RESEARCH ARTICLE

Biological Evaluation of Newly synthesized Spebrutinib Analogues: Potential Candidates with Enhanced Activity and Reduced Toxicity Profiles

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ABSTRACT

Background: Undoubtedly, cancer is regarded as a major concern for researchers alongside the whole humanity for its high mortality rates. At this moment, there must be some researchers working hard to design, synthesize, and biologically investigate the effects of some potential candidates to fight back cancer.

Materials and methods: In previous unpublished work, the authors successfully designed, synthesized, characterized a potential two spebrutinib analogs. Consequently, these analogs were evaluated with the employment of MCF-7, HCT116, and MDCK cell lines.

Results: In respect to the spebrutinib standard, one of these analogs has superior activity against MCF-7 cell line (IC50; 10.744 µg/mL against 13.566 µg/mL for spebrutinib) and an enhanced toxicity profile on madin-darby canine kidney (MDCK) cell line (IC50; 8.653 mg/mL against 4.011 mg/mL for spebrutinib).

Conclusion: The two compounds showed good activity against breast and colon cell lines and enhanced toxicity profile against normal kidney cell line in respect to spebrutinib standard.

Keywords: Biological evaluation, Colon cancer, Breast cancer, Spebrutinib analogs, Tyrosine kinase inhibitor, Ic50, Mcf-7, Hct116, Mdck, Cell Lines

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INTRODUCTION

Cancer is a major concern of researchers as it is the second leading cause of deaths worldwide.[1] Cancer-related mortality has increased by almost (40%) in the past 40 years, although death rates from communicable diseases have improved worldwide as a result of the medical improvements. In the next 15 years, a further 60% rise is expected, with about 13 million people estimated to die of cancer in 2030.[2]

Hence, there is a compelling need to develop new drugs to treat this life-threatening disease. Cancer treatment using conventional chemotherapy is associated with several side effects.[3] In recent years, the development of small molecules such as tyrosine kinase inhibitors (imatinib, spebrutinib, gefitinib, sunitinib, semaxinib, etc.,) in the treatment of cancer helped the scientists in the understanding of molecular mechanisms of this disease.[4] Targeting enzymes involved in the signal transduction pathways of protein kinases that regulates cellular growth and multiplication is one of the approaches in developing the new anticancer drugs.[5,6]

Consequently, scientists have recognized tyrosine kinases as a potential target to suppress or even cure breast cancers.[7] Consequently, many tyrosine kinase inhibitors (TKI) have been developed and tested.[8-10] However, the off-target serious side effects of these TKIs are a major obstacle that has been encountered.[11-14]

For some prestigious journal publishers, biological investigation of newly synthesized potential anticancer candidates is a must.[15] In this work, the authors aimed to biologically evaluate a previously synthesized novel TKI with superior activity and reduced toxicity. These compounds were successfully designed, synthesized, and characterized.

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Table 1: Utilized materials with their manufacturers and countries of origin.

#	*Material*	*Manufacturer*	*Country*
1	Spebrutinib AVL-292 (99.48%)	BLDpharm	CHINA
2	N-(3-((5-fluoro-2-((4-(2-methoxyethoxy)phenyl)amino) pyrimidin-4-yl)amino)phenyl) benzamide (2a) (99.63%)	Medicinal chemistry lab*	IRAQ
3	N-(3-((5-fluoro-2-((4-(2-methoxyethoxy)phenyl)amino)pyrimidin-4-yl)amino)phenyl) pivalamide (2b) (99.69%)	Medicinal chemistry lab*	IRAQ
4	Dimethyl sulphoxide (99%)	CDH	INDIA
5	Cellulose acetate membrane filter pore size 0.2 µm diameter 25 mm.	chm	SPAIN
6	MTT (3.[4,5.dimethylthiazol.2.yl] .2,5.diphenyl tetrazolium bromide)	Roth	GERMANY
7	Celltreat® 96 well cell culture plates	CELLTREAT scientific products	USA

*These compounds were synthesized and characterized in previous unpublished work.

MATERIALS AND METHODS

Materials

The materials used in this work are tabulated in Table 1.

Cell lines

The following types of cell lines were used in this study:

MCF-7 Breast cancer
HCT116 colorectal cancer cells
MDCK kidney normal cells.

Cell lines were obtained kindly from the international cell line collection of Dr. Hamid N. Obied (M.B.CH.B., MSc, PhD Pharmacology, Lecturer and researcher in anticancer at the department of clinical pharmacology, College of Medicine, University of Babylon, and the head of cancer cell research unit in Al-Fadhil foundation for educational services, training and development – branch of Babylon).

Instruments

The instruments used in this work are listed in Table 2.

Methods:

Cell lines preparation:

The cell lines were cultured in medium 1640 (RPMI-1640, Gibco-BRL), with 10% heat-inactivated fetal bovine serum (FBS) (Gibco). Cell lines were allocated in Celltreat® 96 well cell culture plates and incubated to grew at 37°C. The time of cell culture was optimized from 72 hours to be 24 hours and the steps involved in the cell line part of this work are listed below:

MTT stock solution preparation:

A 25-mg was accurately weighed and transferred into a suitable flask. Then a 5-mL of DMSO was added and the MTT was completely dissolved. The 5-mL of 5mg/mL was filtered, into 12.mL centrifuge tube, with 0.22µm sterile filters and the tube was foiled with aluminum sheet as the MTT solution is light sensitive. This solution was kept in the fridge for the preparation of a working solution.

Table 2: Employed instruments with their manufacturers and countries of origin.

#	*Instrument*	*Manufacturer*	*Country*
1	4-digit balance	Sartorius Lab	GERMANY
2	Clean Bench	LabTech	KOREA
3	Incubator UN 55	Memmert	GERMANY
4	Microplate reader 800 TS	BioTek	USA
5	Inverted Microscope	Zeiss	GERMANY

MTT working solution preparation:

According to the protocols, the working concentrations of MTT is 0.5mg/ml. This is 10% v/v of the stock solution. For a final volume of 12 ml of cell-medium with 10% MTT, the following dilutions were performed. A 10.600 ml of cell medium was accurately measured and allocated into a suitable flask. Then a 2.400 ml of MTT stock solution was added to the medium and adequately homogenized. The cell-medium with 10% MTT was ready to be utilized for cell-lines and incubation of 3hrs period.

Preparation of working concentrations from each test chemical for the cell-lines:

A suitable amount of each chemical was dissolved in DMSO to get a stock solution with a concentration of 5mg/mL for each chemical and standard. After several trials on cell-line, the concentration was optimized for a 50µg/mL as the higher concentration from witch a serial dilution was performed. For each standard and synthesized chemical, a 990-µl of the medium was accurately measured and a 10-µL from the 5mg/ml was added and homogenized to get a final concentration of 50 µg/ml and a final concentration of 1% for the DMSO. Serial dilution was performed for each to get the following concentrations (50, 25, 12.5, 6.25, 3.125, and 1.5625) µg/mL.

Stock solution preparation:

An accurately-weighed amount of each synthesized chemical compounds was dissolved in pure DMSO to get a concentration of 5mg/mL. After complete dissolution, the solutions were filtered through a 0.2 µm sterile filter. A 10 µL of the above filtrate was further diluted with 990 µL of RPMI.1640 Medium to get a final concentration of 50 µg/mL. A serial dilution was prepared from the above concentration to get (50, 25, 12.5, 6.25, and 3.125) µg/mL.

Application of the chemicals on the cell-lines:

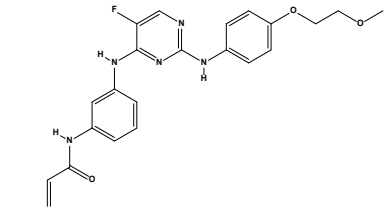
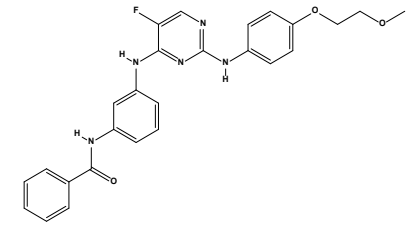
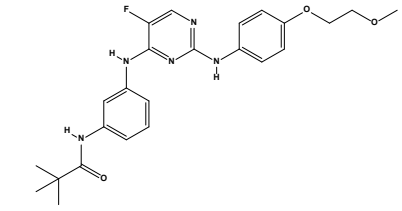
The above serial dilution solutions were added in a 200 µl portions for each well in triplicates and incubated for 24hours. After the incubation period, the plates were visualized with

an inverted microscope and a screenshots was captured for each well. The media were replaced with 10% MTT media and incubated for 3 hours. After the 3 hours' incubation, the media was removed and the wells were washed with phosphate buffer saline (PBS). Finally, a 200.µL portions of DMSO was added into each well and left for 30 minutes and were read with plate reader at 630 nm.

RESULTS

In previous unpublished work, two spebrutinib analogs were successfully designed, synthesized, and characterized. These analogs are tabulated in Table 3.

Table 3: The symbols, IUPAC names, chemical formulas, and the chemical structures of the spebrutinib and the synthesized analogs.

Chemical formula (Code)	*Structure* *Chemical name*
$C_{22}H_{22}FN_5O_3$ (AVL-292) Spebrutinib	 *N*-(3-((5-fluoro-2-((4-(2-methoxyethoxy)phenyl)amino)pyrimidin-4-yl)amino)phenyl)acrylamide
$C_{26}H_{24}FN_5O_3$ (2a)	 *N*-(3-((5-fluoro-2-((4-(2-methoxyethoxy)phenyl)amino)pyrimidin-4-yl)amino)phenyl)benzamide
$C_{24}H_{28}FN_5O_3$ (2b)	 *N*-(3-((5-fluoro-2-((4-(2-methoxyethoxy)phenyl)amino)pyrimidin-4-yl)amino)phenyl)pivalamide

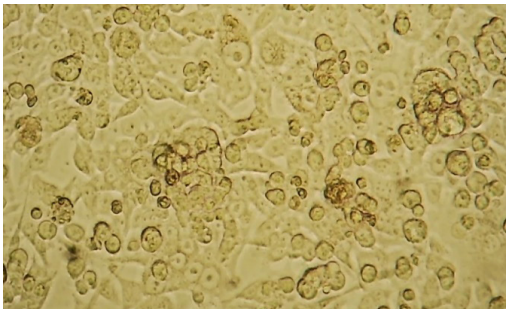


Figure 1: Shows control of colorectal HCT116 cells. An inverted microscope observation post 24-hours' incubation.

Results of the biological effect of the synthesized compounds on the cancerous and normal cell-lines:

Effect on HCT116 colorectal cancer cell-line is shown in Figure 1:

Effect of Spebrutinib is shown in Figures 2, 3, and 4:

Effect of compound 2a is shown in Figures 5, 6, and 7:

Effect of compound 2b is shown in Figure 8, 9, and 10:

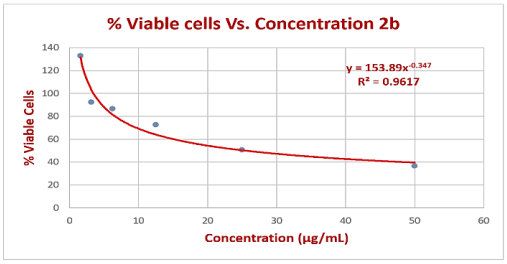


Figure 2: Shows HCT116 colorectal cell line (subjected to 12.5µg/ml of spebrutinib standard). An inverted microscope observation post 24-hours' incubation.

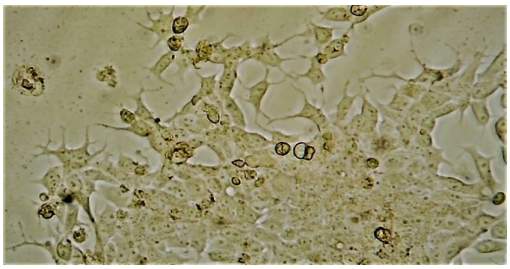


Figure 3: The absorbance of MTT versus concentration of spebrutinib standard after 24-hours' incubation of HCT116 colorectal cell line.

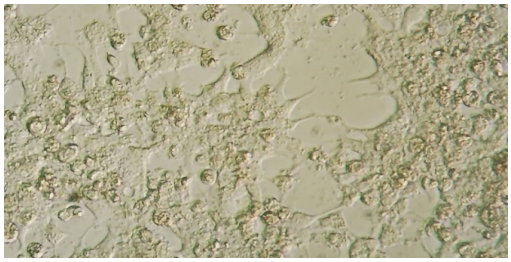


Figure 4: The percent viable cells versus concentration of spebrutinib standard after 24-hours' incubation of HCT116 colorectal cancer cell line.

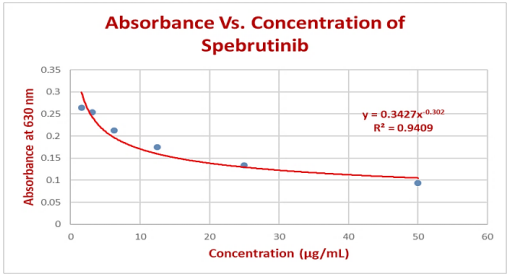


Figure 5: Shows HCT116 colorectal cell line (subjected to 12.5µg/mL of compound 2a). An inverted microscope observation post 24-hours' incubation.

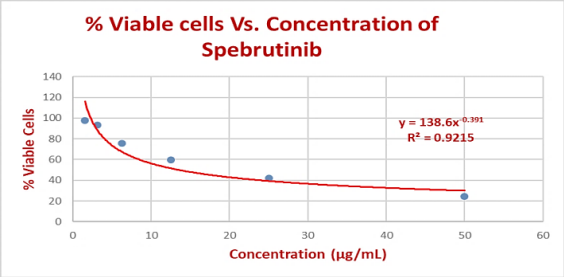


Figure 6: the absorbance of MTT versus concentration of compound 2a after 24-hours' incubation of HCT116 colorectal cancer cell line.

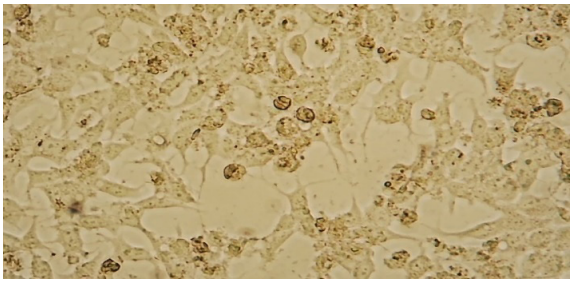


Figure 7: The percent viable cells versus concentration of compound 2a after 24-hours' incubation of HCT116 colorectal cancer cell line.

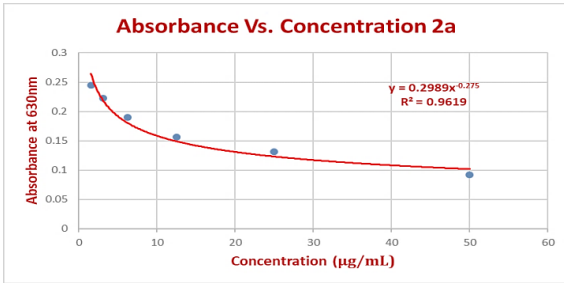


Figure 8: Shows HCT116 colorectal cell line (subjected to 12.5µg/mL of compound 2b). An inverted microscope observation post 24-hours' incubation.

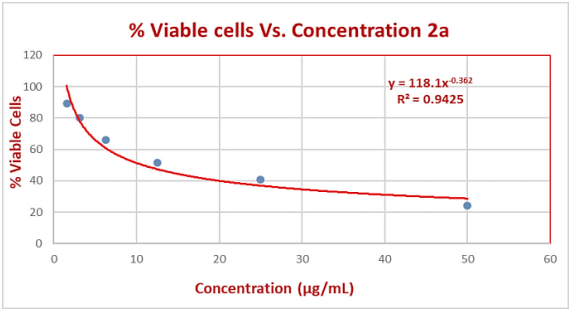


Figure 9: The absorbance of MTT versus concentration of compound 2b after 24-hours' incubation of HCT116 colorectal cancer cell line.

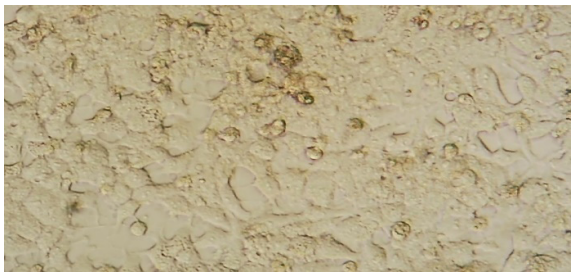


Figure 10: The percent viable cells versus concentration of compound 2a after 24-hours' incubation of HCT116 colorectal cancer cell line.

Effect on MCF-7 breast cancer cell line is shown in Figures 11, 12, 13, and 14:

Effect of Spebrutinib is shown in Figure 11:

Effect of compound 2a is shown in Figures 15, 16, and 17:

Effect of compound 2b is shown in Figures 18, 19, and 20:

EFFECT ON MDCK KIDNEY NORMAL CELL-LINE

The *ex vivo* toxicity of the synthesized spebrutinib analogues

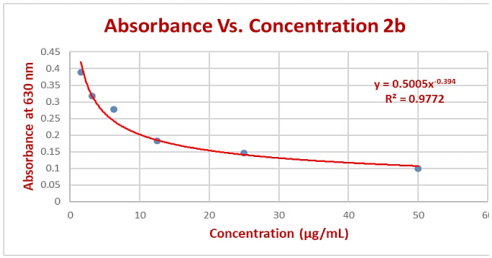


Figure 11: shows control of MCF-7 breast cells. An inverted microscope observation post 24-hours' incubation.

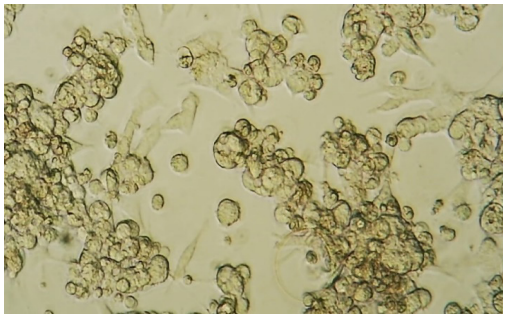


Figure 12: Shows MCF-7 breast cell line (subjected to 12.5µg/ml of spebrutinib standard). An inverted microscope observation post 24-hours' incubation.

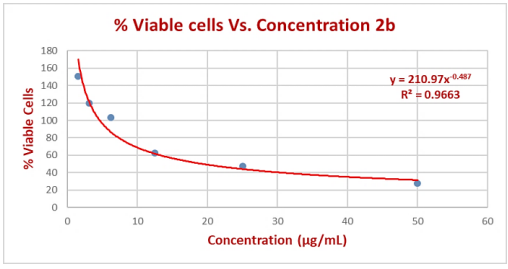


Figure 13: the absorbance of MTT versus concentration of spebrutinib standard after 24-hours' incubation of MCF-7 breast cancer cell line.

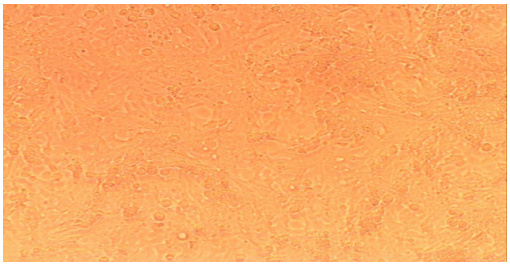


Figure 14: The percent viable cells versus concentration of spebrutinib standard after 24-hours' incubation of MCF-7 breast cancer cell line.

were evaluated by applying these synthesized chemicals on MDCK epithelial cell line (non-cancerous, normal kidney cells as shown in Figure 21. These cells were derived by S. H. Madin and N. B. Darby from the kidney tissue of an adult female cocker spaniel.

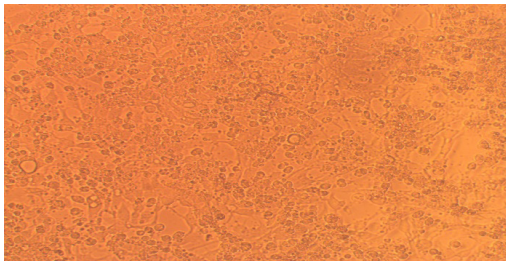


Figure 15: Shows MCF-7 breast cell line (subjected to 12.5µg/ml of compound 2a). An inverted microscope observation post 24-hours' incubation.

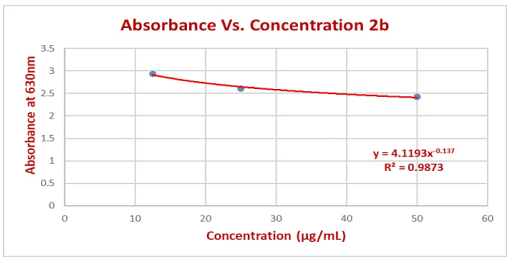


Figure 16: The absorbance of MTT versus concentration of compound 2a after 24-hours' incubation of MCF-7 breast cancer cell line.

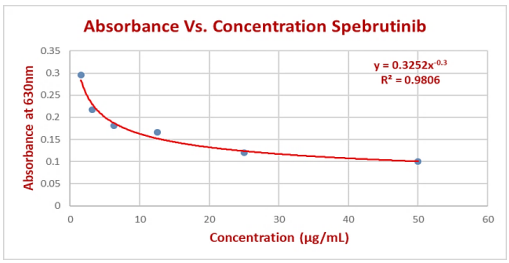


Figure 17: The percent viable cells versus concentration of compound 2a after 24-hours' incubation of MCF-7 breast cancer cell line.

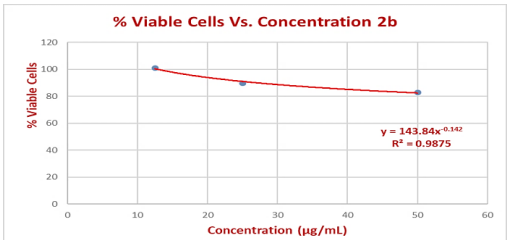


Figure 18: Shows MCF-7 breast cell line (subjected to 12.5µg/ml of compound 2b). An inverted microscope observation post 24-hours' incubation.

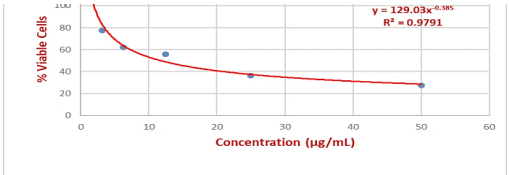


Figure 19: The absorbance of MTT versus concentration of compound 2b after 24-hours' incubation of MCF-7 breast cancer cell line.

Effect of Spebrutinib is shown in Figures 22, 23, and 24:

Effect of compound 2a is shown in Figure 25, 26, and 27:

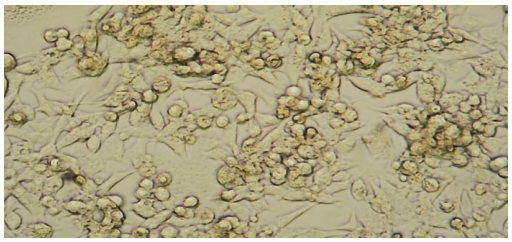


Figure 20: The percent viable cells versus concentration of compound 2b after 24-hours' incubation of MCF-7 breast cancer cell line.

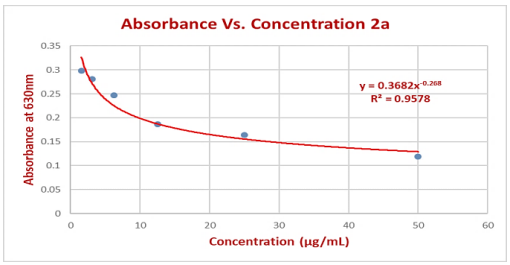


Figure 21: Shows control MDCK kidney normal cell line. An inverted microscope observation post 24-hours' incubation.

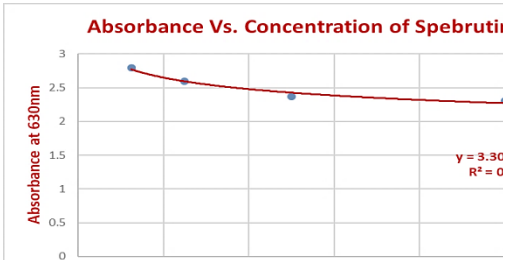


Figure 22: Shows MDCK kidney normal cell line (subjected to 12.5µg/ml of spebrutinib standard). An inverted microscope observation post 24-hours' incubation.

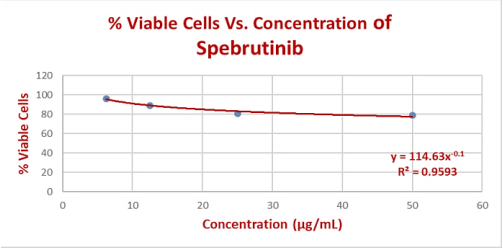


Figure 23: the absorbance of MTT versus concentration of spebrutinib standard after 24-hours' incubation of MDCK kidney normal cell line.

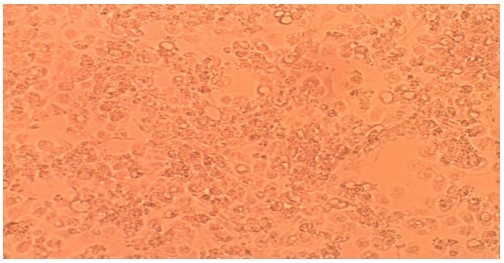


Figure 24: the percent viable cells versus concentration of spebrutinib standard after 24-hours' incubation of MDCK kidney normal cell line.

Effect of compound 2b is shown in Figure 28, 29, and 30:

The IC50 for the synthesized compounds and the spebrutinib standard for the HCT116, MCF-7, and the MDCK cell lines are calculated in Table 4.

DISCUSSION

Results of the biological effect of the synthesized compounds on the cancerous and normal cell-lines:

The time of incubation was optimized to 48 hours instead of 72 hours and eventually 24 hours' incubation period was selected. The concentrations of the synthesized chemicals that were applied to the cells were optimized though. Starting with the highest concentration of 1 mg/L and reaching the optimized high concentration of 50 µg/mL.

Thereafter, the serial dilution performed from the 50 µg/mL was mathematically accepted to give good representative curves. The sketched curves possess good correlation coefficients. Furthermore, the half-maximal inhibitory concentration (IC50) values were mostly in the middle of the curves to exclude any proposed drift in the curves if any. The IC50 is a measure of the effectiveness of the synthesized chemical compound in inhibiting cell growth.

The cell lines were meaningfully chosen and accurately selected. For colorectal cell lines, excluding skin cancers, colorectal cancer is classified as third cancer in occurrence in US for both sexes. In the same context, in the United States, the American Cancer Society has proposed the number of the cases for colorectal cancer for 2019 as (44,180) new cases of rectal cancer and (101,420) new cases of colon cancer [16]. Regarding breast cancer cell line, excluding skin cancers, breast cancer is classified as first cancer in occurrence in the US for American women. In terms of numbers, in the United States, the American Cancer Society has estimated the number of the cases for the breast cancer for 2019 as (268,600) new cases of invasive breast cancer and (62,930) new cases of non-invasive breast cancer.[17]

Notably, to evaluate the selectivity of the newly synthesized compounds toward the cancer cells, it is essential to test their toxicity on normal (non-cancerous) cells.[18-20] A dramatic number of authors utilized the MDCK cell line for cell viability studies.[21-29] According to this fact besides their availability, the MDCK cell lines were selected.

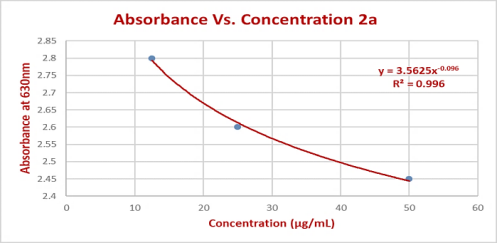


Figure 25: Shows MDCK normal kidney cell line (subjected to 12.5µg/ml of compound 2a). An inverted microscope observation post 24-hours' incubation.

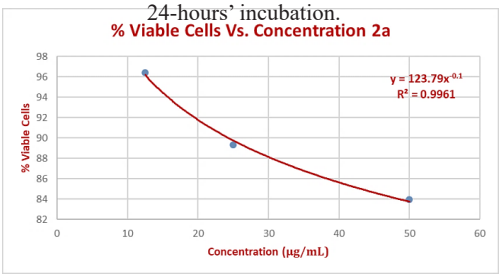


Figure 26: The absorbance of MTT versus concentration of compound 2a after 24-hours' incubation of MDCK kidney normal cell line.

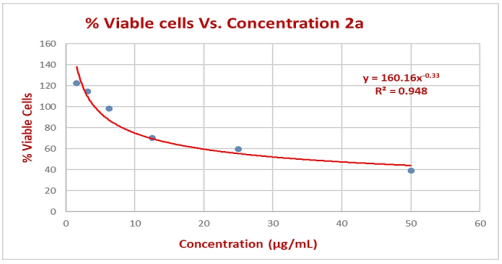


Figure 28: Shows MDCK normal kidney cell line (subjected to 12.5µg/ml of compound 2b). An inverted microscope observation post 24-hours' incubation.

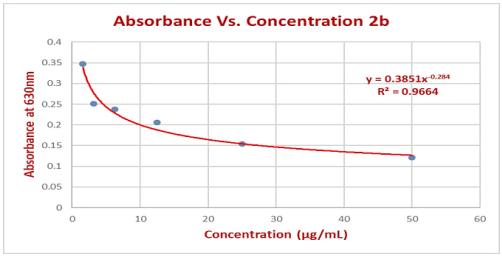


Figure 30: The percent viable cells versus concentration of compound 2b after 24-hours' incubation of MDCK kidney normal cell line.

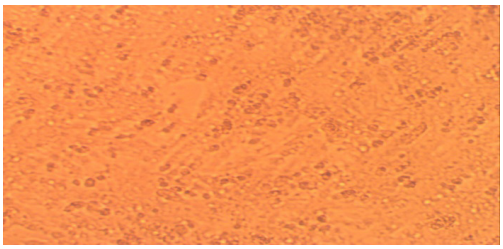


Figure 27: The percent viable cells versus concentration of compound 2a after 24-hours' incubation of MDCK kidney normal cell line.

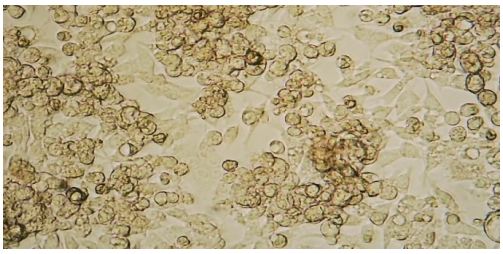


Figure 29: The absorbance of MTT versus concentration of compound 2b after 24-hours' incubation of MDCK kidney normal cell line.

Table 4: A summary for IC50 for the cell lines and chemical compounds specified.

Cell line	IC50 AVL-292	IC50 2a	IC50 2b
HCT116	11.73 µg/mL	34.05 µg/mL	25.53 µg/mL
MCF-7	13.566 µg/mL	10.744 µg/mL	19.23 µg/mL
MDCK	4.011 mg/mL	8.653 mg/mL	1.705 mg/mL

Table 4 shows the net results of this work as it reveals the biological effects of the chemically synthesized compounds on the cancerous and normal cells. This brief comparison gives valuable information. To critically analyze the observed data, it will be categorized for each compound and then for each cell as below:

- For the spebrutinib standard, it shows close values of IC50 for both HCT116 and MCF-7 cell lines with priority for the HCT116 cells. In the same context of spebrutinib, the IC50 for MDCK cells shows approximately 300-fold the concentration required for the activity against both HCT116 and MCF-7 cell lines. This reveals an excellent selectivity toward the cancerous cells rather than the normal non-cancerous cells.
- For compound 2a, the IC50 value for the HCT116 cells is 3-fold its value for MCF-7 cells. In other words, compound 2a has superior activity on MCF-7 cells rather than for the HCT116 cells. Moreover, the IC50 of compound 2a for MDCK shows approximately 250-fold and 800-fold of the concentration required for the activity against the HCT116 and MCF-7 cell lines, respectively. This indicates an excellent selectivity toward the cancerous cells rather than the normal non-cancerous cells with better selectivity toward the MCF-7 cell line.
- For compound 2b, the IC50 value for the HCT116 cells is 1.3-fold its value for MCF-7 cells. Accordingly, compound 2b has better activity on MCF-7 cells rather than for the HCT116 cells. Moreover, the IC50 of compound 2b for MDCK shows approximately 67-fold and 89-fold of the concentration required for the activity against the HCT116 and MCF-7 cell lines, respectively. This indicates an acceptable selectivity margin toward the cancerous cells rather than the normal non-cancerous cells with better selectivity toward the MCF-7 cell line.

  According to the European Medicines Agency (EMA), with respect to the "Nonclinical Evaluation for Anticancer Pharmaceuticals" The EMA concludes the following statement "A common approach for many small molecules is to set a start dose at 1/10 the Severely Toxic Dose in 10% of the animals (STD 10) in rodents.[30] If the non-rodent is the most appropriate species, then 1/6 the Highest Non-Severely Toxic Dose (HNSTD) is considered an appropriate starting dose. The HNSTD is defined as the highest dose level that does not produce evidence of lethality, life-threatening toxicities or irreversible findings".[30] In all the previously discussed findings the proposed therapeutic doses are much lower than the "one-tenth" portion of the STD 10. Accordingly, the therapeutic doses can be reduced to be equal or below the micromolar concentrations recommended by many types of research.[31-33]
- For HCT116 cell line, the IC50 for the spebrutinib standard has the lowest value, followed by compound 2b and lastly compound 2a. Where as, compound 2b has twice the IC50 value in respect to spebrutinib standard, compound 2a has trice the IC50 value for the standard.
- For the MCF-7 cell line, it has the most interesting findings. Compound 2a has the lowest IC50 value, which indicates better cytotoxic activity than the spebrutinib standard. Whereas, spebrutinib has the middle value for IC50 (1.25 times of that of compound 2a). Finally, compound 2b has the highest IC50 value.
- For the MDCK cell line, the IC50 values have a piece of appreciated information. For example, compound 2b has the lowest IC50 value which means the most toxic in respect to compounds 2a and standard. On the other hand, the IC50 for compound 2a has approximately 2-times the IC50 value of that of spebrutinib standard. To sum up, compound 2a has better activity and lower toxicity than the spebrutinib standard.

CONCLUSIONS

In this study, two of the synthesized compounds were found to have biological activity. The biological effects are concluded for compound 2a which has approximately 2-times the IC50 value of that of spebrutinib, hence compound 2a has the lowest IC50 value, which indicates better cytotoxic activity than the spebrutinib standard. Whereas, spebrutinib has the middle value for IC50 (1.25 times of that of compound 2a).

SUPPLEMENTARY MATERIALS

Graphical abstract will be available in the supplementary material's pattern when published.

DATA AVAILABILITY

"The data used to support the findings of this study are available from the corresponding author upon request".

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ABBREVIATIONS

HCT116, Human Colorectal Carcinoma; MCF-7, Michigan Cancer Foundation -7; MDCK, Madin-Darby Canine Kidney; TK, Tyrosine Kinase; TKI, Tyrosine Kinase Inhibitor.

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