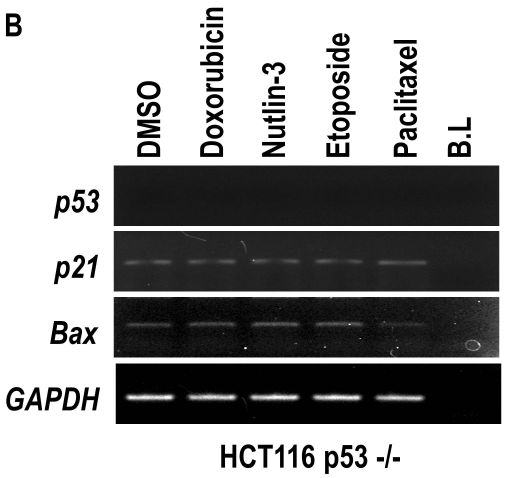
Cont
0.5 μM
1 μM
2 μM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Paclitaxel
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p53
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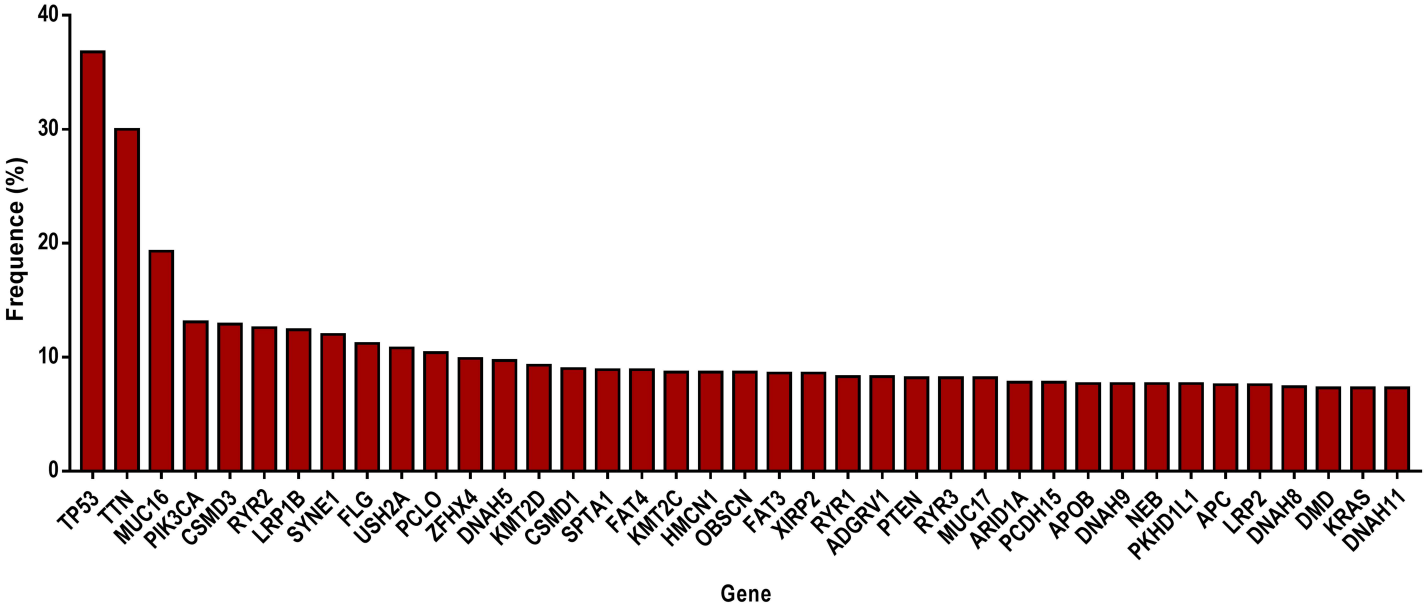
Bax
GAPDH
HCT116 p53 -/-

Figure. 3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30
20
10
0
Frequence (%)
TP53
TTN
MUC16
PIK3CA
CSMD3
RYR2
LRP1B
SYNE1
FLG
USH2A
PCLO
ZFHX4
DNAH5
KMT2D
CSMD1
SPTA1
FAT4
KMT2C
HMCN1
OBSCN
FAT3
XIRP2
RYR1
ADGRV1
PTEN
RYR3
MUC17
ARID1A
PCDH15
APOB
DNAH9
NEB
PKHD1L1
APC
LRP2
DNAH8
DMD
KRAS
DNAH11
Gene