RESEARCH PAPER

THE EXPRESSION OF SLAMF7 LEVELS IN MALIGNANT B CELLS: A NOVEL THERAPEUTIC PATHWAY FOR PATIENTS WITH CLL

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ABSTRACT
Signalling lymphocyte activation molecule (SLAM) F7 is found on the surface of some immune cells including B-lymphocytes. Its activation leads to the proliferation or differentiation of immune cells. The objectives of the study were to measure SLAMF7 expression levels on B-CLL cells, and to upregulate the expression of SLAMF7 with phorbol myristate acetate (PMA) and Bryostatin. The levels of expression of SLAMF7 receptors of B-CLL cells from patients were measured; using immunofluorescence, flow cytometry, confocal microscopy and reverse transcriptase polymerase chain reaction (RT-PCR). The effects of treatments with PMA and Bryostatin were determined from different patients. Different levels of SLAMF7 expression were found to be associated with B-CLL cells from different patients. PMA treatment of B-CLL cells showed more positive SLAMF7 staining with the majority of the extracted B-CLL cell cases, while less positive results were associated with Bryostatin treatment. The study has shown that both PMA and Bryostatin could upregulate SLAMF7. Successful modulation of SLAMF7 expression may provide a novel target for the treatment of patients with CLL.

Keywords: *SLAMF7, RT PCR, Elotuzumab, Bryostatin, PMA*

INTRODUCTION

Signalling lymphocyte activation molecule (SLAM) F7, is among the members of SLAM family of receptors found on the surface of some specific immune cells that regulate several functions of different immune cell types (Murphy *et al., 2002;* Bouchon *et al., 2001;* Veillett *and* Latour, *2003;* Lee *et al., 2007;* Stark and Watzl*, 2006*). According to the Hugo Gene Nomenclature (*2012)*, SLAMF7 contains one immunoglobulin-like C2-type domain. Expression of SLAMF7 can be found in lymph nodes, bone marrow, stomach, trachea, spleen, lungs, appendix, small intestine and peripheral leucocytes (Calpe *et al., 2008*).

Activation of SLAMF7 receptor leads to stimulation of the signal transduction pathway,

which results in the proliferation or differentiation of the immune cell (Cambier *et al*., 1994; Jiang *et al.,* 2005). Currently, a monoclonal antibody, Elotuzumab used in the treatment of multiple myeloma is known to target SLAMF7 (Richardson *et. al*., 2011) and has been successfully targeted with Elotuzumab, a novel drug for the treatment of CLL (Murphy *et al.,* 2002).

Protein kinase C (PKC) activators like phorbol esters and Bryostatins affect the signal transduction pathway, which has enormous effect on a variety of biological responses, which include proliferation and differentiation (Cambier and Ransom*,* 1987*;* King*,* 1988*;* Isakov *et al.,* 1986*;* Muhie *et al.,* 2013). Bryostatins are antipromoters of tumour cells (Hennings *et al*., 1990); hence they are more significant in clinical trials, than phorbol esters like phorbol myristate acetate (PMA), which are carcinogenic (Nishimoto *et al.,* 2013*;* Hennings *et al.,* 1987). The optimal concentration for Bryostatin modulation of SLAMF7 is suggested to be 10nM (Drexler *et al*., 1989), which has been corroborated by Murphy *et al*. (2002). This study aims to upregulate the expression of SLAMF7 receptors with protein kinase C (PKC) activators (Bryostatin and PMA). Successful modulation of SLAMF7 expression may provide a novel target for the treatment of patients with chronic lymphocytic leukaemia.

MATERIALS AND METHODS
Sample population
Between July 2013 and August 2013, consecutive samples of CLL patients were selected for the study. Blood samples were collected from the CLL patients attending the oncology clinic (Department of Haematology, UCL Cancer Research Institute) at the University College of London Hospital, London, UK. They were analysed at the University of Westminster Immunology Laboratory. The samples collected were from twenty-two patients with the malignancy who were from different ethnic backgrounds. Twelve (12) of them who had significant amount of white cell counts ($5x10^5$ cells/ml) were subsequently enrolled into the study.

Stored frozen multiple myeloma cells and some B-CLL cells were obtained from University of Westminster (extracted and stored from previous studies).

LABORATORY METHODS
Whole blood samples collected by venepuncture were processed immediately by extracting the white blood cells (WBC). 8 mls of blood sample was added to equal volume of RPMI 1640 medium with L-glutamine (Sigma-Aldrich Company Ltd, UK) and mixed. 15mls of histopaque reagent (Sigma-Aldrich Company Ltd, UK) was added to 10mls of the blood: RPMI mixture previously constituted. The addition was done carefully drop by drop unto the surface of the histopaque, taking care that it settled on the top of the histopaque. The new mixture was centrifuged at 2500 rpm at 22^oC for 30 minutes. This was to allow all other cells to go through the semi-solidified histopaque, while leaving the white cells on top. Approximately 3mls of B-CLL cells suspended on the surface of the histopaque was carefully transferred into a universal tube, and RPMI 1640 medium was added. Further centrifugation at 1500 rpm at 4^oC and for 5 minutes was done to obtain a pure sample with minimal contamination from other cells. The supernatant was decanted and white cell pellets (B-CLL cells) were resuspended in the remaining medium and counted in the Neubauer chamber. Subsequently RNeasy Minikit (Qiagen, UK) was used to isolate 1μg of its ribonucleic acid (RNA) following the manufacturer's protocol.

Samples that could not be processed immediately were stored in liquid nitrogen. A minimum of $5 x 10^5$ cells/ml was required for selection and running in each experiment.

Cell culture testing
A concentration of $0.5x10^6$ B-CLL cells was cultured using RPMI-1640 as medium in a six-well culture plate (Thermo Fisher Scientific, Sigma-Aldrich). Duplicates of test (wells with PMA or Bryostatin treatment) and control (wells without PMA or Bryostatin treatment)

were prepared following standard protocol, as described by Murphy *et al.* (2002). Cells of test samples were stimulated with a 30 nM concentration of PMA (Sigma, Poole, Dorset), or a 10 nM Bryostatin (Sigma, Poole, Dorset). Additionally, a dose-response testing, using 0 nM, 10 nM, 20nM and 40 nM of Bryostatin was done, followed by 24 hours incubation at 37^0C with antibiotics (L-glutamine and penicillin/streptomycin) and 10% v/v foetal bovine serum (Sigma-Aldrich, UK). The result showed successful proliferation and differentiation of the B-CLL cells after treatment with Bryostatin. The dose-response study also showed that the optimal dose for proliferation and differentiation of the B-CLL cells using Bryostatin was 10 nM.

Immunofluorescence testing
Cells obtained from the culture study were resuspended, washed twice with phosphate buffer saline and 0.1% bovine serum antibody, (staining solution mixture) and transferred into a 5ml falcon tube following the protocol by Murphy et al. (2002). 5 µl of Anti-SLAMF7 purified mouse monoclonal IgG2a (R&D systems, Minneapolis, MN) was added to the suspended pellets. The Anti-SLAMF7 purified mouse monoclonal IgG2a then binds with SLAMF7 receptors (antigen) forming antigen-antibody complex on the B-CLL cells, if present, and served as positive control, while Ig-GA$_2$ which does not contain Anti-SLAMF7 antibody was added in a second falcon tube as negative control, before incubating at 4^oC on ice. After washing with the staining solution mixture the cells were stained with Fluorescein Isothiocyanate (FITC) labelled polyclonal goat anti mouse IgG (Dako Company, Denmark).

Flow cytometry assay
The cells obtained from the immunofluorescence testing were subjected to flow cytometry testing to determine the level of SLAMF7 receptors on the B-CLL cells following standard protocol provided by Dako Company. The results were recorded as mean fluorescence intensity (MFI) and percentage positive of SLAMF7 receptors on the cell surface. Control samples (without Anti-SLAMF7 treatment) showed low expression levels while positive test samples (with Anti-SLAMF7 IgG2a) showed positive results. Flow cytometry was also used to determine the optimal dose response using Bryostatin treatment.

Confocal microscopy
The levels of expression/fluorescence of the SLAMF7 receptors were determined on the B-CLL cell prior to the treatment and after treatment of the cells. This was visualised using a confocal microscope to detect the dye fluorescence on the cell surfaces. The observation was done in the dark room (to avoid sunlight interacting with the dye) using the Leica TCS SP2 confocal microscopy system (Leica Microsystems, Milton Kynes, and UK).

Reverse transcriptase polymerase chain reaction (RT-PCR)
This was performed to identify the presence and level of expressing mRNA of SLAMF7 receptors on B-CLL cells before and after upregulation. This involved RNA extraction using RNeasy Minikit (Qiagen Sample and Assay Technologies, UK), in accordance with the manufacturer's protocol. The pure sample of the white cells was harvested by adding buffer RLT (350 µL) from the Qiagen kit. Samples were then passed through several washes with Qiagen reagent buffers, which were placed in a RNeasy spin column and were centrifuged at $\geq$ 8000 x g for 15s. The resulting total RNA was extracted into approximately 50 µL of RNase free water and measured spectrophotometrically using the Nanodrop automated machine (Thermo Scientific, USA) (at an absorbance of 260nm and 280nm) to determine the right molecular weight for RT-PCR. The optimal RNA ratio read by the Nanodrop Spectrophometer for the samples was 1:1.9 - 2.1. This indicates the purity of the sample being analysed, because a lower ratio than 1.9 suggest the presence of contaminants, such as proteins that absorb UV light. An amount of RNA (1µg) was measured and was reverse-transcribed to produce cDNA. Random primers and oligo-dT,

using the GO TAG SYBR green PCR kit (Promega Corporation, UK) with Biometra PCR machine (Thistle Scientific, UK). The forward primer 5'-GCCAAT GAGTCC CATAAT-3' and the reverse primer 5'-GTATTT GCTGGA TCTTCC-3' (Life Technologies Invitrogen Corporation, UK) were used to amplify the cDNA material. A 2% agarose gel electrophoresis was run, using TRIS/Borate/EDTA (TBE) at 100 volts for 40 minutes. The molecular weight marker of 100bp was used (Promega Corporation, Madison, USA) as a control ladder for the amplified cDNA product. Ethidium bromide was used in staining the DNA to enable the products to be visualised using an ultra violet (UV) Trans Illuminator Imaging System (UVItec Limited, Cambridge, UK).

Ethical Approval
Ethical clearance was obtained from two bodies; the Committee on Human Research Publication and Ethics, School of Life Sciences, University of Westminster and Department of Cancer Studies, UCL Hospital (REC reference number, 09/H07146).

RESULTS
Establishing the optimal dose response of

Bryostatin using cell culture and flow cytometry studies
Initial studies undertaken to establish the optimal concentration of Bryostatin for upregulation of SLAMF7 showed that Bryostatin treatment was effective at an optimal concentration of 10 nM. (Table 1).

The effect of Bryostatin on the receptors of SLAMF7 modulation on B-CLL cells showed that 10 nM produced the highest peak of SLAMF7 expression, but the expression declined after the concentrations were subsequently increased to 20 nM and 40 nM. This trend was observed with all cases of B-CLL patients selected for dose-response testing.

Cell culture and morphological features of B-CLL cells
It was observed that culture plates treated with PMA had the most cells aggregating or forming clusters that led to clumps in certain areas on the plates (Fig 1). Untreated medium had fairly evenly distributed cells on the plate with little evidence of clumping (Fig 1A).

Bryostatin treatment also showed some clump formation (Fig 1C), even though they were in fewer areas, compared to clumping occurring

Table 1: Bryostatin dose response values using flow cytometry

Conc.	JL %Pos	MFI	JH %Pos	MFI	FW %Pos	MFI
0 nM	0.94	5.52	6.94	3.56	13.37	4.47
10 nM	1.32*	5.54*	16.11*	5.31*	12.42*	4.04*
20 nM	0.78	5.19	15.91	5.29	11.81	3.62
40 nM	0.61	5.09	4.02	2.82	3.2	1.87

These were results obtained when the optimal concentration of bryostatin was determined. The B-CLL cells were from three patients; JL, JH and FW. The cells were used in the flow cytometer study, in which the cells under the influence of some stimulators, expressed SLAMF7 receptors, the presence of which was detected by fluorescence. FW could show a high level of expression even in the untreated state. This confirms the high level of expression of these receptors in untreated B-CLL cells. It also confirms the optimal concentration of Bryostatin at 10 nM. The B-CLL cells were from 3 patients, JL, JH and FW.

with PMA. It thus suggests that PMA treatment caused the most increase in induction of the SLAMF7 receptor and cell clumping (Fig. 1B).

The levels of expression of SLAMF7 receptors on B-CLL cells of patients
To determine the level of SLAMF7 receptors on B-CLL cells, the cells were exposed to varied conditions: untreated, PMA and with Bryostatin. The levels of expression of positive SLAMF7 were studied and the results indicated that different B-CLL cells expressed different levels. Two (2) out of the twelve (12) cases (17%) studied showed more than 40% positivity for SLAMF7 cells when untreated (Fig 2). More than 40% (5 out of the 12 cases) of B-CLL cells studied were positive (above 10% positivity) for SLAMF7, 83% (10 out of the 12) of these cases also showed expression level below 10% positivity.

Approximately 50% of all cases studied had two fold increases in the percentage positivity of SLAMF7 expression when the untreated cells were subsequently treated with PMA. However, cells from the 2 B-CLL cases, which had a high percentage positivity of SLAMF7 expression, did not show much increase when treated with PMA. One (1) case, representing 8% of the cases studied, showed a decrease in the level of expression after treatment with PMA. However, there was slight increase of SLAMF7 expression when the same case was subjected to Bryostatin treatment.

Similarly, 3 out of the 12 (25%) studied showed a decrease in modulation with Bryostatin while 9 out of 12 (75%) had elevated positive cells after modulation with Bryostatin. Whilst 75% (9/12) of cases showed greater increase in the level of SLAMF7 modulation with PMA than with Bryostatin, 17% (2/12) showed higher SLAMF7 expression levels with Bryostatin than with PMA with only 1/12 showing equal increase in SLAMF7 modulation with both PMA and Bryostatin drugs.

Expression of SLAMF7 receptors using confocal microscopy
To confirm and establish the results showing high level of expression associated with B-CLL as seen in our study, we further studied the
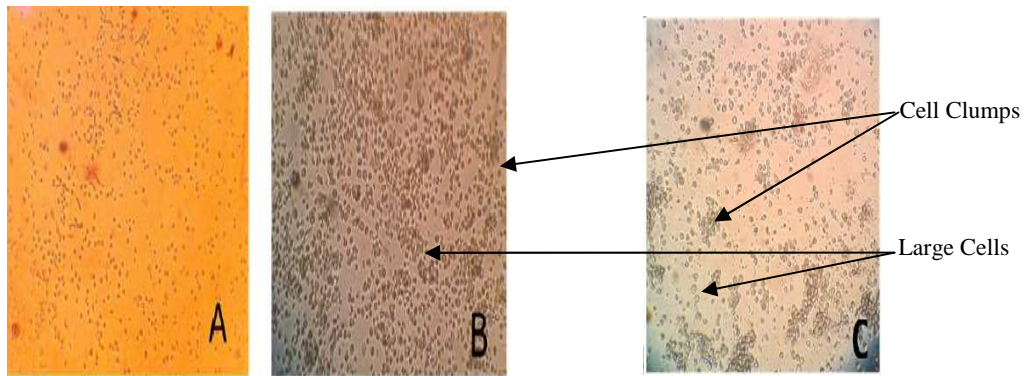

Fig. 1: Morphological changes of B-CLL cells after cell culturing when untreated and treated with PMA and bryostatin
Photomicrographs were taken with Wilovert inverted microscope fitted with Olympus camera after 24 hours of incubation. Photomicrograph A shows cells without treatment used as a negative control where cells do not adhere to each other or form clumps. Photomicrograph B shows high number of cells clustered together in clumps after treatment with PMA. Photomicrograph C show moderate to high number of cells clustered together with some mild forms of clump formations after treatment with Bryostatin
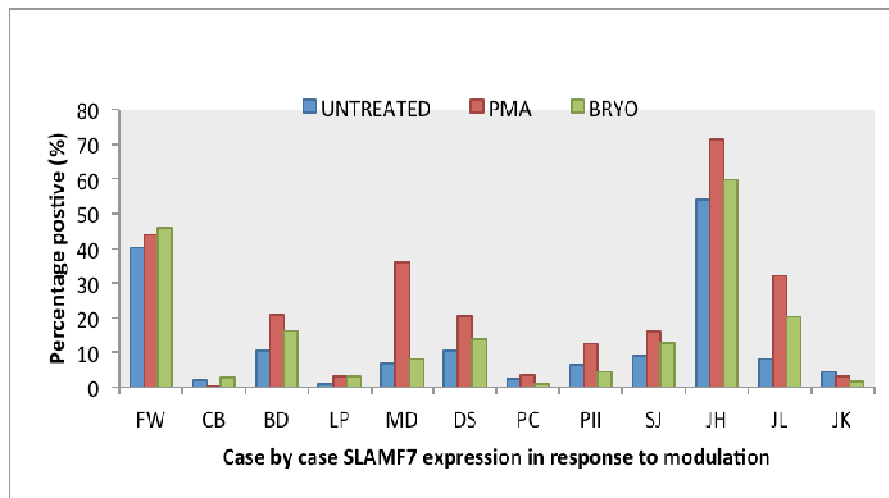
Fig. 2: SLAMF7 expressions in response to exposure to different conditions
B-CLL cells from different patients were exposed to different conditions (untreated, PMA, bryostatin), for the expression of SLAMF7 receptors, which were measured by fluorescence in the flow cytometer. Each peak in the graph, showing percentage SLAMF7 positivity, represents a response generated from the Dako flow cytometer. FW, CB, BD, LP, MD, DS, PC, PII, SJ, JH, JL and JK are codes of B-CLL patients whose white cells were used.

level of fluorescence on selected positive and negative cases recorded from the flow cytometry results. Results obtained confirmed the high level of SLAMF7 expression on some untreated B-CLL cells. Recorded results from the light microscopy (Fig 3A) and from the confocal microscope (Fig 3B and 3C) of case JH showed fluorescence on the surface of the immunofluorescence stained SLAMF7 cells (Fig. 3B) whereas the control staining (irrelevant antibody) was negative (Fig. 3C). However, there were no green patches of fluorescence seen on the negative case BD. This also gave a negative result from flow Cytometry. (Fig.3E).

Expression of SLAMF7 isoforms as measured by RT-PCR
To confirm the expression of two SLAMF7 isoforms in B-CLL cells, we conducted studies using RT-PCR amplification. Selected B-CLL cells without treatment were used and extracted RNA converted to cDNA. Using selected myeloma cells as controls, amplified cDNA product from the control, showed one strong band (396 bp) and a weak band (296 bp) (Fig 4), representing two isoforms associated with myeloma cells (OMLS and MMLS). The test results of B-CLL cells showed two less intense bands, confirming the presence of low levels of SLAMF7 isoforms in B-CLL cells.

DISCUSSION
The study observed that the optimal dose for upregulating B-CLL cells was 10 nM (table 1). This is in agreement with previous observation by Drexler *et al*. (1989) who reported the same concentration for Bryostatin for effective upregulation of SLAMF7 on B- CLL cells. Other studies have established similar concentration for phorbol esters in inducing increase in SLAMF7 receptors on the cell surface (Murphy *et al*., 2002).

B-CLL cells responded to treatment with PMA and Bryostatin by cellular proliferation and aggregation. This was evident as abnormal
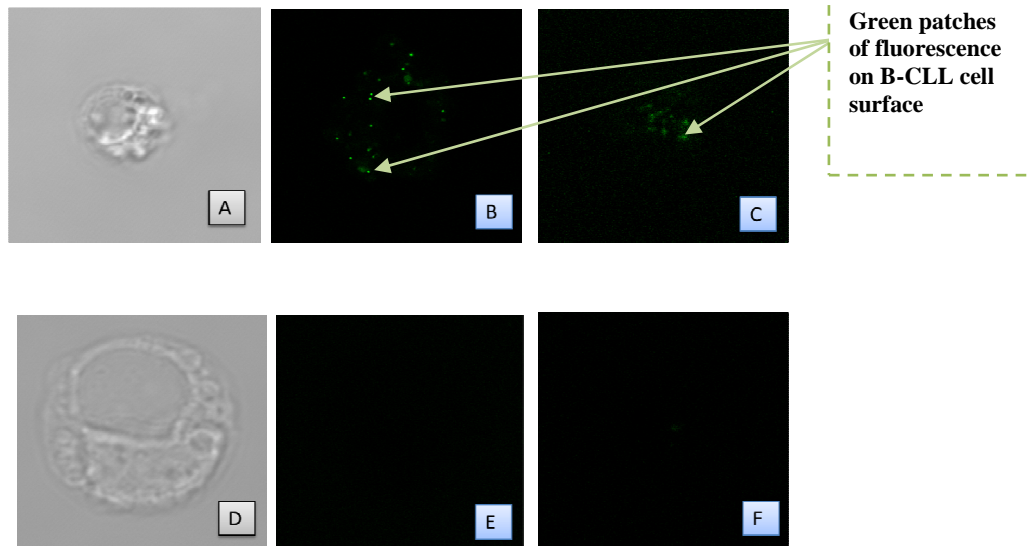
Green patches of fluorescence on B-CLL cell surface

Fig. 3: **Confocal microscopy showing positive and negative immunofluorescence for selected SLAMF7 B-CLL cell.**

Two patients samples measured for high and low expression levels of SLAMF7 with flow cytometry were selected for observation of immunofluorescence under the confocal microscope. Pictures A, B and C represent positive SLAMF7 B-CLL cell with FIT C-labelled dye. These fluoresced with green colour under a confocal microscope as observed in B and C pictures and as a white patch on a black and white background as seen with picture A. Pictures E and F were negative for SLAMF7 expression on the selected B-CLL cell with FIT C-labelled dye.
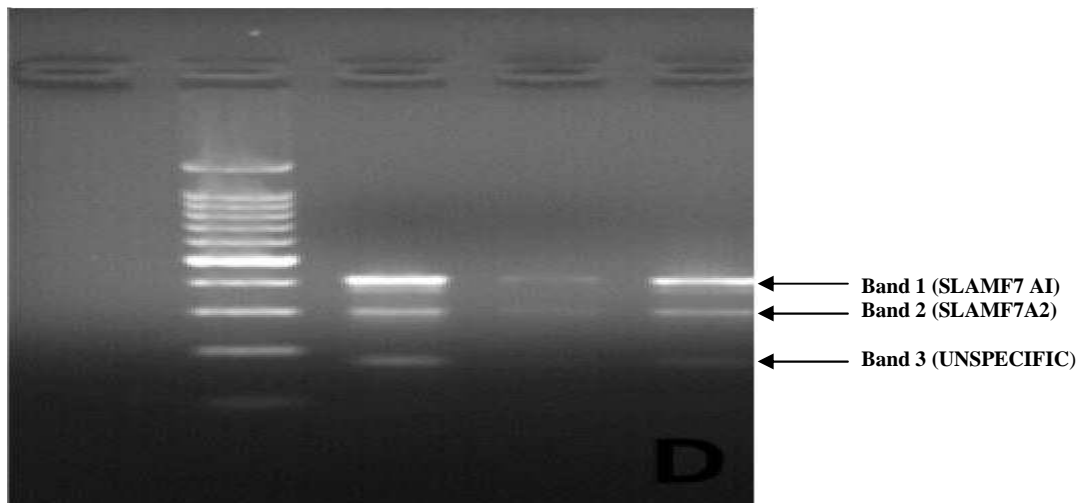
Band 1 (SLAMF7 AI)
Band 2 (SLAMF7A2)
Band 3 (UNSPECIFIC)

Fig. 4: Ethidium bromide-stained gels showing isoforms of SLAMF7 expression

Two multiple myeloma cell lines are shown which had positive expression of SLAMF7. The cDNA of SLAMF7 was run on agarose gel electrophoresis, using a 100kb molecular marker. Lanes 2 and 4 produced a strong and a weak band. OMLS and MM1S were the myeloma cells used as positive control; both show the two bands representing the isoforms of SLAMF7. The cell from patient CB also showed two bands of equal intensity

morphological characteristics, seen as clumps and clusters of enlarged B-CLL cells (Fig. 1). Some B-CLL cells however, did not show any any abnormal morphological characteristics. However, the lack of response of some of the other cells cannot be explained. The characteristic feature of clumped and enlarged cell is similar to observation by Springer *et al.*, (1987) and Springer (1990) who indicated that activation of lymphocytes is coupled with the increased formation of clumps. It was evident however, that PMA induced more cellular proliferation and aggregation, hence more clumping to each other B-CLL cells than Bryostatin (Figs. 1B and 1C).

SLAMF7 receptors have been found to play an important role in the activation of B-CLL cells, thus resulting in proliferation and/or differentiation of these cells (Cambier *et al., 1994;* Jiang *et al., 2005*). In our study, SLAMF7 receptors showed varying levels of expression on B-CLL cells using immunofluorescence, flow cytometry and confocal microscopy, which is in agreement with observations by Williams *et al. (2004) and* Williams *et al. (2013)*. This result is however, contrary to what had been previously suggested, that all B-CLL cells express low levels (below 10% positivity) of SLAMF7 or show complete absence of SLAMF7 expression (Hsi *et al., 2008*).

When B-CLL cells were treated with PMA *in vitro*, the cells were shown to portray varied characteristics, from the change in shape, size, extensions and cell numbers when stimulated by PKC activators (Drexler *et al., 1989*) in the signal transduction pathway. Our study showed 83 % (10 out of the 12 cases) responding positively to PMA treatment, compared with 75% (9 out of the 12 cases) of B-CLL cases that responded to Bryostatin treatment (Fig 2). This is in conformity with observation by Drexler *et al. (1989)*, and Garcia *et al.*(2006). More than 50% of cases however showed different levels of positivity for SLAMF7 expression before treatment with PMA or Bryostatin.

This study as shown in Fig 4, found two isoforms of SLAM receptors, namely SLAMF7 and SLAMF7A2, on the B-CLL cells which are associated with multiple myeloma cells. An observation by Murphy *et al.* (2002) suggests that SLAMF7 is an active form of the two isoforms, which is targeted by Elotuzumab in the current treatment of multiple myeloma. We therefore suggest that the SLAMF7 isoform identified in our study might be a possible target by Elotuzumab for the treatment of CLL patients. Our study found over 50% positivity of SLAMF7 expression in the B-CLL cases studied.

While extensive studies have been done with PMA, Bryostatins represent a newer discovery and provides the advantage of being anti-promoters (Ueno *et al.,* 2012). Differences in the extent and effect of the two PKC activators (PMA and Bryostatin) have been reported (Wender *et al.,* 1988; Kedei *et al.,* 2013a; Kedei *et al.,* 2013b). In contrast to PMA, Bryostatins do not have tumour promoting activity, but rather show anti-neoplastic features (Hennings *et al.*, 1987; Nakagawa *et al.*, 2009). In this study, 10 nM of bryostatin provided an effective concentration at which, activation of the B-CLL cells and SLAMF7 are optimal where 75% of all cases of B-CLL cells studied had increase in SLAMF7 receptors with bryostatin modulation (Drexler *et al.*, 1989). However, beyond this concentration, the SLAMF7 receptors did not increase any further, suggesting that increasing bryostatin beyond 10 nM might not increase the expression of receptors any more. The reason for this phenomenon cannot be explained and might require further investigation.

The two identified isoforms of the SLAMF7 (SLAMF7 and SLAMF7 A2) genes in this study (Fig 4), as have also been previously reported by Murphy *et al.,* (2002), were considered to be involved in the signal transduction pathway, with SLAMF7 noted as the active isoform. Not much information is known on the role of SLAMF7A2 in the immune cell. The

RT-PCR study showed the presence of SLAMF7 and SLAMF7A2 in myeloma cells, (OMLS and MM1S), and also in the B-CLL cells (CB) (Fig 4). However, it is noted that the relative levels of expression of SLAMF7 are much higher in myeloma cells, compared to B-CLL cells (Fig 4). Also in myeloma cells, the relative intensity of band 1 (SLAMF7) is higher than band 2 (SLAMF7A2), whereas in the B-CLL cell, the two bands show equal intensity. These results were consistent with the findings of Murphy *et al.* (2002) and Ju *et al.* (2012) who also observed two equal bands (isoforms) with SLAMF7.

CONCLUSION
The study has demonstrated that SLAMF7 levels of expression vary with different B-CLL cells, and the majority of these cells respond to PKC activators (PMA and Bryostatin) by increase in proliferation, differentiation accounting for some changes in morphological characteristics.

Currently, Elotuzumab, a monoclonal antibody has been used to target high SLAMF7 expressing receptors in cells like myeloma cells. Our study has shown the high expression of SLAMF7 receptors on some B-CLL cells, implying that without treatment with PKC activators, Elotuzumab could be used to treat some B-CLL cells. This could provide a novel treatment for CLL patients.

Our study confirmed possible upregulation of the SLAMF7 receptors on B-CLL cells with PKC activators like PMA and Bryostatin. This could therefore lead to possible successful treatment of CLL patients who express low levels of SLAMF7 receptors. The study further confirms the presence of two isoforms of SLAMF7.

RECOMMENDATION
The study could not measure the level of SLAMF7 expression levels after modulation. It is recommended that further studies be undertaken to investigate the expression levels of SLAMF7 gene after treatment with the PKC activators to determine the effect of this treatment on the levels of expression of the isoforms.

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