Title: Thymidine Kinase 1 Cell Surface Expression in B Cell Lymphoma

Author: Nathaniel Lallatin

Institution: Savoy Pharmaceuticals, Inc.

1303 North Uinta, Provo, UT 84604

Summary

Our Research shows that Thymidine Kinase 1 is found constitutively on the surface of B Cell Lymphoma cells, making it a potential marker for antibody based therapy and changing our understanding of how this highly-controlled protein, thought to exist only within the nuclear membrane and found only in cells that are in the process of mitosis. B Cell Lymphoma cells, or Raji cells as they are also known by the ATCC nomenclature, are known to be of viral etiology whereby they are transformed to cancerous cells by the Epstein Barr Virus, also called **human herpes virus 4** (HHV-4),which is a virus of the herpes family and is one of the most common viruses in humans. It is best known as the cause of infectious mononucleosis. It is also associated with particular forms of cancer, particularly Hodgkin's lymphoma, Burkitt's lymphoma, nasopharyngeal carcinoma, and central nervous system lymphomas associated with HIV.[1] Finally, there is evidence that infection with the virus is associated with a higher risk of certain autoimmune diseases, especially dermatomyositis, systemic lupus erythematosus,[2][3] rheumatoid arthritis,[3] Sjögren's syndrome,[3] and multiple sclerosis.[4]

Background

Thymidine Kinase 1, which is in the class of S-Phase regulated proteins.
TK1 is a cellular enzyme which is involved in the "salvage pathway" of DNA synthesis. In normal growing cells TK1 mRNA rises near the G1-S boundary, peaks in early S phase, and returns in G2 to approximately the level of early G1. It is activated in the G1/S phase of the cell cycle, and its activity has been shown to correlate with the proliferative activity of tumor cells. The reason for this regulation is that in normal cells the *de novo* synthesis of dTMP is achieved through a complex series of reactions in which aspartate and carbamoyl-phosphate are the starting blocks for the biosynthesis of dUDP, which is converted to dTMP by thymidylate synthetase. Dividing cells require a significant intracellular pool of dTTP for cell survival. The *de novo* synthesis of dTTP is expensive to the cell in terms of available resources. The direct conversion of Thymidine to dTMP by TK1 circumvents the *de novo* pathway. This recycling of nucleotides has been termed the “salvage pathway”.

RESULTS

TK1 Deregulation

Malignant cells have lost the strict regulation of TK1 that is observed in normal cells. TK1 activity is a major biochemical marker of cell proliferation and many studies show that TK1 levels are elevated in many different malignancies. The elevation of TK1 levels in malignancies is not simply the result of cellular proliferation but is directly caused by alteration of regulatory mechanisms in cancer cells, which constitutively express TK1 mRNA.

Discovering a tumor marker

To assess whether TK1 could be used as a tumor marker, we performed numerous experiments to determine if our CB001 antibody would differentially bind to the surface of cancer cells but not normal cells. To do this, we used several techniques including immunization of mice with TK1 to develop anti-TK1 antibodies, Murine Monoclonal Antibody Development, Immunohistochemistry (IHC), In-Vitro Fluorescence Microscopy, TK1 Inhibition assay, Complement Mediated Lysis, Antibody Dependent Cell Cytotoxicity, and Flow Cytometry, antibody humanization and toxicity studies.

In-Vitro Fluorescence Microscopy

From the photographs below (see Photos 1–4) it can be observed that virtually all of the cancerous cells have significant amounts of the CB001 antibody bound to their surface, which demonstrates that TK1 is in fact on the surface of these cancer cells.

In contrast, the same experiment performed with normal cells - lymphocytes, fibroblasts, and human tonsil tissue (Photos 4–7) - show negligible binding. We emphasize that Photos 4–5 show that mature lymphocytes are apparently unaffected and that Our anti-Tk1 antibody™ will therefore not compromise the patient's immune function – a complication of most other therapies including chemotherapy, radiation therapy, and Rituxan™.

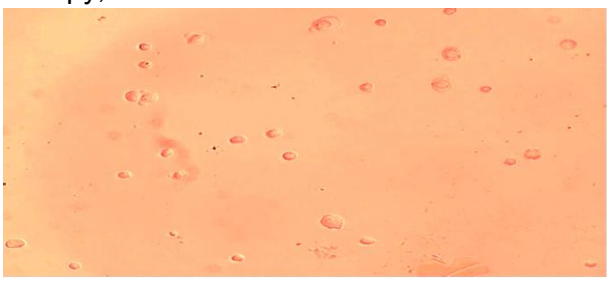


Photo 5: Normal Human Fibroblasts stained with antibody using light microscopy at 100X to verify presence of cells.

Photo 6: Normal Human Fibroblasts stained with antibody using fluorescence microscopy at 100X No staining observed.
Note: Same field of view as Photo 5

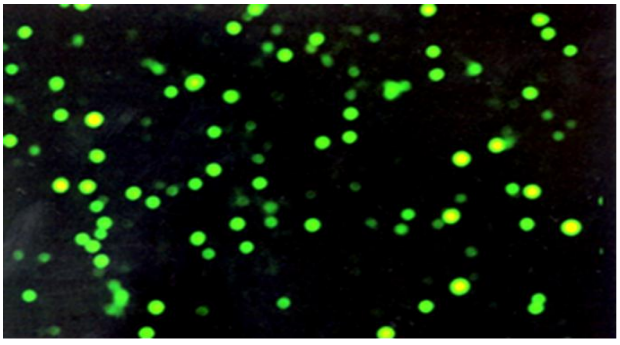


Photo 1: Hepatocellular Carcinoma stained with antibody at 100X magnification

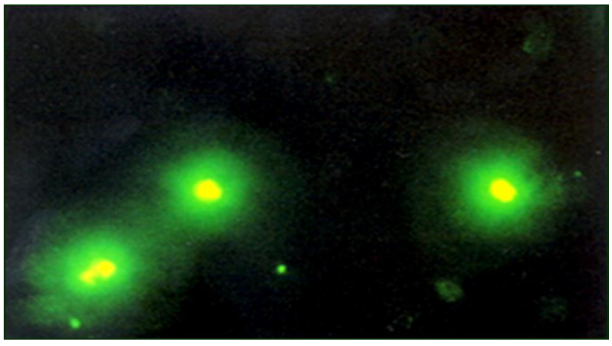
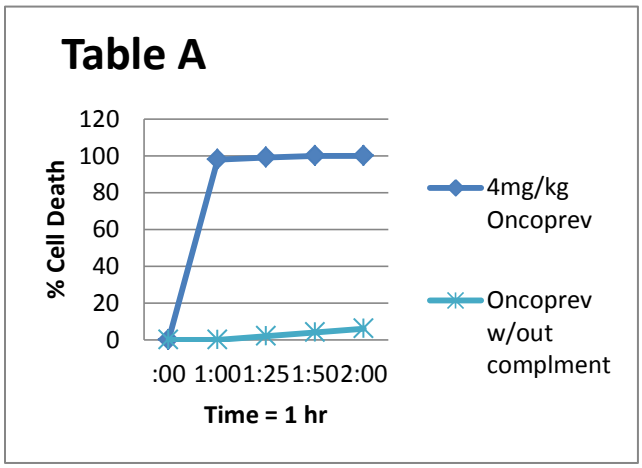


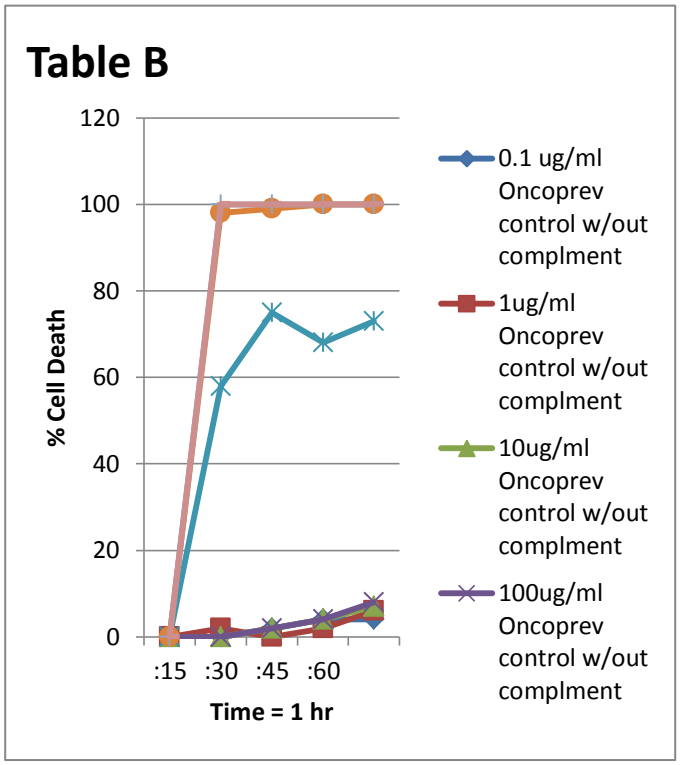
Photo 2: Hepatocellular Carcinoma stained with antibody at 500X magnification

In-Vitro

CDC – Complement Mediated Lysis

Table A - Experimental Cells





4mg/kg Oncoprev	Oncoprev w/out complement	Time
0	0	:00
98	0	1:00
99	2	1:25
100	4	1:50
100	6	2:00

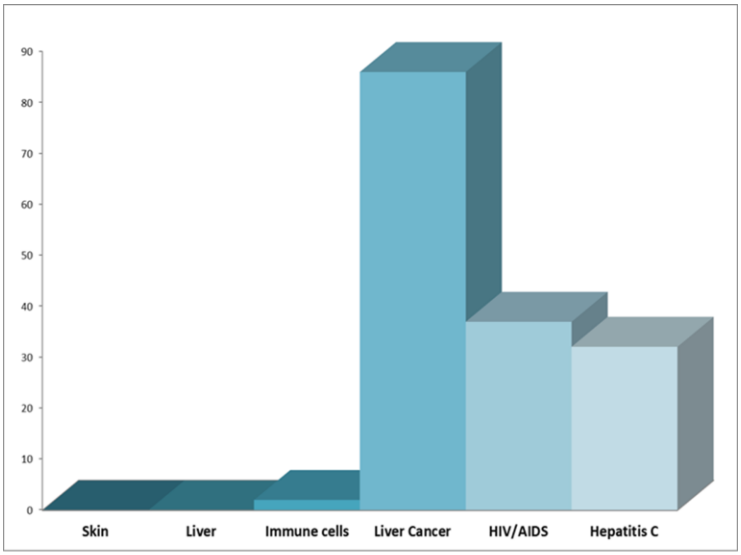
0.1 ug/ml Oncoprev control	1ug/ml Oncoprev control	10ug/ml Oncoprev control	100ug/ml Oncoprev control
0	0	0	0
0	2	0	0
2	0	2	2
4	2	4	4
4	6	7	8

Oncoprev 20% fresh human serum

0.1ug/ml Oncoprev	1ug/ml Oncoprev	10ug/ml Oncoprev	100ug/ml Oncoprev
0	0	0	0
58	98	100	100
75	99	100	100
68	100	100	100
73	100	100	100

Flow Cytometry

Additional assays demonstrate that our selected monoclonal antibodies bind specifically to cells expressing surface TK1. Data from the flow cytometer showed that the OUR ANTI-TK1 ANTIBODY monoclonal antibody selectively binds the following human cancer cell lines: HepG2 (liver cancer), MDA-MB-231 (breast cancer), MDA-MB-435 (breast cancer), H292 (lung cancer), MCF-7 (breast cancer), Jurkat (T-cell lymphoma), and Raji (Burkitt's lymphoma) and. Data also show that OUR ANTI-TK1 ANTIBODY does not bind to the surface of normal lymphocytes, fibroblasts, and human tonsil tissue. Lymphocytes were harvested from control patients without cancer. Cancer cell lines and normal human fibroblasts (BUD-8) were cultured in our lab and harvested prior to staining. Positive and negative control samples were incubated without a primary antibody and experimental samples were incubated with OUR ANTI-TK1 ANTIBODY. Both populations were then incubated with a fluorescent secondary antibody. Experimental samples were compared to control samples and the percent increase of stained cells is shown in the graph and table below. **Results indicate that the OUR ANTI-TK1 ANTIBODY™ monoclonal antibody detects TK1 on the surface of cancer cells, but not on the surface of normal human lymphocytes, fibroblasts, and tonsil tissue.**



Normal cells Cancerous cells

TK1- QUANTITY ON SURFACE AND INTERNALIZATION

Savoy contracted with Targa Therapeutics, San Diego California to conduct trials comparing the quantity of TK1 on the surface of cells to a known antigen – gp240. The results show that TK1 is more common than gp240 and therefore there are more than 1 million copies per cell. The results also show that at least 5,000 to 10,000 copies of Anti-TK1 antibody are internalized by cancer cells; making our antibody a candidate for conjugation to a toxin for the specific targeting and killing of cancer cells via methods other than the immune system.

Breast cancer cells (MDA-MB-435/nu) were grown in 96 well micro-titer plates and stained with Anti-TK1 antibody, and with ZME-018, which binds gp240, acting as a positive control. The photos below demonstrate that Anti-Tk1 antibodies are internalized into breast cancer cells, which makes them a candidate for cell killing by coupling our antibody to a toxin.

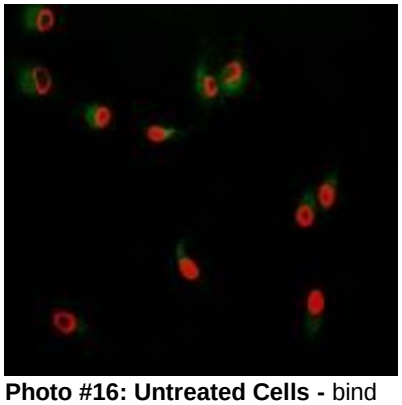


Photo #16: Untreated Cells - bind the secondary reagent nonspecifically

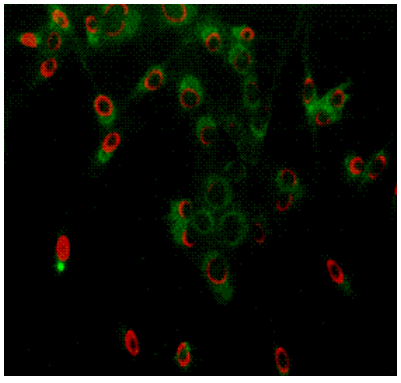


Photo #17: ZME-018 - binds gp240.

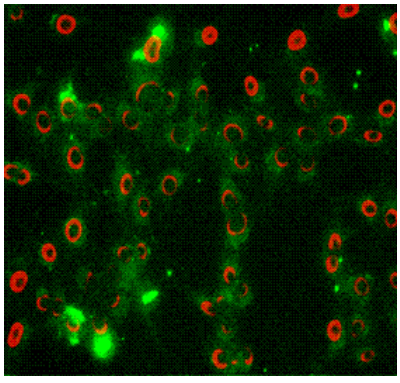
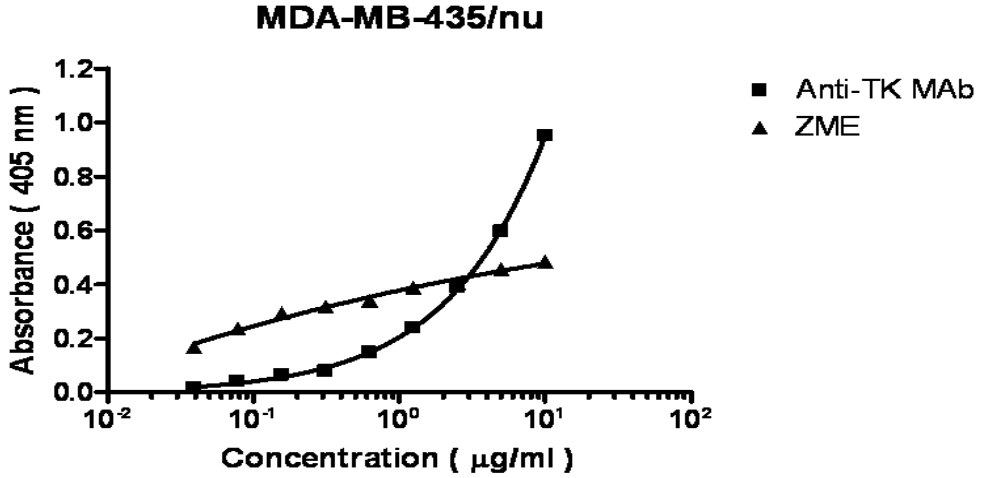


Photo #18: Anti-TK1 antibody - demonstrates internalization.

ELISA

Breast cancer cells (MDA-MB-435/nu) were grown in 96 well micro-titer plates and stained with Anti-TK1 antibody, and with ZME-018, which binds gp240, acting as a positive control. The data below demonstrates that surface TK1 is present in quantities that exceed gp240 at antibody concentrations of 2μg/ml (gp240 has been shown to have between 500,000 and 1,000,000 copies per cell).



DISCUSSION

In B Cell Lymphoma, the normally tightly regulated control of TK1 expression is lost and more than one million copies of TK1 are found on the surface of these cells at any given time, no matter the stage in the cell cycle. This is due to the phosphorylation of pRB and dissociation of pRB from E2F, which leads to the constant expression of the S phase proteins, including TK1, which is able to pass through the nuclear membrane and embed in the cell surface membrane. EBV has multiple proteins (Z, R, LMP-1, EBNA-2,-3C,-5) that could lead to the phosphorylation of Rb by cellular Cdks, and/or may directly phosphorylate Rb through the function of the viral kinase, BGLF-4. TK1 has no known trans-membrane segment and therefore must attach to the viral latent membrane proteins (LMP)-1, necessary for viral proteins to embed in the cell membrane where the viral proteins are able to re-assemble into virions prior to cell lysis.

CONCLUSIONS

Thymidine Kinase 1 found in high quantities on the surface of EBV infected cells and B Cell Lymphoma cells, making TK1 a candidate for monoclonal antibody based therapy**.** We have shown that antibodies directed toward the active site of TK1 not only recognize surface TK1, but are able to induce cell killing via ADCC and CDC. We have also shown that our antibody does not bind to the surface of normal cells at any point during the cell cycle and were therefore unaffected by cell mediated and complement associated cell killing. We therefore conclude that anti-TK1 antibodies have the potential to be used in conjunction with chemotherapy and/or Rituxan, which is a monoclonal antibody that binds to CD20.

References

1. High baselinecserum thymidine kinase 1 level predicts unfavorable outcome in patients with follicular lymphoma.Procházka V, Faber E, Raida L, Langová K, Indrák K, Papajík T. Leuk Lymphoma. 2012 Feb 21.
2. Spectrum of Epstein-Barr virus-related diseases: a pictorial review. Maeda E, Akahane M, Kiryu S, *et al.* (January 2009). "". *Jpn J Radiol* 27 (1): 4–19.

3. Serological thymidine kinase 1 is a biomarker for early detection of tumours--a health screening study on 35,365 people, using a sensitive chemiluminescent dot blot assay. Chen ZH, Huang SQ, Wang Y, Yang AZ, Wen J, Xu XH, Chen Y, Chen QB, Wang YH, He E, Zhou J, Skog S. Sensors (Basel). 2011;11(12):11064-80.pub 2011 Nov 28.
4. High thymidine kinase 1 (TK1) expression is a predictor of poor survival in patients with pT1 of lung adenocarcinoma.Xu Y, Shi QL, Ma H, Zhou H, Lu Z, Yu B, Zhou X, Eriksson S, He E, Skog S. Tumour Biol. 2012 Apr;33(2):475-83. Epub 2011 Dec 6.
5. An increased prevalence of Epstein-Barr virus infection in young patients suggests a possible etiology for systemic lupus erythematosus. James JA, Kaufman KM, Farris AD, Taylor-Albert E, Lehman TJ, Harley JB (1997). ". *Journal of Clinical Investigation* 100 (12): 3019–26.\
6. Epstein-Barr virus in autoimmune diseases. Toussirot E, Roudier J (October 2008). *Best Pract Res Clin Rheumatol* 22 (5): 883–96.
7. Epstein-Barr virus and multiple sclerosis. Ascherio A, Munch M (March 2000). *Epidemiology* 11 (2): 220–4. doi:10.1097/00001648-200003000-00023. PMID 11021623.
8. Regulation of the retinoblastoma proteins by the human herpesviruses . Adam J Hume and Robert F Kalejta (January 2009). *Cell Division* 2009, 4
9. Serum thymidine kinase and soluble interleukin-2 receptor predict recurrence of malignant lymphoma. Wakao D, Murohashi I, Tominaga K, Yoshida K, Kishimoto K, Yagasaki F, Itoh Y, Itoh K, Sakata T, Kawai N, Kayano H, Suzuki T, Matsuda A, Hirashima K, Bessho M. Ann Hematol 2002 Mar;81(3):140-6. Epub 2002 Feb 09.
10. Thymidine Kinase and Thymidine phosphorylase activities in various types of leukaemia and lymphoma. Vertongen F, Fondu P, van den Heule B, Cauchie C, Mandelbaum IM. Tumour Biol 1984;5(6):303-11.