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In vitro Anticancer Activity of Vibramycin Against Human Colorectal Cancer Cell Line (HCT-116)

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Abstract

Drug repurposing, which is defined as the process of searching for new uses for existing FDA-approved drugs outside the scope of their original medical indication, has been gaining popularity in recent years. Several success stories of drug repurposing brought global attention to the already existing drug space for potential off-target effects that may be beneficial to certain diseases such as cancer. Nowadays, antibiotics have been widely recognized in the treatment of cancers. In the current study, we performed a cytotoxicity screening of 12 commercial antibiotics available in the Egyptian market on human colorectal cancer cell line (HCT-116) and also on normal human retinal pigment epithelial cells (RPE-1) for their potential activity as anticancer drugs in 2D model using MTT assay at concentration of (100 µM). Screening results showed that Vibramycin is a highly promising drug against HCT-116 cell line. Further studies on Vibramycin on 2-d and 3-d cultures were done. Ultrastructural examination of HCT-116 cells revealed that the effect of Vibramycin (100 µM) on the spheroid model was inducing apoptosis and spacing the cells from each other. In conclusion, the antibiotic Vibramycin has 'a second life' as an anticancer drug for colorectal cancer.

Keywords: Drug repurposing; Vibramycin; 3-D spheroids; Clonogenic assay; CRC; Antibiotics

1. Introduction

Cancer is one of the most important death causes in the world and it has an increasing trend globally [1]. Colorectal cancer (CRC) is the third most common cancer worldwide [2] and it is considered a leading cause of cancer-related mortality around the world [3]. Colorectal cancer is considered the most frequent malignancy of the gastrointestinal tract in the world [4], and its incidence has increased rapidly in recent decades [5]. CRC refers to cancer of the colon or rectum and most often occurs in the form of adenocarcinoma, which is known to arise from adenoma, a precancerous lesion [6].

Colorectal cancer most cases arise sporadically. Risk factors include increasing age, male sex, previously formed colonic polyps, or previous colorectal cancer, and factors in environment adopted from some lifestyle behaviours that are known to increase cancer risk (e.g.: red meat, high-fat diet, poor diet with an inadequate intake of fibre, obesity, lifestyle that is sedentary, diabetes mellitus, high consumption of alcohol and smoking) [7]. Although there are numerous therapeutic and screening attempts, CRC still remains a major life-threatening malignancy [8, 9, 10].

Drug repurposing (called also drug repositioning or drug reprofiling) is defined as the process of finding new uses for existing FDA-approved drugs outside the scope of their original medical indication [11]. This process has been gaining popularity nowadays [12], because in the last three decades, the FDA approved drugs number has dropped down [13]. The drug repurposing approach is considered a promising alternative process that can accelerate the process of drug development for infectious diseases and many other diseases and disorders [14]. This approach has provided many promising drug candidates for different viral infectious diseases such as Ebola, influenza, dengue, ZIKA, HIV, HSV, CMV infections, and also for many other infectious diseases [15].

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Several success stories of drug repurposing paid a wide attention to the already FDA-approved drug space for possible off-target effects that may be useful for specific diseases such as cancer. Since these drugs have already been used to cure humans, they have firmly established dose regimens with favourable both pharmacokinetics and pharmacodynamics properties and manageable side effects, this makes the already existing drugs useful sources of new anticancer drug discovery [12].

In the past decades, many drugs that were originally approved for treatments other than the treatment of cancer have shown a cytostatic effect on cancer cells [16, 17].

Nowadays, antibiotics have been widely used in the treatment of cancers. By comparing antibiotics with traditional cancer treatments (such as: surgery, radiotherapy, chemotherapy, immunotherapy and targeted therapy), it will be found that using antibiotics for treating cancer had negligible side effects compared to traditional treatments [18]. Moreover, many different antibiotics have been used in cancer treatment, via their antiproliferative, proapoptotic, and antiepithelial mesenchymal transition capabilities [18]. Antibiotics, as mentioned in previous studies, can encourage cancer apoptosis, suppress cancer growth and also prevent cancer metastasis. Thus, the use of antibiotics in the treatment of cancers is growing [19].

The tetracycline class is a significant class of broad-spectrum antibiotics because its members exhibit potent antimicrobial effects. Within this class, a-6-deoxyoxytetracycline, which is also known as the active pharmaceutical ingredient doxycycline (DOX), is currently the most widely used drug due to its high efficacy, low cost of treatment, and low rate of side effects [20]. The generic name is doxycycline with a brand name: Vibramycin [21].

DOX is particularly effective in treating atypical pneumonia brought on by Chlamydia, Legionella, or Mycoplasma, as well as illnesses brought on by Borrelia and Rickettsiae [22, 23]. Overall, it is mostly used to treat respiratory and urinary tract infections [24]. Moreover, **Ghasemi and Ghasemi (2022)** revealed that the antimicrobial drug DOX, which is considered one of the available chemotherapeutic drugs, has been suitable for treating several malignancies such as CRC, since it has anti-tumor properties as well as can help control tumor growth in different mechanisms, such as inhibiting anti-apoptotic and angiogenic proteins [25].

DOX is also considered a semisynthetic tetracycline antibiotic which, in contrast to many other tetracyclines, is almost completely absorbed after oral administration [26,27].

In the process of drug discovery, cell-based assays have been a crucial technique since they are a simple, fast, and cost effective alternatives to large-scale and animal model testing.

Moreover, the most effective assays for discovering new drugs are three-dimensional (3D) cell culture assays, in which cells form spheroids, because they may offer more physiologically relevant information and more accurate predictions for *in vivo* tests [28].

In this study we aim to repurpose Vibramycin as anticancer drug against colorectal cancer cell line *in vitro*, using three-dimensional multicellular spheroid model.

2. Materials and methods

Antibiotics

Twelve antibiotics are selected namely: Augmentin, Benzibiotic, Cefzim, Cephradine, Claforan, Epicocillin, Epigent, Moflox, Neomycin, Omnicef, Unictam and Vibramycin obtained from the Egyptian market. All the antibiotics in capsules and tablets dosage forms (10 mg) were solubilized in DMSO (Dimethyl Sulfoxide).

Cell culture

Human colorectal cancer cell line (HCT-116) and normal human retinal pigment epithelial cells (RPE-1) were kindly provided by Professor Stig Linder, Oncology and Pathology department, Karolinska Institute and Hospital, Stockholm, Sweden. Cells were cultured in DMEM F-12 medium supplemented with 10 % FBS and 1 % penicillin/streptomycin. Cells were incubated in 5 % CO_2 and 95 % humidity at 37 °C.

In vitro cytotoxicity screening: 2D-monolayer model

To determine their cytotoxicity, twelve antibiotics (100 µM) were added to 96-well microtiter plastic plate containing 100 µl of HCT-116 cell suspension which were seeded at concentration of 10000 cells per well, in fresh complete growth medium at 37 ºC for 48 h and 120 h under 5 % CO_2. Doxorubicin was used as a reference drug at a concentration of 100 µM. Treatments were done in triplicates. Media was then removed and MTT solution was added to each well. The plates were incubated for 4 hours at 37C, 5% CO2 in a humidified incubator. Sodium dodecyl sulfate (SDS) was added, and the plates were incubated in the dark at room temperature overnight. Then, the absorbance was measured using a microplate multi-well reader at 595 nm.

The same procedures were applied on the active antibiotics, which were active against HCT-116 cell line, against the normal cells RPE-1 and the cell suspension were seeded at concentration of 20000

cells per well. All other steps were repeated by the same way at the same conditions.

Determination of IC_{50} values of Vibramycin

The highly active antibiotic possessing ≥ 55 % cytotoxicity on HCT-116 cell line was selected (Vibramycin) and different concentrations were prepared for dose response study (100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µM) using the MTT assay for 48 h and 120 h [29]. The results were used to calculate the IC_{50} values using probit analysis and utilizing the prism software.

Clonogenic Assay

HCT-116 cells were seeded in 96-well microtiter plastic plate at concentration of 10000 cells per well and treated with IC50 of Vibramycin. Then, HCT-116 cells were harvested using trypsinization to produce single-cell suspensions which were seeded into six-well plates. Then, HCT-116 cells were incubated for 5 days at 37 °C and cell growth of six-well plates was stopped simultaneously.

Fixation and staining of colonies were performed using a mixture of 6 % glutaraldehyde and 0.5 % crystal violet for at least 30 min. then, plates were rinsed in tap water.

The plates with colonies were left to dry in normal air at room temperature and colonies were counted [30].

Generation of 3D-multicellular HCT-116 spheroids and cytotoxicity bioassay using acid phosphatase (APH) method

A cell suspension (200 µl), containing 10^4 cells of HCT-116, was added to each well of poly-HEMA-coated round bottom 96-well plates. Plates containing cell suspension were then centrifuged at 1000 rpm for 10 minutes. The plates were incubated under the standard cell culture conditions at 37° C, 5 % CO_2 in humidified incubator. Cells were incubated for 5 days until they formed proper spheroids of about 500 µm diameter.

Screening was performed by exposing spheroids to Vibramycin (100 µM) for incubation period 5 days and to Cisplatin (50 µM) as a positive control under the same conditions. For negative control spheroids, untreated spheroids were incubated with 0.5 % DMSO (w/v) under the same conditions.

At the end of incubation, cytotoxicity was determined using the acid phosphatase method [31]. After washing twice with PBS, spheroids were lysed in assay buffer which consists of 100 µl of 0.1M sodium citrate, 0.1% Triton X-100, pH 5, *p*-nitrophenyl phosphate (2 mg/ml) (Pierce Biotechnology Inc., Rockford, IL) and incubated for 90 min at 37 °C. At the end of the incubation, 10 µl 1N NaOH stop solution was added to each well. In this assay intracellular acid phosphatase release the yellow chromophore *p*-nitrophenol. Absorbance of each well was measured at 405 nm. The released chromophore measured at 405 nm is directly proportional to the number of viable cells.

Cytotoxicity was calculated according to the following equation:

% cytotoxicity = $[1- (AV_X / AV_{NC})] \times 100$

AV: the average, X: the absorbance of sample, NC: the absorbance of negative control.

Imaging of HCT-116 spheroids

Images of HCT-116 spheroids were taken at the end of incubation period (prior to the APH assay) in cytotoxicity experiment to verify the activity of Vibramycin against HCT-116 spheroids compared to control spheroids by using an Olympus inverted microscope with an attached Olympus SC100 Camera applying Cellsens software program.

Transmission Electron Microscope (TEM) for HCT-116 spheroids

Untreated HCT-116 spheroids and treated spheroids with Vibramycin (100 µM) were fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 2 h, then post-fixed for 2 h with 2 % cold OsO_4 dissolved in distilled water, dehydrated in a graded series of ethanol alcohol, and embedded in Epon. Ultrathin sectioning was done using an ultra-microtome (MT-7, RMC Company) and grids stained with uranyl acetate and lead citrate according to **Reynolds (1969)** [32] for examination under a transmission electron microscope (JEOL, JEM-1200 EXII Electron Microscope).

Statistical analysis

Each experiment was performed at least three times and error bars represent standard deviations (SD). Determining of IC_{50}s was performed by probit analysis and utilizing the prism software.

3. Results

Cytotoxicity and Anti-cancer screening of antibiotics at 100 µM against human colorectal cancer cell line (HCT-116) in 2D model after treatment for 48 hrs

As demonstrated in Figure (1), the present data declared the inhibitory effect of some of the tested antibiotics on the growth of HCT-116 at concentration of 100 µM after treatment for 48 hrs in increasing order. The highest cytotoxic effect was with Vibramycin (64.53 %), while Cefzim, Cephradine, Claforan, Epicocillin, Moflox and Unictam showed no inhibitory effect on HCT-116 cells proliferation.

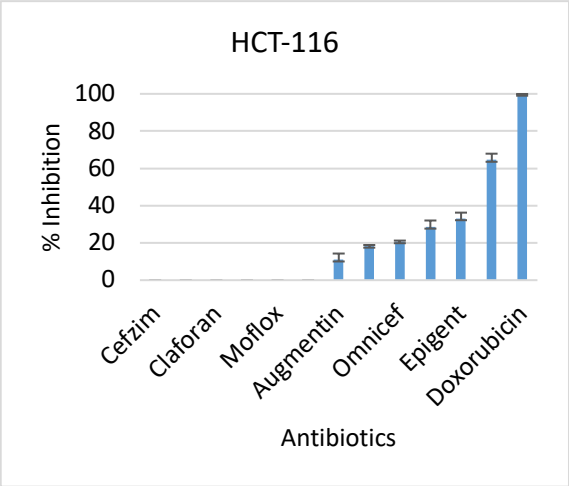

Fig. (1): Schematic representation of average % cytotoxicity of the 12 selected antibiotics and doxorubicin on cell viability/proliferation on HCT-116 cell line after 48 h, evaluated by MTT assay. Values are expressed as mean ± SD, n = 3 at a concentration of 100 µM.

Cytotoxicity and Anti-cancer screening of antibiotics at 100 µM against human colorectal cancer cell line (HCT-116) in 2D model after treatment for 120 hrs

As illustrated in Figure (2), the present data declared the inhibitory effect of all antibiotics on human colorectal cancer cell line (HCT-116) at 100 µM after treatment for 120 hrs, except Cefzim and Unictam which showed no cytotoxic effect on these cells, in increasing order. The most cytotoxic effect was recorded with Vibramycin (91.43 %) and the least cytotoxic effect was with Claforan (0.21 %).

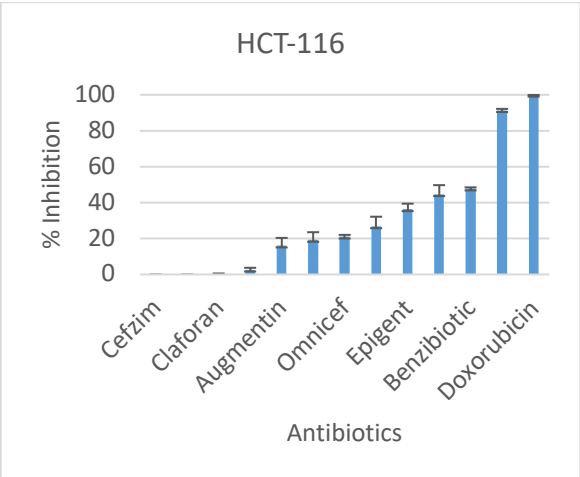

Fig. (2): Schematic representation of average % cytotoxicity of the 12 selected antibiotics and doxorubicin on cell viability/proliferation on HCT-116 cell line after 120 h, evaluated by MTT assay. Values are expressed as mean ± SD, n = 3 at a concentration of 100 µM.

Cytotoxicity of antibiotics at 100 µM against normal human retinal pigment epithelial cells (RPE-1) in 2D model after treatment for 48 hrs

Counter screening on normal human retinal pigment epithelial cells (RPE-1) to assist the safety of the 6 antibiotics namely: Augmentin, Benzibiotic, Omnicef, Neomycin, Epigent and Vibramycin, which have inhibitory effect on HCT-116 cell line after treatment for 48 hrs, showed that none of these antibiotics have inhibitory effect at 100 µM on RPE-1 cells compared to positive control (doxorubicin) ensuring their safety on normal cells.

Cytotoxicity of antibiotics at 100 µM against normal human retinal pigment epithelial cells (RPE-1) in 2D model after treatment for 120 hrs

As demonstrated in Figure (3), the present data declared the counter screening inhibitory effect of 10 antibiotics namely: Benzibiotic, Neomycin, Vibramycin, Augmentin, Claforan, Epicocillin, Epigent, Cephradine, Omnicef and Moflox, which have inhibitory effect on HCT-116 cell line after treatment for 120 hrs, on the growth of RPE-1 in increasing order, the most cytotoxic effect is with Moflox (14.22 %). On the other hand, Benzibiotic, Neomycin and Vibramycin showed no cytotoxic effect on normal human cells (RPE-1) at 100 µM ensuring their safety on normal cells.

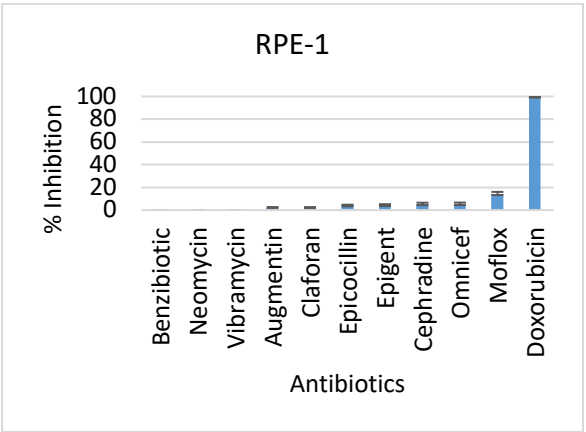

Fig. (3): Schematic representation of average % cytotoxicity of 10 antibiotics and doxorubicin on cell viability/proliferation on RPE-1 cells after 120 h, evaluated by MTT assay. Values are expressed as mean ± SD, n = 3 at a concentration of 100 µM.

Cytotoxicity and estimation of IC_{50} by using serial dilutions (100, 50, 25, 12.5, up to 0.78 µM) of Vibramycin against HCT-116 human cancer cell line after 48 hrs and 120 hrs in 2D model.

The above-mentioned screening data of the twelve antibiotics confer that Vibramycin was the most effective antibiotic on human colorectal cancer cell line (HCT-116) and the safest drug on human

normal cell line (RPE-1) with zero inhibitory effect after treatment for 48 and 120 hrs. Hence, it has been selected for proving its inhibitory toxicity at different concentrations and IC_{50} estimation on the HCT-116 human cancer cell line.

As observed in Table (1) and Figure (4), the present data declared the inhibitory effect of Vibramycin on human colorectal cancer cell line (HCT-116) with IC_{50} = 45.86 µg/ml after 48 hrs and IC_{50} = 40.86 µg/ml after 120 hrs.

Table (1): The IC_{50} values of Vibramycin against HCT-116 cell line after treatment for 48 and 120 hrs.

Vibramycin	48 h	120 h
IC_{50}	45.86±1.23 µM	40.86±6.67 µM

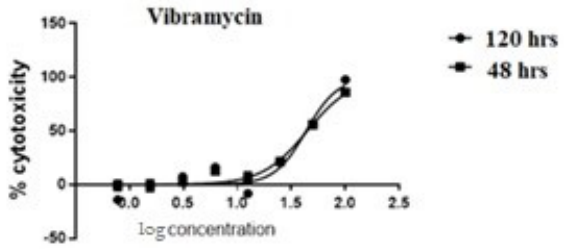

Fig. (4): Dose response curve for Vibramycin on HCT-116 cells after 48 and 120 hrs with estimated IC_{50} = 45.86 µM and 40.86 µM, respectively.

Clonogenic assay of the IC_{50} of Vibramycin on HCT-116 cells versus untreated control HCT-116 cells

As demonstrated in Figure (5), clonogenicity of control HCT-116 cells and treated HCT-116 cells with IC_{50} of Vibramycin, respectively, it was clear that treatment with the IC_{50} of Vibramycin has significantly affected the number of the resulted viable colonies as compared to the untreated control, indicating its high potential cytotoxic effect with % clonogenicity inhibition = 80 % through counting the colonies.

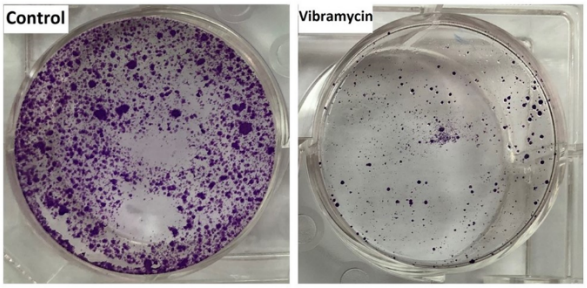

Fig. (5): Image indicating clonogenicity of control HCT-116 cells versus treated HCT-116 cells with IC_{50} of Vibramycin.

Data of 2D cytotoxicity and clonogenic assay has proved that Vibramycin is the promising anticancer drug and selective on human colorectal cancer (HCT-116) cell line that should be tested on 3D model. Hence, Vibramycin has been applied at a concentration of (100 µM) using cisplatin as positive control.

Imaging of HCT-116 spheroids

Microscopic Imaging was performed at the end of incubation period (prior to the APH assay) in cytotoxicity experiment to verify the activity of Vibramycin against HCT-116 spheroids compared to control spheroids which were formed in proper shape (Fig. 6). As shown in Figure (6) it was observed that the Vibramycin affected the formed spheroids by affecting proliferation of the outer layer which led to the loss of integrity by spacing the cells from each other.

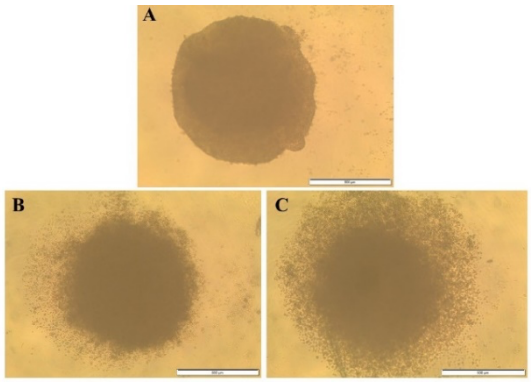

Fig. (6): Inverted microscope images of HCT-116 spheroids prior to the APH assay. (A) Control untreated HCT-116 spheroid of size = 500 µm in diameter showing the outer proliferating layer and the inner hypoxic core. (B) Treated HCT-116 spheroid with Vibramycin (100 µM) resulting in spacing the cells from each other and loss of integrity. (C) Positive control treated HCT-116 spheroid with Cisplatin (50 µM).

Cytotoxic effect of Vibramycin (100 µM) on human colorectal cancer cell line (HCT-116) in 3D model using acid phosphatase assay.

As shown in Table (2), the percentage of cytotoxic effect of Vibramycin at concentration of 100 µM was 17.61 through application of acid phosphatase assay. This cytotoxic effect being demonstrated as affecting on proliferation of the outer layer of HCT-116 spheroids led to destroying the outer layer cells.

Table (2): % cytotoxic effect of Vibramycin as measured by activity of acid phosphatase.

Drug/Concentration	% Cytotoxic activity
Vibramycin (100 µM)	17.61 ± 0.4
Cisplatin (50 µM)	56.01 ± 0.17

TEM analysis of spheroids of HCT-116 cells

Low-magnification TEM imaging in Figure (7) indicated that control HCT-116 spheroid cells were very close to each other with almost no distances between them and that the cell-to-cell border was not clear in some cells. Most of these cells were connected, even through many intercellular junctions (microvilli and filopodia). It was noticed that their nuclei were prominent and the cytoplasm amount was small (A and B). Numerous mitochondria and many rough endoplasmic reticulum cisternae (RER) were also present (C). On the other hand, treatment of HCT-116 spheroids with Vibramycin (100 µM) resulted in spacing the cells forming spheroids from each other and decreasing the number of microvilli and formation of apoptotic cells with malformed nuclei (D). Results also revealed the formation of membrane blebs in the plasma membrane of HCT-116 cells (D, E and F) and numerous apoptotic bodies were also observed (E). In addition, presence of many lysosomes (E and F), few rough endoplasmic reticulum cisternae (RER) with nill ribosomes in the cytoplasm (E) and some empty and swollen mitochondria were also observed (F).

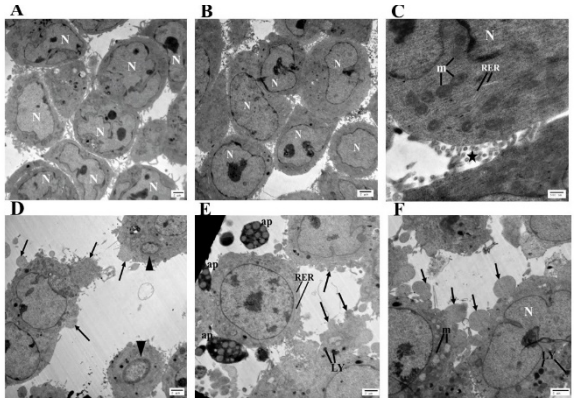

Fig. (7): Transmission electron micrograph (TEM) of HCT-116 control spheroids (A, B and C) and HCT-116 treated spheroids with Vibramycin (100 µM) (D, E and F). (A and B) Low-magnification images showing HCT-116 cells that formed spheroids were very close to each other with almost no distances between them with many microvilli. Prominent nuclei (N) and small cytoplasm amount were observed. (C) High-magnification image showing prominent nucleus (N), cell surface with apparent microvilli along the intercellular space (star). Presence of numerous mitochondria (m) and many rough endoplasmic reticulum cisternae (RER). (D) Low-magnification image showing HCT-116 cells that formed spheroids treated with Vibramycin. The cells were distant from each other with few microvilli. Note the formation of membrane blebs (arrow) and the presence of apoptotic cells with malformed nuclei (arrowhead). (E) Image showing the formation of membrane blebs (arrow), presence of many lysosomes (LY) and few rough endoplasmic reticula (RER). Note the presence of apoptotic bodies (ap). (F) Image showing irregular shaped lobulated nucleus (N), formation of membrane blebs (arrow), some empty and swollen mitochondria (m) and many lysosomes (LY).

4. Discussion

The present study is considered as one of the recent approaches of colorectal cancer therapy by re-purposing antibiotics. Twelve antibiotics, namely: Augmentin, Benzibiotic, Cefzim, Cephradine, Claforan, Epicocillin, Epigent Moflox, Neomycin, Omnicef, Unictam and Vibramycin have been screened for their potential activity as anticancer drugs in 2D model using MTT assay at constant concentration of (100 µM) using human colorectal cancer cell line (HCT-116) in comparison with normal human cell line (RPE-1) after 48 and 120 h. Ultimately the best effective antibiotic (Vibramycin) has been furtherly investigated for the 3D model using acid phosphatase assay and transmission electron microscope imaging.

The tested antibiotics showed selective cytotoxicity toward the cells of HCT-116 colon cancer spheroids where Vibramycin was the most potent. When comparing the investigated antibiotics to the doxorubicin (positive control), it is clear from the results obtained that all of them are safe to be used against the normal cells RPE-1.

It has been reported that, by comparing to 2D model, cellular reactions responding to drug treatments in 3D model resembled those that take place in vivo [33]. Also, resistance to anticancer drugs of cultured cells is more increasing in 3D models than in 2D models. For example, a study on cell proliferation of ovarian cancer in spheroid model after treatment with paclitaxel revealed the reduction of cells by almost 50 %, compared to 80 % in the monolayer model [33].

Another study resulted in the following data, the drug sensitivity of HCT-116 colon cancer cells was found to be lower in a spheroid culture than in a monolayer culture in response to 6 common anticancer medicines, regardless of their various mode of action. This may imply that elevated drug resistance was linked to the phenotypical changes brought about

in 3D-formed spheroid [33]. These 3D culture studies' more pronounced drug resistance was linked to signals entering the cellular decision-making process through interactions between nearby cells and the extracellular matrix. The decreased spheroid diffusion and hypoxia, which activated genes involved in cell survival and drug sensitivity, may also be to blame for the increased drug resistance in 3D spheroid culture. The same chemo-resistance that was developed in 3D spheroids is also seen *in vivo*.

In a third study, which applied on liver spheroid tumor cells, it was found that cancer cells' drug resistance was also influenced by stromal cells [33].

In accordance with results of our cytotoxicity screening and by comparing the results of both 2D-monolayer and 3D-multicellular spheroids models of the anticancer activity of Vibramycin and by TEM investigation, we can conclude that Vibramycin induced its anticancer activity through apoptosis to give the best results in the monolayer model, moreover, the % clonogenicity inhibition = 80 %. However, in the multicellular spheroid model a cytotoxic activity = 17.61 % was induced which in terms reflects that Vibramycin affected only the proliferation of the outer layer of HCT-116 spheroids and that led to destroying the outer layer cells without capability to penetrate the 3D model. This observation was confirmed by the results of inverted microscope images.

Moreover, results of our TEM investigation revealed the presence of few RER with nill ribosomes, which are responsible for protein synthesis, in the cytoplasm of treated cells, compared to control cells. This led to impairment in protein synthesis demonstrated as following: the ribosomes, which are the sites of protein synthesis in living cells, are composed of proteins and RNA. The mechanism of action of doxycycline in bacterial replication was supposed to be as following: in a crucial interaction for translation, the 30S subunit, which is the smaller subunit of the ribosome present in prokaryotes, including bacteria, is where the beginning of proteins takes place at the 3′ ends of the 16S rRNA [34, 35, 36]. Tetracyclines like doxycycline, which bind to the 16S rRNA region of the ribosome and hinder the binding of tRNA to the mRNA-30S bacterial ribosomal subunit which are essential for the transport of amino acids for protein synthesis, are thought to inhibit translation. The commencement of protein synthesis by polyribosome formation is thus prevented as a result of the aforementioned processes. Additionally, this has a bacteriostatic action and prevents bacterial multiplication [37, 38].

5. Conclusion

Understanding the potential of the drug reprofiling strategy, which is a time and money-effective route, is to produce broad range of anticancer treatments and this will help us meet our needs by repositioning the currently FDA-approved drugs. Drug repurposing has already produced excellent results with the medications that have been effectively repurposed, and this strategy may open up new avenues for overcoming the difficulties associated with the emergence of drug resistance. In this article, this method has already demonstrated the viability of creating novel anticancer medications such as, the antibiotic Vibramycin and its ‘second life’ as an anticancer drug for colorectal cancer. The repurposing strategy has provided promising candidates in treating cancer and can be further investigated to get through the drug discovery bottleneck for many diseases including cancers.

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Conflict of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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