Drug Re-Purposing - Can Anti-Allergy Drugs also be Used to Treat Cancer?

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Abstract

Medulloblastoma (MB) constitutes the commonest malignant childhood brain cancer, and it is the leading cause of death in infants under 1 year. Treatment approaches, such as surgery, radiation, and chemotherapy, have significantly enhanced in patients' clinical outcome with approximately more than 60% of 5-year survival. Nevertheless, the majority of cancer patients deal with long-term side effects. So, it is important to develop novel therapeutics with lower toxicity and increased efficacy. The present project was conducted to examine if the anti-allergy drugs can inhibit the cancer growth. More specifically, eight anti-allergy compounds, designed by the chemists at University of Central Lancashire (UCLAN), were evaluated for their anti-cancer activity against medulloblastoma. The effect of each testing drug was tested with the use of MTT assay after three days of incubation on medulloblastoma ONS76 cell line. From the extraction of the results of these anti-allergy compounds, it was found that only three of them (CL1-45-1, CL1-56-1, CL1-57-1) were more active, as they significantly inhibited the cancer growth. But, the CL1-42-1 compound displayed the greatest anti-cancer activity against the medulloblastoma ONS76 cells with $IC_{50} = >10 \mu M$ ($IC_{50} = 0 \mu M$). This leads to the conclusion for further investigation of the anti-allergy compounds in order to consider them as potential anti-cancer agents.

Keywords: Anti-Allergy Drugs; Cancer; Anti-Cancer Agents, Drug-repurposing

Abbreviation

Cancer

Cancer is a complex disease which is based on abnormal growth of malignant tumors. Uncontrolled growth and reproduction of cells characterize cancer [1]. It is also supported that cancer constitutes the second leading cause of death worldwide, accounting for more than ten million novel cancer cases and more than eight million deaths [2]. Furthermore, according to global statistics, the rates of cancer will be increased in the next years. For instance, more than twenty million people will be diagnosed with cancer in the next six years [3].

Types of cancer

There are more than one hundred different types of cancer [1]. The cancer types are usually named for the tissues or organs where the tumors form. For instance, brain cancer occurs in the cells of the brain, while lung cancer occurs in the cells of the lung. In addition, cancer can be characterized by the type of cell that formed it, such as squamous cell or epithelial cell. Three of the categories of cancer are presented below:

Leukemia

Leukemia constitutes a type of tumour which is developed in the blood-forming tissue of the bone marrow. This type of cancer does not form solid tumours. Numerous white blood cells are abnormally developed in the bone and blood marrow, however [4]. Acute, chronic, lymphoblastic, and myeloid are four of the common types of this cancer category.

Multiple myeloma

Multiple myeloma is also characterized as Kahler disease and/or plasma cell myeloma [1]. This type of cancer is developed in plasma cells. The plasma cells are also named as myeloma cells. They form tumours in bones and are presented in the bone marrow.

Melanoma

This specific cancer category occurs in cells that become melanocytes. Melanocytes are described as cells that make melanin. The majority of melanomas are formed on the skin. Melanomas are also formed in different tissues, such as the eye.

Paediatric cancers

In the developed countries, pediatric cancers constitute the most common cause of death in children [5]. Approximately more than two hundred thousand children of all ages have cancer diagnosis every year [6]. However, it is supported that the brain and solid nervous system (CNS) cancers can be occurred more frequently in patients with a percentage of 20% of pediatric cancers, compared to leukemia which constitutes the commonest type of tumor [5].

Medulloblastoma is considered to be the most common malignant pediatric brain tumor. This type of pediatric cancer occurs in six children per million under the age of nine and two children per million up the age of fifteen [4]. Totally, approximately four hundred patients have been diagnosed by medulloblastoma in the United States of America (USA), eighty in the United Kingdom (UK), and more than five thousand cases worldwide every year [4,6].

Significant progress has been done in order to understand the pathogenesis and treatment of childhood cancers. Somatic mutations and their role in oncogenesis have been discovered by the growing capacity of the analysis of genetic and epigenetic abnormalities in cancer cells. Mutations in approximately 1.6% of the thousands of coding human genes have been involved in carcinogenesis with the number of cancer genes growing to more than four hundred. In addition, pediatric cancers involve many of these genes [7]. Hopefully, the next generation of extremely parallel, single-molecule DNA sequencing systems will recognize many more genetic transformations in cancer cells [8]. There has been notable progress in the survival rates for childhood cancer patients in conjunction with these biological developments. From the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute, the survival rates for the majority of pediatric cancers vary from 61.7% in 1975 - 1977 to 81.4% in 1999 - 2006. Except for gliomas, the 5-year comparative survival rate has surpassed the percentage of 55% for all the types of pediatric cancer. This progress can be credited to the enlistment of numerous patients in well-structured prospective clinical trials, steady care, improved risk assessment, and the advancement of medications coordinated at particular targets, which have mostly been accomplished using preclinical models. Between 1975 and 2007, the death rates for a combination of all pediatric cancers reduced by approximately more than 50%. Based on the National Cancer Institute, this led to a decrease of over than 60% in the death rates of the following three diseases: acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), and non-Hodgkin lymphoma (NHL), with the first two being among the most curable pediatric cancers. In comparison with the previous statement, the

survival rates for children with different pediatric cancers have not significantly improved the last years, according to studies of the National Cancer Institute.

Medulloblastoma

Medulloblastoma (MB) constitutes the commonest malignant childhood brain tumor and is a leading cause of death worldwide [9]. Approximately more than 15% of young children (5-9 years) are affected by this type of cancer [10]. In contrast, this phenomenon is not extremely frequent in medulloblastoma adult patients (1 - 2%) [11]. Medulloblastoma constitutes an aggressive embryonic type of tumour, and it is presented in the cerebellum of the posterior fossa (Figure 1) [13].

Figure 1: Representation of medulloblastoma in cerebellum of posterior fossa [13].

It is considered that medulloblastoma is separated into several different molecular pathways: sonic hedgehog (SHH), wntless (WNT), and notch [14]. Each of these pathways plays a crucial role based on the proliferation of the cells, the gene mechanisms, and the intracellular communications [14].

In addition, it has been declared that the growth of medulloblastoma can be activated by these pathways [15]. Furthermore, in 2016, it is claimed that approximately more than 35% of patients can perform metastasis during diagnosis [9].

Medulloblastoma subgroups

According to studies, medulloblastoma is considered to be a heterogeneous disease and is classified into four different molecular subgroups [16]. The subgroups are classified as follows: Wnt, SHH, Group3, and Group 4 [11,19-22]. It is supported that specific mutations characterize the tumours from different molecular subgroups. Tumours are characterized by different signaling pathways by having differentiation in cells' origin [11,17,23]. Recently, sci-

entists have discovered a more detailed classification within each medulloblastoma subgroups that divides medulloblastoma into twelve subtypes (two for Wnt, four for SHH, three for Group 3, and three for Group 4) [24].

WNT subgroup

Wnt (**Wingless tumours**) constitutes a family of growth factor receptors that are included in cell control mechanisms and in embryogenesis [23]. Wnt is considered to be the rarest molecular medulloblastoma subgroup, accounting for approximately 10% of tumours that belong to Wnt signaling pathway [17,23]. Also, more than 95% of cancer patients can survive in this molecular medulloblastoma subgroup [11]. Children between the ages of ten and twelve years are affected mostly by the Wnt tumours compared to infants (< 4 years) that have not any effects from this medulloblastoma subgroup. According to studies, patients from this subgroup have leaky blood vessels with the lack of markers combined with an intact blood-brain barrier [16]. Wnt subgroup can be divided into two subcategories: Wnt α and Wnt β . The first subcategory mostly occurs in children in comparison with Wnt β [24]. The Wnt β type occurs in adults. It is supported that the tumours of Wnt subgroup arise from the lower rhombic lip and often are developed in a central location [25]. The majority of Wnt tumours activate CTNNB1 mutations, which encode β -catenin and display Wnt signaling [11]. Furthermore, DDX3X has mutations enriched in Wnt subgroup and is considered to encode an ATP-dependent RNA helicase [11,19-22]. Approximately 15% of Wnt tumours identify TP53 mutations [26]. Different and less common mutations are found in sporadic tumours such as APC, AXIN1, and AXIN2 [11], by playing an important role in the Wnt signaling pathway.

SHH subgroup

Approximately 30% of medulloblastoma tumours contribute to this molecular subgroup, sonic hedgehog (SHH). The cells for subgroup SHH are cerebellar granule neuron precursors (CGNPs) [25]. Normal CGNP proliferation during development requires this signaling pathway. SHH constitutes a combination of deletions or mutations of pathway genes (SMO, PTCH1, SUFU) and amplifications of downstream transcription activators (GLI1 and GLI2) [27]. Even though the 5-year survival in this molecular medulloblastoma subgroup is approximately more than 60%, patients with metastatic disease, GLI2, MYCN amplifications, or lack of the 14q chromosome [30] and specifically patients with TP53 mutations, have < 50% survival [31]. Furthermore, SHH tumours are divided into four subcategories: SHH α , SHH β , SHH γ , and SHH δ . The first of these subcategories exists in patients at the ages of four and seventeen and the last subcategory occurs in adults (> 17 years). The two other subcategories, SHH β and SHH γ , occur in infants from

0 - 3 years [24,27]. SHH γ medulloblastoma tumours have an excellent prognosis after treatment with chemotherapy. In contrast, metastases often occur in SHH β medulloblastoma patients. Moreover, TP53 mutations are contained by SHH α tumours. Adults or children patients with wild-type TP53 have intermediate prognosis [23,27]. It is interesting the fact that mutations in telomerase reverse transcriptase (TERT) promoters are developed in SHH δ tumours. Better treatment outcomes associate with these mutations within this specific medulloblastoma subgroup [24,32].

Group 3

Approximately more than 20% of medulloblastoma patients belong to this molecular subgroup, by characterizing with metastasis at diagnosis. This molecular medulloblastoma subgroup, Group 3, is found in children, infants, and mostly in males rather than females. Because of lack of the molecular pathogenesis of two medulloblastoma subgroups, Group 3 and Group 4, they are categorized based on similar transcription profiles [32]. In comparison with the other molecular medulloblastoma subgroups, Group 3 has the worst prognosis with percentage less than 50% of 5-year survival [17,24]. Group 3 has also been characterized as MYC subgroup [33]. However, MYC expression (mRNA or protein) cannot inform subgroup classification and does not have prognosis. As a downstream target of Wnt signaling pathway, MYC is also observed in Wnt molecular medulloblastoma subgroup [34]. In addition, SMARCA4 constitutes one of the most frequent mutated genes with percentage of 11%. This gene encodes a chromatin remodeling factor. Group 3 tumours are divided into three subcategories: Group 3 α , Group 3 β , and Group 3 γ . Group 3 α mostly occurs in medulloblastoma patients of this group. Group 3 β tumours enrich the amplification of OTX2, GFI1, and GFI1B genes [12,24]. In contrast, Group 3 γ tumours constitute a combination of MYC amplification. Finally, it seems interesting to report that OTX2 gene has shown transcriptional up-regulation to the MYC oncogene [17]. Approximately more than 20% of tumours in this molecular subgroup have isochromosome 17q. But, it is observed gain of chromosome 1q and/or loss of chromosomes 5q and 10q in Group 3 medulloblastoma in comparison with tumours in Group 4 [25].

Group 4

Group 4 is considered to be the commonest medulloblastoma subgroup. Approximately 40% of patients belong to this molecular medulloblastoma subgroup. Tumours of this group constitute a classic histology [19]. It is supported that Group 4 is connected with the isochromosome 17q of approximately 80% of medulloblastoma patients [30]. Tumours from this subgroup occur mostly in children at the age of ten, by carrying a 70% prognosis with only

5-year survival, in comparison with infants (0 - 3 year) that they have less effect [23]. Group 4 does not have differences with Group 3 molecular subgroup. However, they have few MYC amplifications [12,24]. KCNA1 has been reported as an immunohistochemical biomarker for tumours of this molecular subgroup. Also, neuronal differentiation involves in genes, and transcription profiles represent development in Group 4 tumours [17]. Furthermore, Group 4 tumours are divided into four subcategories: Group 4 α , Group 4 β , Group 4 γ and Group 4 δ . The second subcategory, Group 4 β , is enriched for SNCAIP duplications, while Group 4 α is enhanced for MYCN amplifications. Also, two of these medulloblastoma subcategories, Group 4 α and Group 4 γ , are both enhanced for CDK6 amplifications [24]. Children at the age of six and eight years are characterized by the Group 4 tumours with a peak incidence in males. In contrast, female patients of Group 4 tumours obtain high prevalence (approximately 80%) of the X chromosome in tumor tissues [30]. Therapeutic results of Group 4 medulloblastoma patients are intermediate.

Medulloblastoma cell lines

Generally, the cell lines have the ability to easily grow and be relatively uniform, by performing less variability in comparison with the primary cultures [35]. Approximately more than forty medulloblastoma cells lines have been discovered in the last decades [36]. With the molecular medulloblastoma subgroups, the preclinical data can be easily linked to patients' tumours after the classification of the subtype of each cell line [37]. Moreover, as it is shown above (Figure 2) there is not variety in the genetic features of the numerous cell lines. For instance, the cell lines in Group 3 have MYC amplification and approximately 50% of the cell lines from SHH molecular medulloblastoma group (UW-228 and DAOY) have a mutation in TP53. However, TP53 function can be suppressed across MDM2 overexpression [38,39].

MYC amplifications and functional inactivation of TP53 constitute features of high-risk aggressive and invasive medulloblastoma. This can lead to the fact that the most active subtypes of cancer are suitable to grow *in vitro*. An example of *in vitro* option is the MED6 cell line. While a mutation existed in the codon 37 of CTNNB1, it is believed that this cell line (MED6) displayed a wild-type sequence for the gene [40]. In addition, two more cell lines, D425 and D458 perform an interesting situation. The D425 cell line refers to TP53 mutation, compared to that derived from the metastasis (D425), which is TP53 wild-type [38,41]. Both of these cell lines have the similar R72P polymorphism. The TP53 mutation is not essential in order to propagate the metastatic cell line. It is remarkable to report that the MYC and TP53 inactivation contribute to patients' cancer recidivism [42]. Also, the loss of data based on the p53 sta-

Figure 2: Organizational chart of medulloblastoma cell lines classified by molecular subtype. Wnt is indicated with blue, SHH is indicated with red, Group 3 is indicated with yellow, Group 3/4 is indicated with green-yellow, Group 4 is indicated with green, and unclassified is indicated with grey [39].

tus of the original biopsies makes it difficult to demonstrate if the D425-D458 occasion constitutes a real biological phenomenon or a fact of cell culturing. According to studies, three established cell lines (D283, D721 and USP-13-MED) are considered to belong to two different medulloblastoma subgroups: Group 4 [43] and/or Group 3 [44,45].

The three previous cell lines display OTX2 overexpression consistent with group 3 and group 4, while D283 and D721 perform MYC overexpression at the protein level and mRNA. Because of the similarities of group 3 and group 4, it remains uncertain the classification of these cell lines into these two medulloblastoma subgroups [31]. Furthermore, there is a lack of *in vitro* models for the tumours of two molecular medulloblastoma subgroups, Wnt and Group 4 in comparison with the subgroups SHH and Group 3. However, it has been reported the use of medulloblastoma cell line (MED5R) with catenin mutation derived from a rare situation of recurrent large-cell anaplastic WNT tumour [40]. Also, it has found two different cell lines, CHLA-01-MED and CHLA-01R-MED, from the same cancer patient, which belongs to Group 4 molecular subgroup. Approximately more than 40% of medulloblastoma cancers belong to Group 4. To conclude, some of the cell lines were not subcategorized or characterized in the medulloblastoma research. Further research of the cells would benefit to characterization and subtype classification with the use of methylation or gene expression.

Medulloblastoma treatment

A combination of surgery, chemotherapy, and radiation constitutes the current treatment for medulloblastoma. This has led to a medulloblastoma transformation from a cureless disease to a manageable situation. For instance, there is a survival of children over five years old (70%) and over fifteen years old (58%) [5]. However, it is supported that the rise in survival causes numerous side effects. More specifically, approximately more than 20% risk of cerebellar mutism syndrome (CMS) is carried by the medulloblastoma surgery [46], consisting of the following three side effects: balance, total loss of speech, and reduced coordination. Also, several defects in motor and cognitive functions are caused after the children's recovery. Furthermore, the approach of craniospinal radiotherapy is used for the treatment of the brain and spine in medulloblastoma to be prevented the medulloblastoma metastasis in the cerebrospinal fluid (CSF) [47]. It is essential to note that children under three years old avoid being cured by radiotherapy because of the possible damage to the brain development by the irradiation of the central nervous system (CNS) [14]. Radiation causes many consequences in younger patients. Spinal growth and endocrine dysfunction are considered to be two of the radiotherapy's toxicities. The combination of these two side effects can cause premature ageing and reduced independence for patients [48]. In addition, the reduction of radiation dose in cancer patients constitutes an important manner in order to be decreased the severity and incidence of brain's side effects, by offering alternative adjuvant treatments. In comparison with the above statement, this ability can be offered by chemotherapy, which is the second adjuvant cancer treatment. It is believed that patients can survive by the treatment of chemotherapy [18]. Specifically, approximately more than 80% cancer patients have not performed metastasis at diagnosis in the last five years according to studies [49]. To conclude, new treatments for younger population need to be discovered in order to treat this type of disease and reduce the side effects [14]. Based on recent studies, targeted therapies that affect the death of tumour cells have been developed for medulloblastoma patients. These therapies suspend the growth of chemo resistant cells by crossing the blood-brain barrier (BBB), which is considered to be an important barrier for the treatment of medulloblastoma [14].

Drug repurposing

Drug repurposing (DR) constitutes an effective procedure for the drug discovery and has attracted the interest from universities and pharmaceutical companies. It is also called as "drug recycling or drug repositioning". Drug repositioning constitutes a promising process in order to overcome the obstacles in the development and discovery of novel drugs through the identification of new thera-

peutic applications. It is supported that candidates of this approach have established designing methods, pharmacokinetic properties, clinical trial data [50] and phase IV safety information [51] which constitute an advantage to develop drug with less chance to fail [52]. Approximately more than forty drugs have been repurposed for novel therapeutic uses [50,53-55]. Many stories are successful about repositioned drugs until today. More specifically, two well-known drugs were developed by the use of drug repurposing method: sildenafil and thalidomide. Sildenafil (Viagra) was the first drug of this approach. Sildenafil had an inhibition of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 (PDE5) and it was firstly discovered by the pharmaceutical company Pfizer to treat the coronary artery disease, but it failed in phase II clinical trials [28]. After this failure, sildenafil was directed to treat erectile abnormalities because of its effects in penile erections [29,52]. In 1998, sildenafil was marketed after the approval from the Food and Drug Administration. In contrast, in 1957, thalidomide was discovered by a German pharmaceutical company. This drug was firstly discovered in order to treat the morning sickness, but it was discovered to cause severe side effects in pregnant women. Approximately ten thousands children in different countries were born with body abnormalities by the use of this drug causing deaths a few months after birth. It was obvious its immediate withdrawal from the market [56]. Years after this failure, it was finally discovered thalidomide's anticancer activity for the angiogenesis inhibition in animal models [57]. As a consequence, thalidomide was considered to therapeutically affect the diseases of prostate cancer and myeloma [58,59]. In 2006, it was approved by the FDA for the treatment of myeloma. Based on studies, numerous drugs have been proven efficient for the treatment of different disease than aimed at first indications, like minoxidil, everolimus, thalidomide, nelfinavir [29,52,60,61].

Aim and Objectives

In this research project, eight anti-allergy compounds, supplied by the University of Central Lancashire (UCLAN), were tested for their anti-cancer activity against medulloblastoma ONS76 cell line. In order to be addressed this aim, the following objective was used: The screening of each anti-allergy drug in the medulloblastoma ONS76 cell line with the use of MTT assay after 72 hours of treatment.

Methods

Cell culture of ONS76 cell line

For culturing of ONS76 cell line, the cells were grown *in vitro*. In order to be prevented the culture's death, the cell line was sub-cultured by the removal of media and by the cells' transformation from a previous culture into fresh growth media. This process was

efficient in order to propagate the cell line. After it, the cells had to be brought into suspension, and they were released from the flask with the use of trypsin.

More specifically, the cultures were examined with the use of an inverted microscope (Nikon, Eclipse TS100) in order to be observed approximately more than 80% confluency and confirmed the lack of any contaminants. Firstly, the work surface inside the cabinet was sanitized with 1% virkon (Fisher Scientific, UK) and 70% ethanol (Fisher Scientific, UK). Also, the materials, which were put in the cell culture hood, were sterilized by spraying them with 70% ethanol (Fisher Scientific, UK). With the use of 10 ml pipette, the cell culture media was discarded from the cells' vessel and then it was put into a waste pot. Furthermore, 5 ml of pre-warmed sterile PBS (phosphate buffered saline) (Fisher Scientific, UK) were added to the flask by rocking it backward and forward. Then, it was poured to the waste beaker. This washing process of the cell monolayer was repeated twice in order to be removed any remaining media containing serum. Next, 1.5 ml of the pre-warmed dissociation reagent, trypsin-EDTA (Sigma-Aldrich, UK), was pipetted into the flask, ensuring its complete cell layer coverage. Moreover, the flask was then prepared for 3 - 5 minutes incubation to be ensured the cells' separation from the flask's wall. After 3 minutes of incubation, the flask was gently tapped to be released any remaining attached cells, following cells' observation for detachment with the use of microscope. 8 ml of RPMI 1640 culture media (Labtech, UK), mixed with 10% Fetal Bovine Serum (FBS) (Labtech, UK) and 1% Penicillin-streptomycin (PS) (Labtech, UK) were added to the culture vessel to be diluted and inactivated the trypsin. The cells were removed to a 15 ml tube with the use of 10 ml pipette. The tube was centrifuged at 1200 rpm (Fisher Scientific, Accuspin™ 400) for approximately five minutes to be obtained a cell pellet. After centrifugation, cell culture media was carefully transferred from the tube and discarded to the waste container. Also, the amount of 5 ml of media was added to the tube for the resuspension of the cells with the use of 10 ml pipette. New culture flask was labelled with the name, date, passage number (the number of times the cell line had been sub-cultured) and split dilution. 1 ml of cells was added to the new flask containing 10 ml of media. To conclude, the flask was incubated at 37°C in 5% CO₂ humidified environment.

Cell quantification using trypan blue

The dye exclusion test (trypan blue) was utilized to be determined the number of viable cells in a cell suspension. The cell suspension was usually mixed with dye and visually investigated in order to be determined the dye absorption or exclusion from the cells. Also, optimum growth was maintained by the use of a consistent number of cells, and results with better reproducibility were given by the cell counting.

More specifically, for the procedure of quantification, the cells had to be in suspension using trypsin/EDTA, as previously described. The cell suspension had to be well mixed by flask's gentle agitation containing the cells. The amount of 20 µl of cell suspension was added in an eppendorf tube. Then, 20 µl of Trypan blue (Sigma-Aldrich, UK) was pipetted in the tube in order to be prepared a 1:1 dilution of the cell suspension in Trypan blue (dilution factor = 2). The sample was well pipetted up and down several times. In addition, the haemocytometer and glass cover slip were sprayed with 70% ethanol (Fisher Scientific, UK). The coverslip was humidified with exhaled breath, and it was carefully put into the counting chamber using light pressure. After it, 20 µl of the mixture were transferred from the tube under the coverslip. Furthermore, the grid on haemocytometer (Figure 3) was noticed under the microscope with the use 100x magnifications.

Figure 3: Counting chamber grid. There were four main grids (A, B, C, and D) within the overall grid, which were each made up of sixteen smaller squares. The cells were separately counted in each section [54].

Furthermore, the cells were counted in one square at a time, without counting the same cell twice. The way that the cells were counted on grid is indicated in the above figure (Figure 4). After the procedure of counting, the coverslip and haemocytometer were immediately sprayed with 70% ethanol (Fisher Scientific, UK), and the tube was appropriately discarded in the biohazard waste.

In addition, the average number of cells was counted from all sections based on the following equation:

$$\text{Average cell count} = \frac{\text{cell count A} + \text{cell count B} + \text{cell count C} + \text{cell count D}}{4} \quad (1)$$

The previous equation (1) was multiplied by the dilution factor (x2). The cells were then multiplied by 10⁴ in order to be obtained

Figure 4: The counting system. In each section, it was usually included the cells that were on the top and left side of the grid (ticked), while excluded those on the bottom and right side (crossed) [54].

the cell concentration (cells/ml) as indicated in the below equation (2):

$$(1) \times 2 = \text{total number of cells} \times 10^4 \text{ cells/ml} \quad (2)$$

Finally, the amount of media that it was needed to be added in the flask was determined using the below equation (3):

$$\text{volume of media (ml)} = \frac{25000}{(2)} \text{ cells/ml} \quad (3)$$

Preparation for MTT assay in 96 well plates

The cells had to be in suspension for their evaluation in the MTT assay. Following the process of cell quantification, the cell suspension and cell culture media were prepared with density of 25,000 cells/ml. More specifically, the amount of 100 μ l of cells' mixture was added in a 96 well plate (1000 - 25000 cells/well) with the use of a multi-channel pipette. Then, the 96 well plate was incubated overnight at 37°C prior to drug addition.

Preparation of drug dilution

The eight testing compounds were provided by the chemists at the University of Central Lancashire (UCLAN). For the preparation of drug dilution, each solid compound had to be pre-weighted and solubilized in dimethyl sulfoxide (DMSO) (Fisher Scientific, UK). The solubilisation procedure was performed by vortexing in order to be ensured the complete dissolution of each drug.

More specifically, on the lab bench, each solid compound was weighted approximately 5 mg into a bijoux and added in an eppendorf tube. Then, the amount of 1.3ml of dimethyl sulfoxide (DMSO)

(Fisher Scientific, UK) was added in each compound for the preparation of drug stock solution. The mixture was vortexed until dissolved. Once dissolved, the sterile cabinet was sanitized with 1% virkon (Fisher Scientific, UK) and 70% ethanol (Fisher Scientific, UK). Into the working surface of cabinet, each drug stock dilution was filtered with the use of 0.22 μ m sterile filter in a separate sterile eppendorf tube, using a sterile 10 ml syringe. Each drug was then labeled with compound name, date, molecular weight, and DMSO stock concentration (Table 1) and stored at room temperature for next use.

Drug serial dilution

Generally, this specific method was used for the reduction of a dense culture to a more usable concentration of cells. More specifically, the cell culture hood was sanitized with 1% virkon (Fisher Scientific, UK) and 70% ethanol (Fisher Scientific, UK). The materials, which were put in the cell culture hood, were sterilized with 70% ethanol (Fisher Scientific, UK). A sufficient amount of pre-warmed media was discarded into a reservoir. With the use of a multi-channel pipette, 150 μ l media were added to a 96 well plate (Figure 5).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 5: Schematic representation of a 96 well plate displaying the wells to which 150 μ l of diluent were added.

Furthermore, the stock solutions of the testing anti-allergy drugs were prepared by adding 20 μ l of each compound and 980 μ l of media in an eppendorf tube. Then, 300 μ l of this mixture were added in duplicates in column 1 of a 96 well plate (Figure 6).

For the preparation of drugs' serial dilution, 150 μ l of the mixture were removed from the first column and added to the second

Compound Name	Compound Structure	Molecular Weight	DMSO drug solution (mM)
CL1-40-1 1-(2,3-dihydro-1 <i>H</i> -inden-4-yl)-3-(3,4,5-trimethoxyphenyl)urea		342.39	11.2
CL1-42-1 1-(1-methyl-1 <i>H</i> -indol-5-yl)-3-(3,4,5-trimethoxyphenyl)urea		343.38	11.2
CL1-45-1 1-(4-hydroxynaphthalen-1-yl)-3-(3,4,5-trimethoxyphenyl)urea		369.38	10.4
CL1-47-1 1-(quinolin-5-yl)-3-(3,4,5-trimethoxyphenyl)urea		353.37	10.9
CL1-49-1 1-(6-hydroxynaphthalen-1-yl)-3-(3,4,5-trimethoxyphenyl)urea		368.38	10.4
CL1-52-1 1-(1 <i>H</i> -indol-7-yl)-3-(3,4,5-trimethoxyphenyl)urea		341.36	11.3
CL1-56-1 1-(anthracen-9-yl)-3-(3,4,5-trimethoxyphenyl)urea		402.44	9.6
CL1-57-1 1-(anthracen-1-yl)-3-(3,4,5-trimethoxyphenyl)urea		402.44	9.6

Table 1: The testing compounds with their chemical structures, molecular weight, and dimethyl sulfoxide drug solution. Each compound had different drug stock concentrations, which were estimated with their molecular weight and weighted mass.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 6: Schematic representation of a 96 well plate in which 300µl of drug stock solution of each testing compound were added in two replicates in column 1. For instance, drug 1 was shown with red, drug 2 was shown with green, drug 3 was shown with blue, and drug 4 was shown with purple.

column by mixing it several times with the use of a multi-channel pipette. This procedure was repeated until the eleventh column (Figure 7) and the 150 µl mixture was then discarded in a waste beaker.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 7: Schematic representation of a 96 well plate displaying the serial dilution of the testing drugs.

Moreover, 10 µl of the positive control solution, vincristine, were mixed with 490µl of cell culture media in an eppendorf tube. 100 µl of this mixture were added in the wells of column 12 (A12-D12). The previously prepared cell plate was removed from the

incubator. With the use of a multi-channel pipette, 100 µl from the drugs' 96 well plate were poured to the relevant wells in the ONS76 cell plate. The tips were changed for the removal of column 12. After that, the plate was put in the incubator.

Making MTT stock solution

The amount of 0.15g of Thiazolyl blue (MTT powder) was weighted into a bijou and added in an Eppendorf tube. The MTT was then dissolved in 5 ml phosphate buffered saline (PBS) (Fisher Scientific, UK). The tube was vortexed until completely dissolved. After this, the hood was sanitized with 1% virkon (Fisher Scientific, UK) and 70% ethanol (Fisher Scientific, UK). Into the hood, the MTT solution was filtered and sterilized with the use of a 0.2 µM syringe. Finally, the MTT solution was added in small tubes and stored at the fridge (-20°C) for storage until the next use.

MTT assay

The MTT procedure was actually a test for the cells' cytotoxicity. It was defined as a cell death assay, as it was a reduction step of the MTT dye to formazan (purple color in the wells). The purple coloration was the determination of the cells alive.

More specifically, the working area into the cell culture hood was sanitized with 1% virkon (Fisher Scientific, UK) and 70% ethanol (Fisher Scientific, UK). A MTT reagent tube was defrosted from the freezer. It was then put in the cell culture hood after spraying it with 70% ethanol (Fisher Scientific, UK). The MTT was discarded in a reservoir. With the use of a multi-channel pipette, 50 µl of MTT solution were poured in a 96 well plate. It was then put into the incubator for three hours at 37°C in a 5% CO₂ environment. After the time of incubation, it was observed purple formazan crystals in the 96 well plate. In addition, the culture media was removed from the wells by tipping out with the use of a multi-channel pipette, and it was discarded in a biohazardous container. The purple crystals must not be disturbed from the bottom of each well. Moreover, a sufficient amount of dimethyl sulfoxide (DMSO) (Fisher Scientific, UK) was added into the reservoir. 200 µl of it were added to each well by the use of multi-channel pipette so as to be solubilized the crystals from the formation of the reduction of MTT. Finally, after the addition of the DMSO, the plate was read at the absorbance of 540nm with subtraction at 690nm using Omega fluostar program on the computer. The normalization of the data, extracted from this program, was calculated as follows:

$$\frac{\text{compound/drug}}{\text{positive control (vincristine)}} \times 100 \quad (4)$$

The results of average cell viability (%) with the drug concentrations ranging from 0.01 μM to 10 μM were extracted using the Graph Pad Prism7 software and indicated in the next section.

Results

In the present research project, a collection of eight anti-allergy compounds, designed by the chemists at the University of Central Lancashire (UCLAN), were evaluated against ONS76 human cancer cell line and screened for their anti-cancer activity against medulloblastoma (MB). The results of testing anti-allergy drugs were estimated with the use of MTT assay after three days of incubation. In this section, the results were determined by the average cell viability of three biological replicates of each testing anti-allergy compounds in different drug concentrations. Schematic representation of the first two drugs is presented below (Figure 8). The concentrations of the drugs are ranged from 0.01 to 10 μM . The CL1-40-1 compound has more significant effect on cells' viability, decreasing from 101% to 67% after treatment, in comparison with the CL1-42-1 compound. The cells have not been affected by the CL1-42-1 drug. In addition, in the next graph (Figure 9), two anti-allergy compounds have different anti-proliferative effect on ONS76 cells, compared to the compounds in the previous graphs (Figure 8). The CL1-45-1 drug has a stable decrease between the drug concentrations of 0.313 μM and 10 μM . It is also observed a significant reduction ranging from 90% to 25% after treatment. In contrast, the CL1-47-1 compound has an increase up to 100% from 0.039 μM to 2.5 μM .

Figure 8 and 9: Average cell viability of the four following compounds: CL1-40-1, CL1-42-1, CL1-45-1, and CL1-47-1 with concentrations ranging from 0.01 to 10 μM , determined by the MTT assay after 72 hours of incubation. The data represents the mean values and the standard deviation of three independent replicates of medulloblastoma ONS76 cell line.

In figure 10, the cell viability is not significantly affected by both compounds, CL1-49-1 and CL1-52-1. As it is observed, the mean values are ranging from 92% to 101%. The anti-proliferative activity of the CL1-56-1 and CL1-57-1 compounds against medulloblastoma cell line (ONS76) is observed below (Figure 11). These drugs have significantly inhibited the medulloblastoma cell growth. Based on the figure 11, CL1-56-1 and CL1-57-1 have a stable decrease between 0.313 μM and 5 μM , ranging from 73 - 86% to 53%.

According to the previous figures (Figure 8-11), only the CL1-45-1 compound seems to have a significant effect after the treatment. So, among the eight testing compounds, the CL1-45-1 drug has the maximum growth inhibition in the medulloblastoma cells. Also, two of the drugs, CL1-42-1 and CL1-49-1 have the lowest effective dose in the cell growth.

Finally, the values of the half maximal inhibitory concentration (IC_{50}) are indicated in table 2. It is evident that the CL1-42-1 com-

Figure 10: Average cell viability of the two following compounds: CL1-49-1 and CL1-52-1 with concentrations ranging from 0.01 to 10 µM, determined by the MTT assay after 72 hours of incubation. The data represents the mean values and the standard deviation of three independent replicates of medulloblastoma ONS76 cell line.

Figure 11: Average cell viability of the two following compounds: CL1-56-1 and CL1-57-1 with concentrations ranging from 0.01 to 10 µM, determined by the MTT assay after 72 hours of incubation. The data represents the mean values and the standard deviation of three independent replicates of medulloblastoma ONS76 cell line.

pound has displayed the greatest anti-cancer activity against the ONS76 cells with $IC_{50} = >10 \mu M$ ($IC_{50} = 0 \mu M$). Moreover, two of the eight compounds, CL1-40-1 and CL1-45-1, have the lowest inhibitory activity against the medulloblastoma cells after three days of treatment. However, all of the eight compounds have displayed inhibitory activity: some of them are more active and the others are less active against the cancer cells.

Testing compounds	IC ₅₀ (µM)
CL1-40-1	1.445
CL1-42-1	>10
CL1-45-1	1.355
CL1-47-1	0.1902
CL1-49-1	0.07651
CL1-52-1	0.7791
CL1-56-1	0.2418
CL1-57-1	0.3304

Table 2: Inhibitory activity of the testing anti-allergy compounds after three days of incubation of MTT assay.

Discussion and Conclusion

Medulloblastoma (MB) constitutes the commonest malignant childhood brain tumor and is a leading cause of death worldwide [9]. Medulloblastoma is an aggressive embryonic type of tumour

and it is presented in the cerebellum of the posterior fossa [13]. Invasive treatment approaches have been used in order to significantly enhance the patients' clinical result. Most of the patients usually suffer from long-term side effects [14]. Thus, it is important to develop new therapeutic approaches with less toxicity and increased efficacy. In this research project, eight anti-allergy drugs were tested for their anti-cancer activity against medulloblastoma ONS76 cell line with the use of MTT assay after three days of incubation.

Specifying the *in vitro* activities of the testing anti-allergy compounds against medulloblastoma ONS76 cell line with the use of MTT assay was important to evaluate the effect on cancer proliferation. The outcomes were estimated with the calculations of the IC_{50} values, which are the concentrations of each anti-allergy drug demanded to manage the half inhibition of cancer cell growth. MTT assay constitutes a cytotoxicity testing, which decreases MTT tetrazolium salts from the compounds by mitochondrial dehydrogenases, to a colorful formazan production [48]. It leads to a measurable product that is equal with the number of viable cells, enabling the anti-cancer growth of the eight anti-allergy drugs to be evaluated. Interpretation of the MTT assay results determined three of the eight anti-allergy compounds to be the most active and have significantly inhibited the cancer cell growth. More specifically, the CL1-42-1 compound presented the greatest inhibitory activity against the medulloblastoma ONS76 cell line with IC_{50} value less than 10 μM ($IC_{50} = 0 \mu M$). In addition, two of the anti-allergy compounds, CL1-40-1 and CL1-45-1, have not significantly inhibited on the anti-cancer growth of ONS76 cells with IC_{50} values 1.445 μM and 1.355 μM , respectively. Furthermore, it is important to note that these anti-allergy compounds were designed and tested for the first time, without knowing other approaches about the reliability of the results and if they can be considered as potential anti-cancer agents.

Potential Limitations

Numerous efforts have been made in order to reduce the drugs' effects during the procedure of cancer treatment, such as the prevention of effects on the nearby tissues and cells, the efficacy in the lesion, and the increase of drug accumulation, developing with this way new drug delivery and targeting systems [62]. In addition, there are so many experimental steps for the cancer's therapy. These methods are as follows: radiation, chemotherapy, surgery, and immunotherapy. These methods are often accompanied by severe side effects. Limited biodiversity, toxicity, restriction in metastasis constitute some of the side effects [27]. Furthermore, chemotherapeutic agents include cytotoxic drugs which perform promising outcomes for the treatment of cancer. These agents

involve topoisomerase inhibitors. For instance, vincristine and docetaxel constitute microtubules acting agents, while cisplatin and carboplatin are alkylating agents [35]. In the present study, eight drugs were investigated for their anti-cancer activity against medulloblastoma ONS76 cell line with the use of MTT assay. There were several limitations in this approach. As it is known, cancer cells can multiply rapidly under normal physiological conditions. The anticancer drugs in this report targeted these cancer cells. Another limitation was the testing of only eight of these anti-allergy compounds. It is unclear whether more than eight of these drugs would be efficiently able to inhibit the cells' growth by leading to medulloblastoma treatment. Finally, based on research findings, anti-allergy drugs possess a promising option for the better and less toxic cancer treatment with proper anti-cancer agents [30].

Future Prospects

In the present research project, the anti-allergy compounds, which perform promising anti-cancer activity, should be presented into further examination in order to discover their possibility of considering them as anti-cancer agents. This additional testing could include the screening of the most active anti-allergy drugs in different molecular subgroups of medulloblastoma. It would be efficient in Group 3 tumors. This medulloblastoma subgroup is considered to be the most aggressive and highly metastatic, approximately more than 40%, in comparison with the other medulloblastoma subgroups (Group 4 = 35%, SHH = 15%, Wnt = 10%) [14]. In addition, it would be beneficial to screen these anti-allergy compounds in anti-cancer cell lines and not in human cancer cell lines, such as ONS76 and DAOY. This approach could be valuable in order to determine if the compounds have cancer cell specificity, which constitutes an important characteristic for efficient anti-cancer treatments, resulting in fewer side effects [52]. Furthermore, as it has been described in a previous section, the cells were grown *in vitro* after treatment with the use of MTT assay. An alternative option for the anti-cancer activity of these compounds could be the screening *in vivo* in order to observe if this type of drugs has the capacity to cross the blood brain barrier of medulloblastoma patients. This is an important barrier for the treatment of medulloblastoma. To investigate it, pharmacokinetic assays should be presented after the intravenous injection of these anti-allergy drugs into mouse models bringing medulloblastoma xenografts.

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