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Screening and Repositioning of the Drugs That Possess Binding Affinity towards Notch 1 Receptor Thereby Inhibiting Cell Proliferation in Leukaemia

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Abstract

Cancer is one of the life threatening disease of 20th century and spreading further with increasing incidence in 21st century. Leukemia's are the type cancers of bone marrow and blood mostly identified in adolescent and young adult population. They are also challenges regarding the effective delivery of the therapy specific to the adolescent and young adult group. Signalling pathways have been investigated for their role in cancer pathogenesis. Notch receptors may contribute to malignancies as both oncogenes and suppressors. The dysregulation of the notch receptor signalling pathway has been associated with disorders, including cancers. The main focus of this research was on notch 1 receptor which has maximum contribution in suppressing leukemia. In activation of notch 1 receptors to gain a function, mutations were identified in more than 50% of T-ALL cases. Gamma secretase inhibitors were first tested small molecule drug candidates to block the signalling of notch receptors. Furthermore, monoclonal antibodies. However they have been challenging in clinical trials due to primary resistance/dose limiting on target toxicities. Success in targeting these receptors could be achieved by repositioning the drugs -where we investigate the effective method to identify new indications of existing medications, which failed to show much therapeutic benefits. The drug bank database was used as a resource for the pharmacological and molecular information of the compound to facilitate the discovery, screening and the prediction of binding interactions of the candidate drug and its analogues.

Keywords: Cancer; Signalling pathway; Notch receptors; Dysregulation; Leukemia; Screening; Docking predictions; Binding interactions

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Introduction

ECancer is one of the life threatening disease of 20th century and spreading further with increasing incidence in 21st century. The increase in incidence is probably due to the change in habits, lifestyle and increased life expectancy. Cancer is a condition where cells grow abnormally in an organ or tissue and do not stop their progression until cancerous mass is removed surgically, chemotherapy/another type of cancer specific medication, radiation therapy or cancer cells shrink/disappear on their own [1].

Leukemia's are the type cancers of bone marrow and blood mostly identified in adolescent and young adult population. They are also challenges regarding the effective delivery of the therapy specific to the adolescent and young adult group [2].

Signaling pathways have been investigated for their role in cancer pathogenesis. Notch receptors may contribute to malignancies as both oncogenes and suppressors [3]. It was reported that Human-T-Lymphoblastic leukemia (T-LL) was the first disease associated with notch signaling dysfunction (Paganini and Ferrando) [4]. Notch receptors are a class of specific trans membrane proteins that mediate cell contact dependent signaling and characterized by epidermal growth factor (EGF)-like repeats in the extracellular domain (Kovall). Four different isoforms of notch receptors are expressed in mammals (Notch–1,2,3 and 4) and two families of functional ligands, Delta-like (DII1-4) and Jagged (JAG1-2) [4].

The dysregulation of the notch receptor signalling pathway has been associated with disorders, including cancers. Thus, this pathway has been studied as potential target for treating chronic lymphoblastic leukemia and T-ALL, an aggressive type of lymphoid malignancy that affects adolescents and children [5].

The main focus of this research was on notch 1 receptor which

has maximum contribution in suppressing leukemia. Notch 1 receptors which have highly conserved cell signalling system. Primary function of the receptor is: when the ligand binds to the receptor, the receptor undergoes S2 cleavage at extracellular portion and then undergoes S3 cleavage, gets separated from the membrane of the cell and there by transfers nucleus(nuclear translocation) and binds to the DNA to exhibit gene transcription, cell proliferation, respiratory functions etc. Hence, if this function could be inhibited eventually led to novel medications in treating cancer [6].

In activation of notch 1 receptors to gain a function, mutations were identified in more than 50% of T-ALL cases (Andersson and Lendahl). These mutations consists of single amino acid substitution in the extracellular negative regulatory region(NRR mutations), or at the C- terminal PEST domain(proline, glutamate, serine, and threonine) [7].

Gamma secretes inhibitors were first tested small molecule drug candidates to block the signaling of notch receptors. Furthermore, monoclonal antibodies. However they have been challenging in clinical trials due to primary resistance/dose limiting on target toxicities [6,8]. For instance, Brontictuzumab was halted in phase 1b trial due to in tolerance in patients with colorectal cancer although it showed anti-cancer effect against Notch 1 receptor [9].

All these incidences together have raised the need to discover novel selective antagonists for notch 1 receptor which are effective and safe (Xue) [10]. Success in targeting these receptors could be achieved by repositioning the drugs-where we investigate the effective method to identify new indications of existing medications, which failed to show much therapeutic benefits [11].

The drug bank database was used as a resource for the pharmacological and molecular information of the compound to facilitate the discovery, screening and the prediction of binding interactions of the candidate drug and its analogues [12,13].

The overall aim of this research work is to determine the candidate drug among a library of 500 compunds, that specifically inhibit the constitutive signalling on notch 1 receptor through drug discovery strategies that combine molecular pharmacology (screening, signalling and toxicology assays) and computational biology approaches(in silico ligand docking and structural activity relationship(SAR analysis) which eventually led to refining novel assay for notch 1 receptor, and developing a method for screening for the final potential in a collaborative team work manner.

METHODS

Cell culture

Required cells that contain notch 1 receptors were available at Coventry university laboratory cultured beforehand for the research. Media was aspirated from the flask, 5 ml of PBS was added to wash the cells and maintain pH. PBS was then aspirated and 1 ml of trypsin is added to incubate the cells for 15 min so that the cells will detach from the surface of the flask. To confirm where the cells were detached from the surface, flask was placed under the electron microscope and observed if the cells were floating. Once the ideal float is achieved 10 ml of media is added to the cells and centrifuged for 5 min at 1600 rpm. Supernatant was aspirated without disturbing the pellet 10 ml of PBS was added to the cells and suspended the pellet to maintain the pH. After resuspension of cells in the PBS cells were centrifuged again for 5 min at 1600 rpm. Supernatant of PBS was aspirated and 3 ml of media was added to the cells and suspended the pellet. In an eppendroff tube 20 µl of cells were taken and to this 20 µl of tryphan blue is added to count the cells in the+notch receptor cell line.

Screening and signalling assessment

In consideration of Perkin Elmer lance assay kit manufacturers suggestions-lance assay was performed to the+notch receptor cell line.

Cell plates (+NR, -NR)

89 µl of stimulation buffer+1 µl of cells in each well and incubated for 1 hour. After incubation 10 µl of drug is added into cell plates using multi-channel pipette and incubated for 30 minutes (LYSATE). Meanwhile prepared detection mix: 1230 µl of detection buffer+10 µl of EU+10 µl of biotin notch 1 ATP and incubated in room temperature for 15 minutes.

Assay plate:

30 µl of antibody+40 µl of lysate is added into each cell and incubated for 30 minutes. After incubation 12 µl of detection mix is added screened in microplate format as per kit manufacturers instructions.

Toxicology assay

CyQUANT™ XTT Cell Viability Assay

Please refer to Thermo Fisher's CyQUANT™ XTT Assay kit instructions.

Data analysis

The values of EC50 and LC50 were determined though AAT Bioquest website. Microsoft Excel was utilised to normalise the data, and to conduct a paired T-test.

RESULTS

Compound screening

Using Perkin Elmer assay, human metastatic small cell lung carcinoma cells were exposed to almost 500 drugs (10 µl of drugs at 100 mM) and seeded in five 96 well plates containing 1000 cells per well. The drugs were initially screened to differentiate between the drugs that specifically stimulated Notch-1 in Notch positive and notch negative cells. Based on conditional formatting as a result, only three drug candidates followed the criteria as shown in Figure 1.

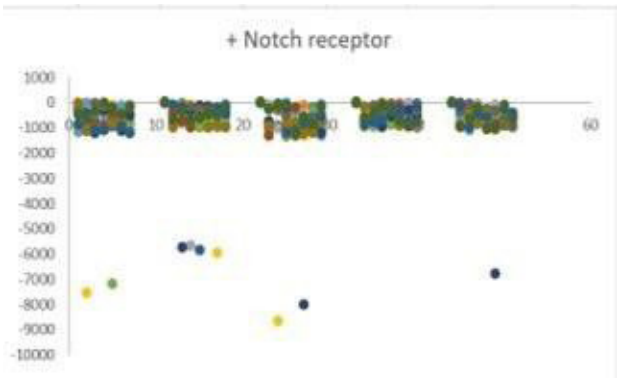


Figure 1 Screening results on Notch-1 positive cells. The yellow objects represent the three candidate drugs that stimulated Notch-1 positive cells, whereas the other objects represent the drugs that worked on cells however couldn't show specificity towards the receptor

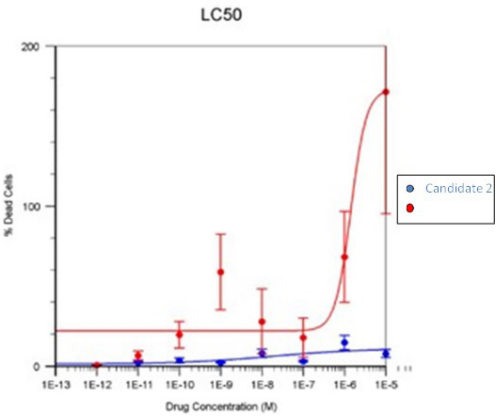


Figure 3 The toxic effects of the drug candidate 2 and H2O2 as control on Notch-1 positive cells. Toxicity of drug candidate 2 LC50 2.07 X 10^-8(M), in addition H2O2 LC 50 7.55 X 10^-7(M). X-axis determining the drug concentration (M) and Y- axis determining the percentage of dead cells

Toxicity profile of the candidate drugs

To estimate the lethal concentration (LC50) value, XTT Cell viability assay was conducted. Notch positive cells were exposed to different concentrations of the drug candidate 1 compound. Hydrogen peroxide (H2O2) was used as the positive control in this study (Figure 2).

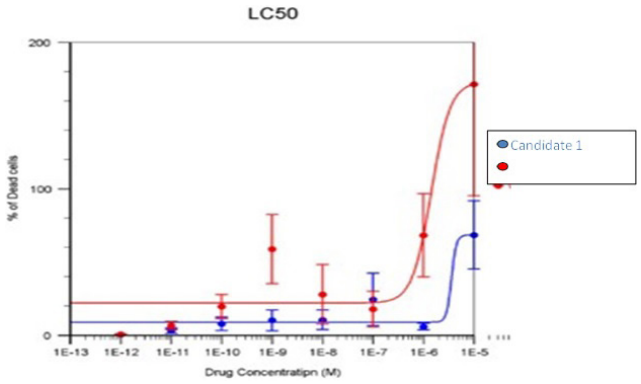


Figure 2 The toxic effects of the drug candidate 1 and H2O2 as control on Notch-1 positive cells. Toxicity of drug candidate 1 LC50 3.94 x 10^-6(M), in addition H2O2 LC 50 7.55 X 10^-7(M). X-axis determining the drug concentration (M) and Y- axis determining the percentage of dead cells.

To estimate the lethal concentration (LC50) value, XTT Cell viability assay was conducted. Notch positive cells were exposed to different concentrations of the drug candidate 2 compound. Hydrogen peroxide (H2O2) was used as the positive control in this study (Figure 3).

To estimate the lethal concentration (LC50) value, XTT Cell viability assay was conducted. Notch positive cells were exposed to different concentrations of the drug candidate 3 compounds. Hydrogen peroxide (H2O2) was used as the positive control in this study (Figures 4-17).

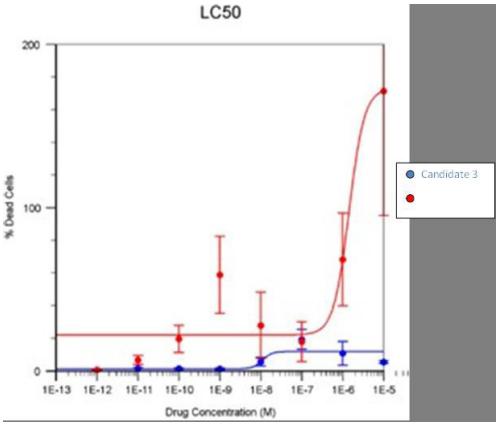


Figure 4 The toxic effects of the drug candidate 2 and H2O2 as control on Notch-1 positive cells. Toxicity of drug candidate 2 LC50 1.07 X 10^-8(M), in addition H2O2 LC 50 7.55 X 10^-7(M). X-axis determining the drug concentration (M) and Y- axis determining the percentage of dead cells

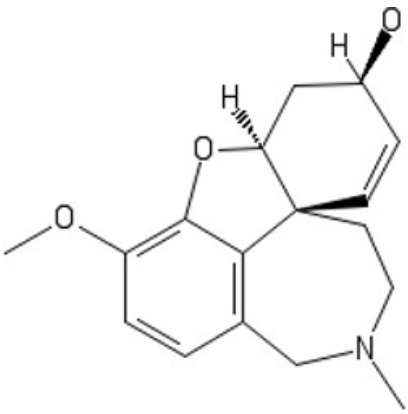


Figure 5 illustrates the compound structure of the parent drug with potency shift of -8 and 100% efficacy..

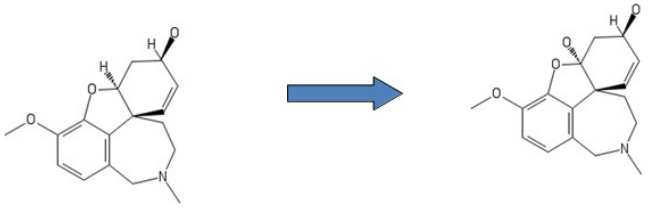


Figure 6A Represents the repositioning of the parent compound to create analogue 1 the SAR analysis of analogue 1 to determine its efficacy. Substitution of Hydrogen atom in parent compound with Oxygen in the first analogue

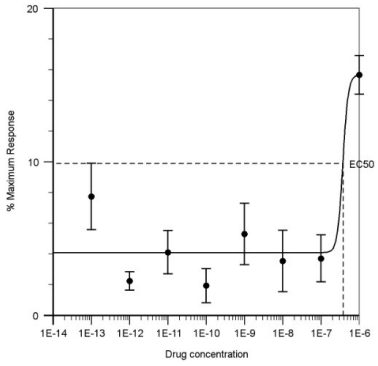


Figure 6B Represents the repositioning of the parent compound to create analogue 1 the SAR analysis of analogue 1 to determine its efficacy. Illustrates the SAR analysis with EC50 2.95 X 10^-7 (M) of analogue 1 where we observed significant decrease in efficacy from 100% to 35% and no major shift in potency

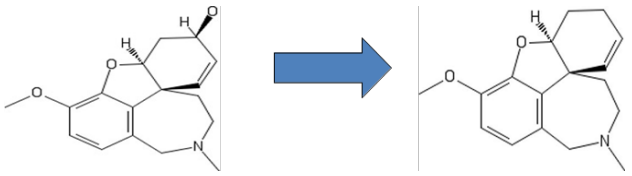


Figure 7A Elimination of the Hydrogen and Oxygen atoms in parent compound to obtain analogue 2.

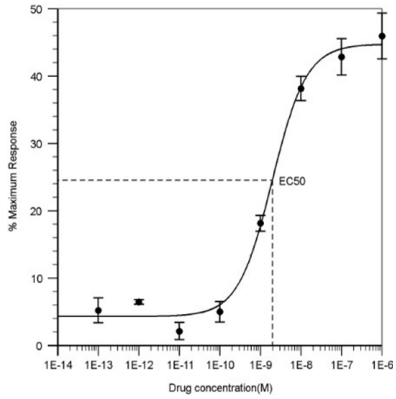


Figure 7B Illustrates the SAR analysis with EC50 2.07 x 10^-9(M) of analogue 2 where we observed significant decrease in efficacy from 100% to 50% and potency shift from -8 to -6. X-axis determines the drug concentration (M) and y-axis determines the % of maximum response

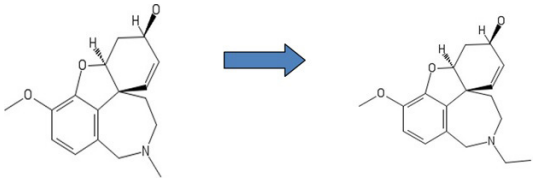


Figure 8A Addition of a carbon atom in parent compound to obtain analogue 3

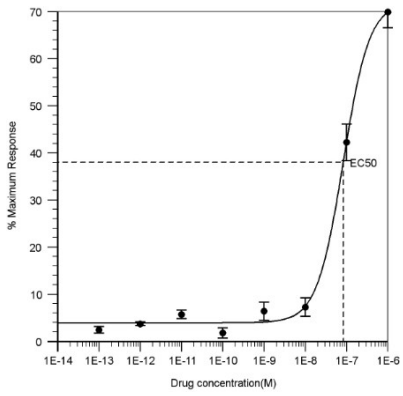
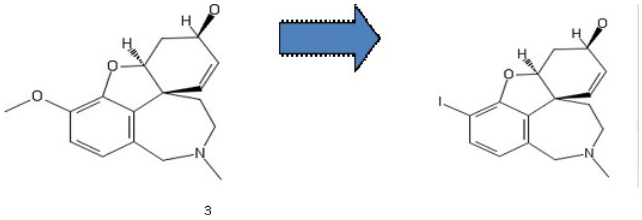


Figure 8B Illustrates the SAR analysis of analogue 3 with EC50 1.43 X 10^-7(M). Minute decrease in efficacy from 100% to 89% is observed with increasing potency shift from -8 to -9. X-axis determines the drug concentration (M) and y-axis determines the % of maximum response



3

Figure 9A Substitution of the Oxygen atom with Iodine ion in parent compound to obtain analogue 4.

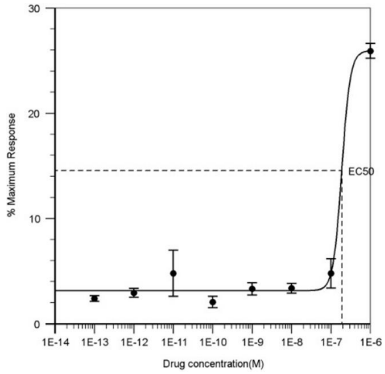


Figure 9B Illustrates the SAR analysis of analogue 4 with EC50 4.1 X 10^-7(M). Significant decrease in efficacy from 100% to 42% is observed with decreasing potency shift from -8 to -7. X-axis determines the drug concentration (M) and y- axis determines the % of maximum response

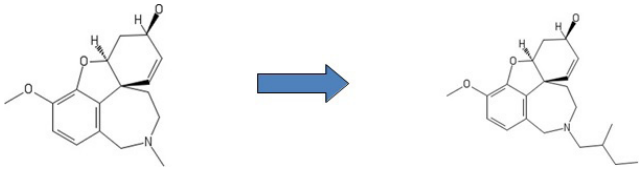


Figure 10A Addition of hydrogen bond at Nitrogen atom in parent compound to obtain 5th analogue.

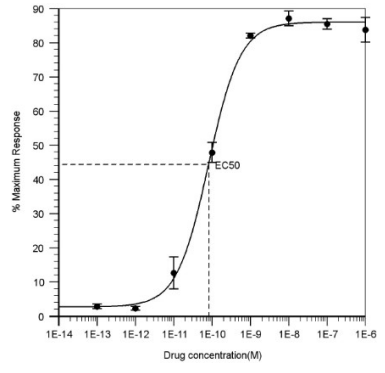


Figure 10B Illustrates the SAR analysis of analogue 5 with EC50 1.05 X 10^-10(M). No Significant difference in efficacy is observed with increasing potency shift from -8 to -11. X-axis determines the drug concentration (M) and y-axis determines the % of maximum response.

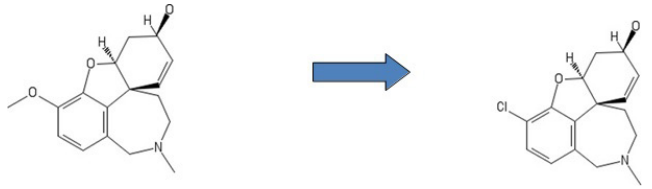


Figure 11A Substitution of the Oxygen atom at Carbon 1 with Chlorine atom in parent compound to obtain analogue 6.

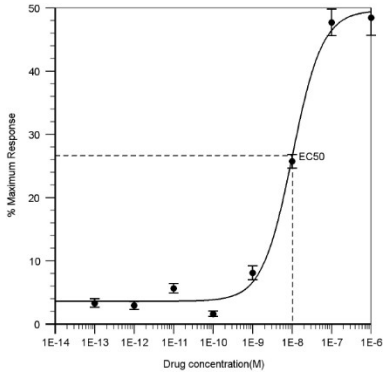


Figure 11B Illustrates the SAR analysis of analogue 6 with EC50 9.02 X 10^-9(M). Significant decrease in efficacy from 100% to 56% is observed with decreasing potency shift from -8 to -7. X-axis determines the drug concentration (M) and y-axis determines the % of maximum response.

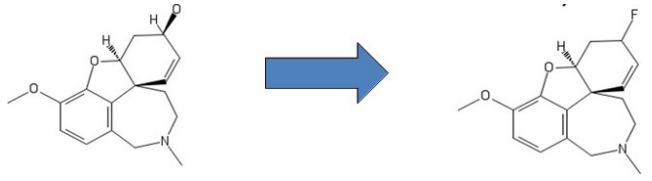


Figure 12A Elimination of Hydrogen and Oxygen bonds and substitute with Fluorine in parent compound to obtain analogue 7.

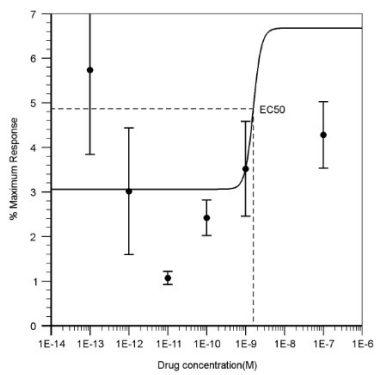


Figure 12B Illustrates the SAR analysis of analogue 7 with EC50 2.57 X 10^-9(M). significant decrease in efficacy from 100% to 10% is observed with decreasing potency shift from -8 to -6. X-axis determines the drug concentration (M) and y-axis determines the % of maximum response

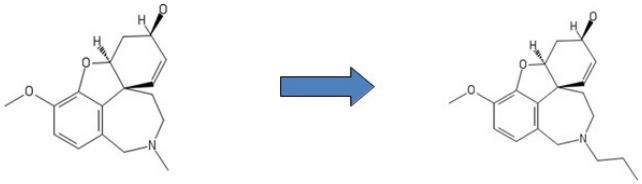


Figure 13A Extension of the NH chain in parent compound to obtain analogue 8.

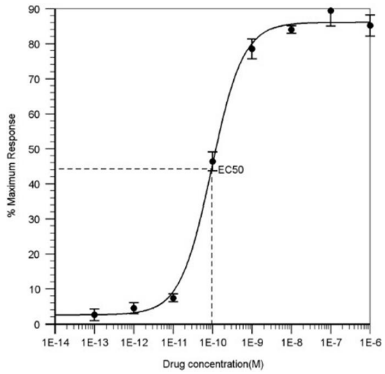


Figure 13B Illustrates the SAR analysis of analogue 8 with EC50 1.22 X 10^-10(M). No significant difference in efficacy is observed with increasing potency shift from -8 to -9. X-axis determines the drug concentration (M) and y-axis determines the % of maximum response.

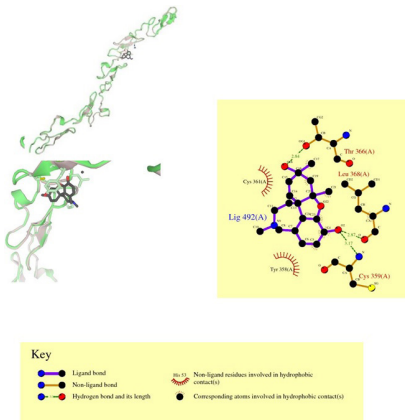


Figure 14 Docking results of the parent compound to Notch-1 receptor. (B) Binding interactions between parent compound and amino acid residues of Notch-1 receptor. For instance, hydrogen bond was formed between Cys 359 of the receptor and O2 group of the ligand, as well as between Thr 366 and oxygen atom of the ligand.

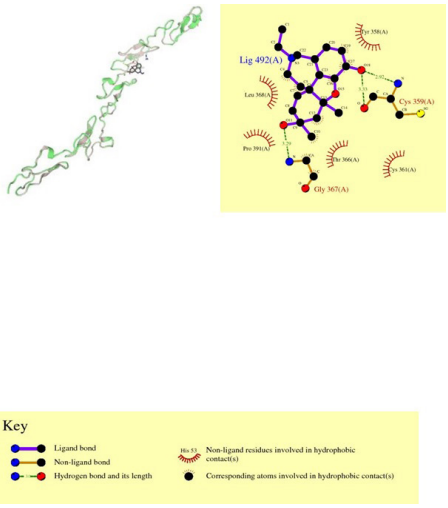


Figure 15 Docking results of analogue 3 to Notch-1 receptor. (B) Binding interactions between analogue 3 and amino acid residues of Notch-1 receptor. For instance, hydrogen bond was formed between Cys 359 of the receptor and Oxygen atom at of the ligand, as well as between Gly 367 and oxygen atom of the ligand.

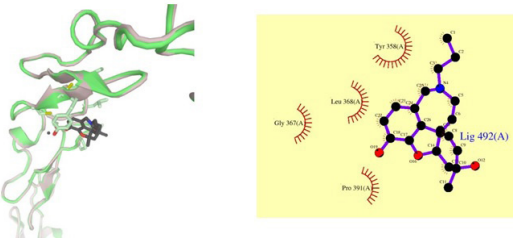


Figure 16 Docking results of analogue 5 to Notch-1 receptor. (B) Binding interactions between analogue 3 and amino acid residues of Notch-1 receptor. For instance, bond was formed between oxygen atom of ser 356 of the receptor and O2 group of the ligand

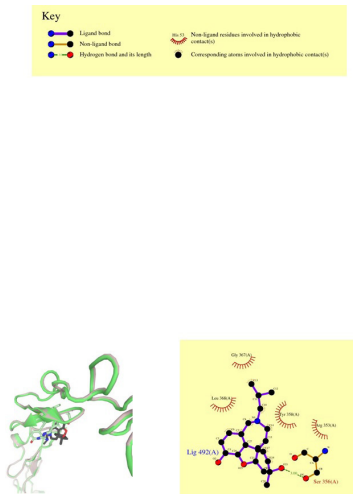


Figure 17 Docking results of analogue 8 to Notch-1 receptor. (B) Binding interactions between analogue 3 and amino acid residues of Notch-1 receptor

Stones	WEEK1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6
screening- 500 s n Elmer Notch Assay ch receptor(NR) cell						
screening rugs n Elmer Notch Assay & -NR cell ology XTT Assay didate drugs						
ture Activity ionship nt compound+ ogues 1-4)						
ture Activity ionship (analogues 5-8)						
d docking						

Figure 18 Time line of the developed strategy illustrated in the Gantt diagram.

DISCUSSION

Identifying molecular pathways that are associated with diseases is crucial for developing new treatments and improving existing therapies (Lindner and Link). This work was done in a period of six weeks as demonstrated on the Gantt diagram below (Figure 18). Each assay was done in replicates to gain more robust data and to reduce the errors

The drugs were firstly screened from a library containing almost 500 compounds to identify the potential drugs which had an effect on Notch-1. The three candidate drugs were selected based on the conditional formatting option in Excel software. It was observed that the compounds which specifically activated Notch-1 receptor had very low stimulation values in Notch positive cells (for instance less than -3000)-(Figure 1). The cells used in this study were extracted from the branchial lymph node cluster of a metastatic small cell lung carcinoma, which exhibited a 19 overexpression for Notch-1 receptor. The genotype of the cells had a mutation at the Cdomain of Notch-1 receptor.

Perkin Elmer assay was used to measure the response of the drugs by calculating the EC50 values, the test was sensitive enough as it aided in excluding the drugs which did not cause a response. The quality of the assay was evaluated by the Z-factor, which is a parameter that is used in High-throughput screening (HTS) assays by comparing the mean and the standard deviations of the samples to the positive control in the plate (Bray and Carpenter) . The calculated Z-factor was in the optimal range ($0.5 \leq 0.78 \leq 1$) which showed that the test was robust and very good.

After narrowing down the number of drugs from 500 to 3, the LC50 values were calculated for the candidates (Figure 3) to determine the toxicity and therapeutic index which is defined as the ratio between the lethal dose of the drug to its effective dose.

Based on the initial signaling assay, drug candidate 2 is the most promising to be the lead compound as it blocked the constitutive signaling of the Notch-1. Thus, it acted as an inverse agonist because it had the lowest response. Moreover, the drug was safe as it had a low LC50 concentration.

To validate the pharmacological data obtained in the first four weeks, computational biology approach was used afterwards by the means of molecular docking and SAR analysis of the parent compound and its analogues. The level of significance was statistically determined by conducting a paired T-test.

Structure-Activity Relationship (SAR) is a tool that provides information about the molecular structure of the compounds and its relation to their biological activity. Lead optimisation studies aim, for example, to enhance the potency, improve bioavailability and reduce toxicity in the early stages of drug discovery. It was clear that extension of Carbon in parent compound had an important position. Conversely the efficacy and potency where increased (Figure 8A, 10A and 13A). The short come of the project where toxicology assay for the effective analogues couldn't be performed due some inconvenience.

Ligand docking was performed as a computational method to predict the binding interactions at the receptors pocket. Hydrogen bonding was formed between cys359 receptor and parent compound, as well as between Thr 366 and parent compound (Figure 14). We could also observe the hydrogen bonding between cys 359 of receptor and analogue 3, and as well as between Gly 367 and analogue 3. These tye of interactions where lost in analogue 5. Whereas analogue 8 did not show any affinity towards the receptor.

According to the pharmacological and computational findings, it is suggested that analogue 5 could be a better choice with 100% efficacy and with increasing potency from-8 (parent compound) to 11(analogue5). Thus, expanding this work in the future can be achieved by determining the key functional group of the compound, as well as identifying biomarkers for Notch-1 inhibition and eventually taking the project further into clinical

studies to indicate if the drug had the expected anti-leukemic effects from the preclinical trials findings.

Since the project involved teamwork, the members communicated through different professional online platforms namely, Slack and Lab archive respectively. Communication was a key in the progression, where each member understood every process was taken and what the next task will be. It also helped in overcoming issues related to having different point of views by discussing and sharing information. The team had faced another problem on the second week of screening, the cells were mistakenly centrifuged using RCF (relative centrifugal force) unit settings instead of RPM (round per minute), the cells were ruptured and eventually died. However, this problem was solved by obtaining live cells from another through negotiation but due to lack of time screening was not successful and thereby lost 1 week in the given schedule.

We do have many techniques such as liposome targeting, luciferase method to identify the drugs effectiveness in future researches. A new approach of modulating ligand and receptor activity can be helpful in future researches [14].

Conclusion

Combining multidisciplinary approaches (practical lab work and in silico methods) to screen candidate drugs that work specifically on Notch-1 receptor was proven to be a valid approach that allows efficient and rapid characterisation of SARs to identify the final candidate drug. Additionally, working as a team had facilitated the drug discovery process. Further investigation of Notch signalling pathway is required and it could be a promising strategy to understand cancer formation and may provide guidance in developing effective and safer leukaemia treatments in the future..

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