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Molecular Inhibition of Phagocytosis: Systematic Review of Immune Response

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ABSTRACT

There are many viral variants that bind to sialic acid rather than cleave it on a human macrophage, which inhibits a macrophage's phagocytosis; for example, the D151G mutant of the influenza virus. Sialic acid must be cleaved in order to initiate phagocytosis, so viral variants that cleave sialic acid can help hasten the cleansing process of the immune system. The process of researching began with the understanding of how sialic acid synthesis occurs and how it inhibits phagocytosis. Next is the understanding of how the enzyme Neuraminidase-1 works in a human macrophage. The function of Neuraminidase-1 is to cleave sialic acid allowing for phagocytosis to occur. Next is the analysis of the molecule 12-*O*-tetradecanoylphorbol-13-acetate which is able to cause more Neuraminidase-1 to be produced and sent to the surface of human macrophages to cleave sialic acid. It has the potential of being used as a drug to stimulate Neuraminidase-1 production and specific activity, so sialic acid can be cleaved therefore the macrophage is able to phagocytize the virus. *In vitro* results show us that it is able to happen; however, this has not been tested *in vivo* which shows that the results obtained do not account for the biological factors that occur in the human bodies. 12-*O*-tetradecanoylphorbol-13-acetate has the potential to be used as a drug to speed up the breakdown of viruses that bind to sialic acid, but more research needs to be made with much progress being made.

Introduction

Humans are able to take in food using their mouth. Phagocytosis, the mechanism by which leukocytes in the immune system engulf pathogens, allows macrophages to take in their "food." The human immune system has numerous types of cells contributing to the death of an invading species in the human body by either breaking it down or neutralizing it. One type of cell is the macrophage which engulfs the foreign substance through phagocytosis and breaks it down using enzymes from its lysosome. Sialic acid(SA), located at the plasma membrane, is a carbohydrate that, if bound to a macrophage, inhibits phagocytosis. In order for phagocytosis to occur, SA must be cleaved via an enzyme neuraminidase because the inhibiting effect SA exerts on the macrophage will vanish. Some mutants of viruses, for example, the influenza virus, have this enzyme and cleaves sialidase to promote phagocytosis. However, mutants that do not have the neuraminidase enzyme will not possess a significant contribution to the stimulus for phagocytosis. This may lower the speed at which macrophages take in the virus whether it is for the adaptive or innate immune system. However, an organic compound 12-*O*-tetradecanoylphorbol-13-acetate(PMA) has been found to increase the concentration of neuraminidase-1, a type of neuraminidase specific to macrophages. This article is an analysis connecting the use of PMA as a drug being the solution to the viral binding to sialic acid problem.

Sialic Acid Synthesis

SA synthesis in human macrophages is what prohibits them from initiating phagocytosis. “Sialic acids are a family of negatively charged monosaccharides with a nine-carbon backbone”(Kooner et al. 2019). “There are three main types of Sias in vertebrates, namely N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc), and deaminoneuraminic acid (Kdn)”(Liao et al. 2020). The enzyme (UDP-N-Acetyl)-2-epimerase/N-acetylmannosamine kinase(GNE) catalyzes UDP-N-Acetyl to N-acetyl-D-mannosamine(ManNAc) and phosphorylates ManNAc to ManNAc-6-P. Consequently, ManNAc-6-P is dephosphorylated by N-acetylneuraminic acid synthase and N-acetylneuraminic acid phosphatase to turn into Neu5Ac(Fig. 1), the most abundant SA in humans. “The Neu5Ac synthesized in the cytosol is transferred into nucleus and used to form CMP-Neu5Ac, the activated form of Neu5Ac, by CMP-sialic acid synthetase (CSS)”(Kooner et al. 2019). Since many SA residues are found in glycoconjugates, many types of sialyltransferases transfer SA to molecules and bond with galactose, GalNAc, or another Sia residue. Now that the sialoglycoconjugates have formed, they are ready to exert their function on macrophages. Most of the SA that inhibits phagocytosis in macrophages requires sialoglycoconjugates emphasizing that the SA synthesis onto glycoproteins is essential to the inhibition of phagocytosis.

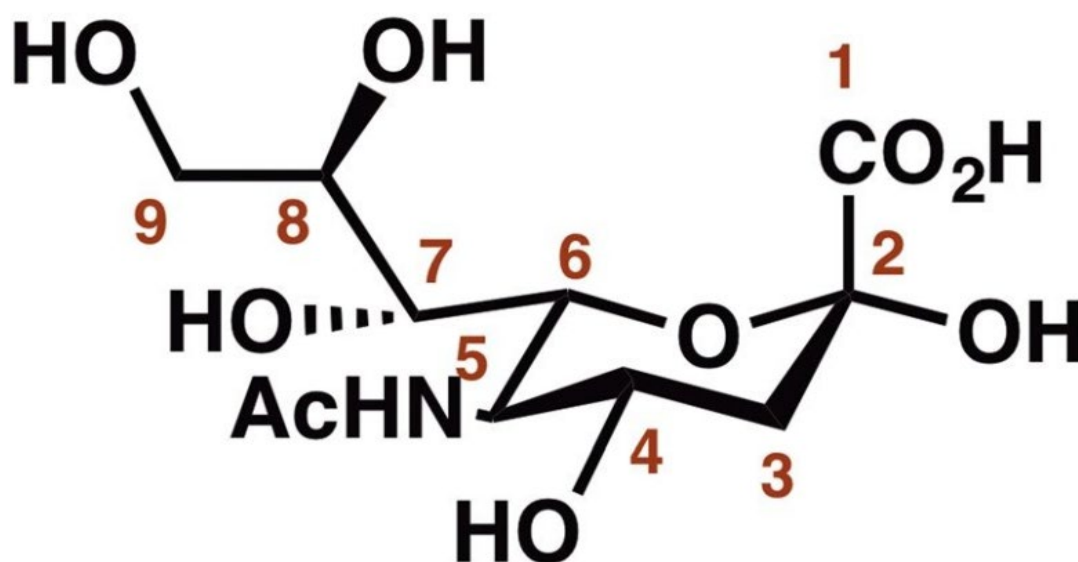

Figure 1. Structural Formula of Sialic Acid. This type of Sialic Acid is the Neu5Ac type, which is the most abundant sialic acid in humans.

Phagocytosis Inhibition

In the innate immune system, SA has been shown to inhibit phagocytosis through the complement system. The complement system is a protein cascade in the immune system that ultimately leads to a pathogen’s death. There are three ways to activate the complement system, but eventually, all the pathways combine at the C3 protein. The C3 protein splits into C3a which mediates inflammation and C3b which acts as an opsonin(Fig. 2), a molecule that binds to the foreign substance making them more prone to phagocytosis. Complement factor H(CFH) negatively inhibits C3 convertase formation which reduces the level of the alternative pathway activation in the complement system. CFH competes with complement factor B(CFB) to bind to C3 either to inhibit or produce C3 convertase, which helps in cleaving C3 to C3a and C3b. When SA binds to CFH, it causes the inhibition effect on the complement pathway and therefore phagocytosis. CFH can only bind to SA residue α2.3-linked Neu5Ac emphasizing the importance that cleaving macrophage SA residues are needed for phagocytosis to

occur. Not only bounded SA can inhibit the complement system, but free SA can inhibit cleavage of C3 and CFB as well as decrease the level of C3 in the body. Removing this inhibition effect decreases the CFH protein's effects and favors CFB rather than CFH to bind to C3b. This allows for the production C3 convertase to form to amplify the amount of C3b produced for phagocytosis. Thus, SA exerts inhibitory effects on phagocytosis via binding to CFH which binds to C3b blocking the production of C3 convertase, but that is not the only way it can inhibit phagocytosis.

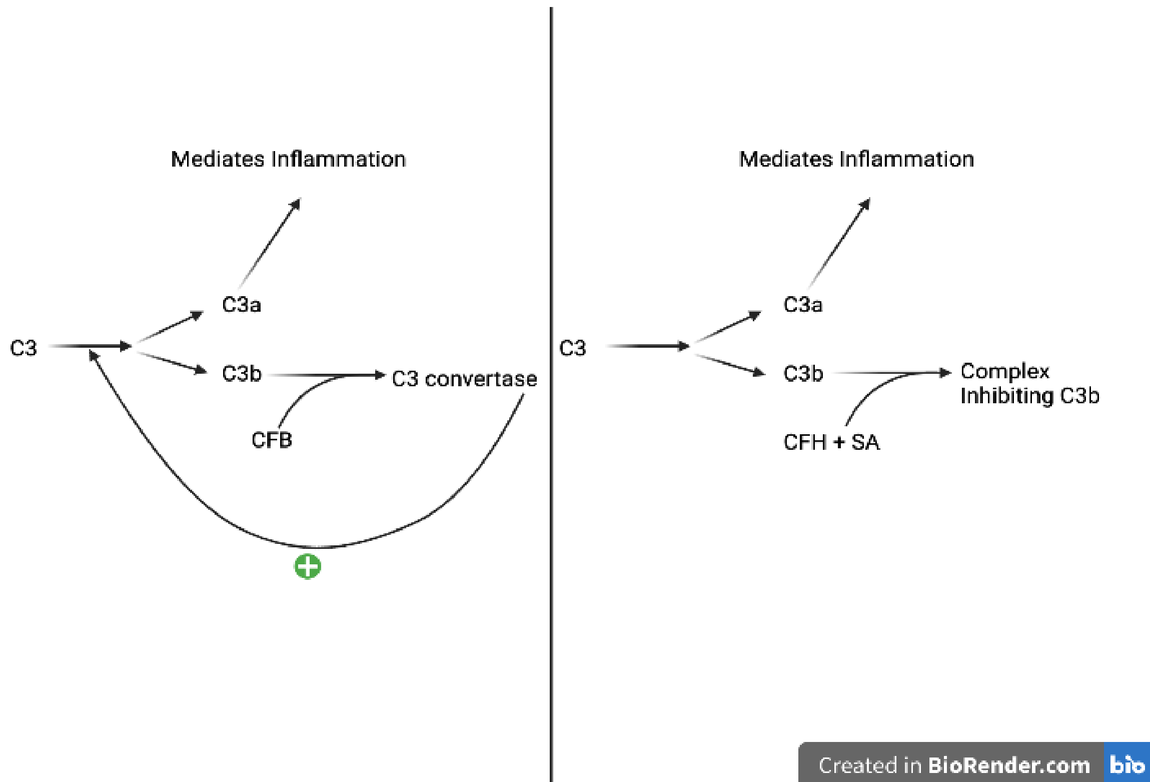

Figure 2. The left side shows that CFB binding with C3b converts to C3 convertase which exerts a positive feedback on the catalytic process of C3 to C3a and C3b. More C3b will be produced, and since C3b is an opsonin, phagocytosis will be more enhanced. The right side shows what happens when CFH and SA bind to C3b, and it inhibits C3b. The positive feedback on C3 catalysis will not occur.

In the adaptive immune system, SA inhibits phagocytosis by changing the conformation of Fc receptors and through the interactions that sialoglycans have. Fc receptors(FcRs) are receptors bound to the Fc domain of the IgG antibody produced during a response to a pathogen. There are many types of Fc receptors, but specifically for macrophages, the type of Fc receptor is FcγRI. The Fc portion of the IgG antibody is associated with glycans, a chainlike structure of carbohydrates. The glycan changes the conformation of the Fc which changes the binding affinity to the Fc receptors. There are two types of FcRs: Type-I FcRs and Type-II FcRs. Each type binds to the Fc domain of IgG at a different location; Type-I FcRs bind at the CH2 domain, and Type-II FcRs bind at the CH2-CH3 interface(Fig. 3). These constant domains(CH2 and CH3) in the Fc have an open or closed confirmation, and if it is closed in a specific area, that area cannot bind to an FcR. If the glycan contains SA, the Fc changes its shape to a closed conformation that allows it to bind to Type-II FcRs but cannot bind to Type-I FcRs.

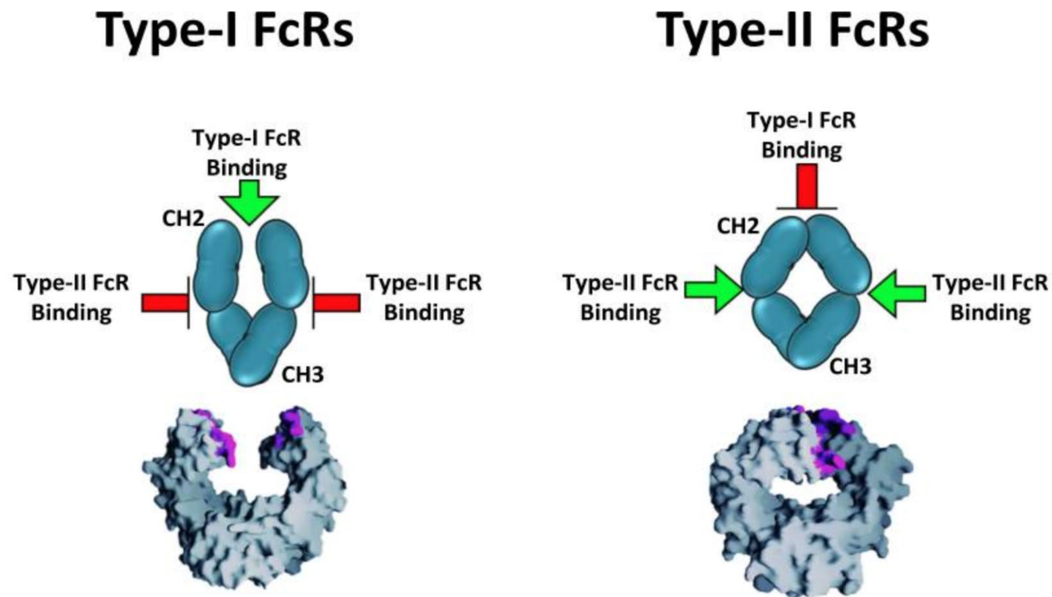

Figure 3. Fc domains alternate between conformations that depend on the type of FcR binding to it. If the Fc domain is sialylated, the domain opens up at the CH3-CH2 interface revealing binding sites for Type-II FcRs. If the Fc domain is non-sialylated, Fc opens up at the CH2 domain allowing for Type-I FcRs to bind to them(Liao et al. 2020).

Since macrophages only contain the Type-I FcR Fc**γ**RI, sialylated IgG cannot bind to macrophages. Activated Type-I FcR is required to initiate phagocytosis as well as many other molecules. These molecules include sialic acid-binding immunoglobulin-like lectins(SIGLECs), immunoreceptor tyrosine-based activation motifs(ITAMs), and immunoreceptor tyrosine-based inhibitory motifs(ITIMs). SIGLECs are Sia-recognizing receptors that bind to glycans and directly interact with sialoglycans; therefore, it can be deduced that SIGLECs bind to the sialoglycans that are bound to the macrophages and to the Fc domain. ITAMs are a necessity to activate immune cell activities like phagocytosis. SIGLECs hold intracellular ITIMs which then counteract the effects of ITAM therefore including phagocytosis initiation. “The counteraction is initiated via phosphorylation of ITIMs by SRC family kinases after ligand binding. Then, the ITIM tyrosine recruits tyrosine phosphatases, such as SRC homology region 2 domain-containing phosphatase-1 (SHP-1/PTPN6) or SRC homology region 2 domain-containing phosphatase-2 (SHP-2), which dephosphorylate the signaling molecules in the ITAM-signaling cascade to suppress the activation of the respective immune cells”(Liao et al. 2020). Without SA, this cascade would not happen as SIGLECs will not be able to bind to ITIMs since they need to bind to sialoglycans in order to be activated, and if SA is cleaved, sialoglycans would not appear in the first place. Non-sialylated glycans would not be able to inhibit the Type-I receptor binding to the Fc, and the glycans will not be able to bind to SIGLECs. Cleaving SA on macrophages will induce phagocytosis by augmenting the amount of the C3b opsonin, allow Type-I receptors to bind to the Fc domain of IgG, and inhibit SIGLEC binding to the glycans SA was previously attached to.

Neuraminidase Function

Neuraminidase contributes to the activation of phagocytosis in macrophages. Simply put, neuraminidase is an enzyme with the function to cleave SA. With SA gone, macrophages are able to initiate the “period when the [pathogen] is in contact with the cell, although engulfment has not started”(Richards and Endres 2014) and then engulf its target. There are numerous types of this enzyme including those found in viruses and in human cells.

One type that will be focusing on is found in human macrophages, neuraminidase-1(Neu-1). Neu-1 is a glycosidase "responsible for the cleavage of terminal SA residues from glycoproteins, glycolipids, and oligosaccharides"(Albrecht et al. 2020). It is usually found at the plasma membrane of macrophages with two potential transmembrane domains. Its transmembrane domains(TM1 and TM2) have been tested using point mutations within the domains which caused "substantial disruption of mNEU1 dimerization and decrease of its sialidase activity"(Albrecht et al. 2020). These results strongly favor the idea that dimerization of the transmembrane domains increases its function as a sialidase. Four amino acid residues "are critical for mNEU1 dimerization and sialidase activity: A_{319}, G_{321}, G_{328}, and V_{330}"(Albrecht et al. 2020). The amino acid sequence and interactions within Neu-1 contribute to its function to cleave SA.

Influenza Neuraminidase

The influenza virus contains the enzyme neuraminidase in many kinds of mutated forms that may pose a danger to our cells. Some forms of neuraminidase have so low enzymatic activity that it only binds to SA rather than cleaving it. One mutant that will be discussed is the TZ205 influenza virus variant. This virus contains a mutant of neuraminidase called D151G. The difference between this mutant and the wild type influenza neuraminidase is that the wild type D151 has the amino acid aspartic acid in its 151st position, but the mutant D151G has glycine instead of aspartic acid in the same position.

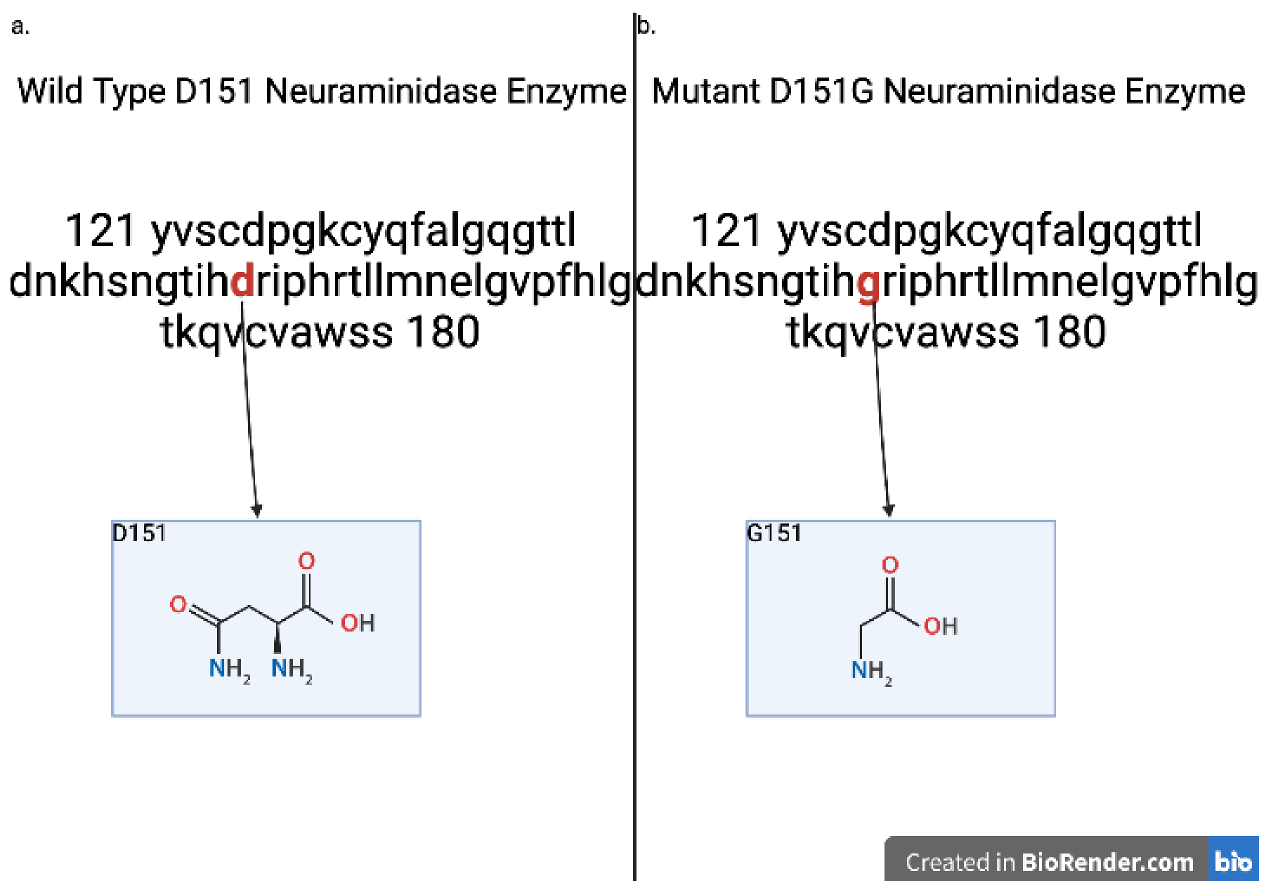

Figure 4. Both sides contain the amino acid sequence of each viral neuraminidase enzyme from its amino acid in the 121st position to the 180th position. Underneath the arrow holds the box with the amino acid difference between the sequences in the 151st position with the left side **(a).** Aspartic acid and **(b).** Glycine. Both boxes show the full amino acid rather than an amino acid residue on a protein.

A way to distinguish each variant is by the protein Hemagglutinin. Hemagglutinin(HA) is a protein that binds influenza virus to the SA glycoproteins. Its activity is only observed in the variants that bind to SA because the wild type cleaves and releases the molecule rapidly; therefore, HA is not needed in wild type activity. To compare the two enzymes, they have been compared to each other using different substrates to bind to the enzymes. When 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid(HEPES) binds to the D151 wild type enzyme, the aspartic acid forms van der Waals interactions with HEPES; however, when the D151G mutant binds to HEPES, HEPES moves 0.6 Å towards the position 150 to form van der Waals interactions with the α-carbon of G151. When tested with 3'siallyactosamine(3'SLN), the complex formed with the mutant showed "remarkably intact and well-ordered features for all three saccharides, Sia-1, Gal-2, and GlcNAc-3, in the NA active site"(Zhu et al. 2012). In the wild type, SA hydrogen bonds with arginine residues at position 118, 371, and 292 with a glycosidic oxygen directed away from the binding site which is hydrogen bonded to Asp151(Fig. 5a). This allows Asp151 to be a stabilizer for the transition state of the cleavage reaction. When 3'SLN was tested in the mutant, the Sia-1 saccharide hydrogen bonded to everything mentioned above for the wild type except Asp151(Fig. 5b). Since Asp151 is gone and replaced, there is no hydrogen bond in the 151st position and shifts towards active site base to hydrogen bonds with Glu119. The carboxylate(COO-) group in 3'SLN rotates from axial(vertical binding) to pseudo equatorial(horizontal binding) due to new charge interactions with the arginine residues. Gal-2 saccharide from 3'SLN in mutant forms hydrogen bonds with Val149 with O_4 from Gal-2, and there are no observed hydrogen bonds or van der Waals interactions between neuraminidase and GlcNAc-3. After the reaction between the wild type neuraminidase and SA, the pyranose ring of SA is refined to a boat configuration, and SA is expelled by switching anomers, isomeric forms of monosaccharides that form cyclic rings, which changes it from a β-anomer to a more thermodynamically stable α-anomer. β-anomers and α-anomers are isomers of each other.

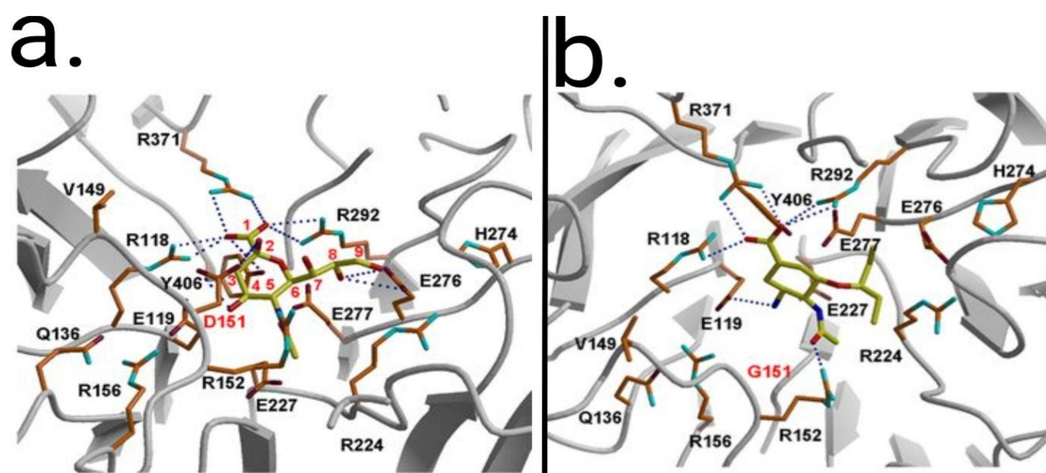

Figure 5. (a). The wild type D151 neuraminidase enzyme. SA hydrogen bonds with R118, R371, and R292. The glycosidic oxygen is hydrogen bonded to D151. **(b).** The mutant D151G neuraminidase enzyme. No hydrogen bonding occurs with the G151 residue which changes the SA interactions with the enzyme(Zhu et al. 2012).

Furthermore, the difference between the mutant and wild type neuraminidase can be expressed kinetically as well. The K_D value, the dissociation constant, of mutant binding to 3'SLN is 30.0 ± 5.5 μM while the wild type showed no results because no binding was detected. The k_{cat}, catalytic efficiency, and K_m, the half number of substrates needed for maximum velocity in an enzyme, "values are 26.8 s−1 and 29.0 μM for TZ205 D151 NA, respectively, and 0.167 s−1 and 0.824 μM for its D151G mutant"(Zhu et al. 2012). These values

favor the idea that the mutant has less enzymatic activity. It matches the idea that the main function of neuraminidase is to cleave SA, but the mutant only binds to it which shows decreased enzymatic activity. These differences between the interactions and kinetics provide the reason why the D151G mutant is only able to bind to SA residues rather than cleave them. It emphasizes the importance of Neu-1 in macrophages because when influenza virus binds to macrophages, the SA residue needs to be cleaved before phagocytosis can occur. Without Neu-1, SA will just be bound to the influenza virus allowing it to persist in the body longer than when it cleaves the molecule, so it slows down the cleansing process of the immune system. Influenza variants containing neuraminidase mutants like D151G may bind to SA on macrophage instead of cleaving them due to its different interactions and kinetics compared to the wildtype showing the need of macrophage Neu-1 to take action.

12-O-tetradecanoylphorbol-13-acetate(PMA)

12-O-tetradecanoylphorbol-13-acetate(PMA) has the capability of stimulating the production of Neu-1 in macrophages to initiate phagocytosis of pathogens (Figure 6).

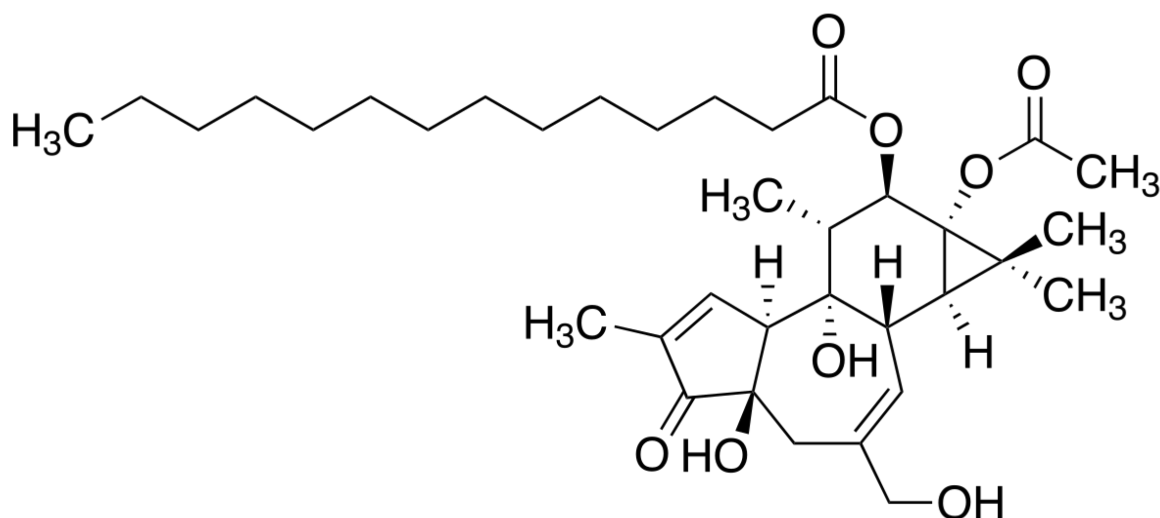

Figure 6. Structural formula of PMA.

The THP-1 line of monocytes differentiate into macrophages, and when this process occurs, more Neu-1 is found at the surface of the plasma membrane(Fig. 7).

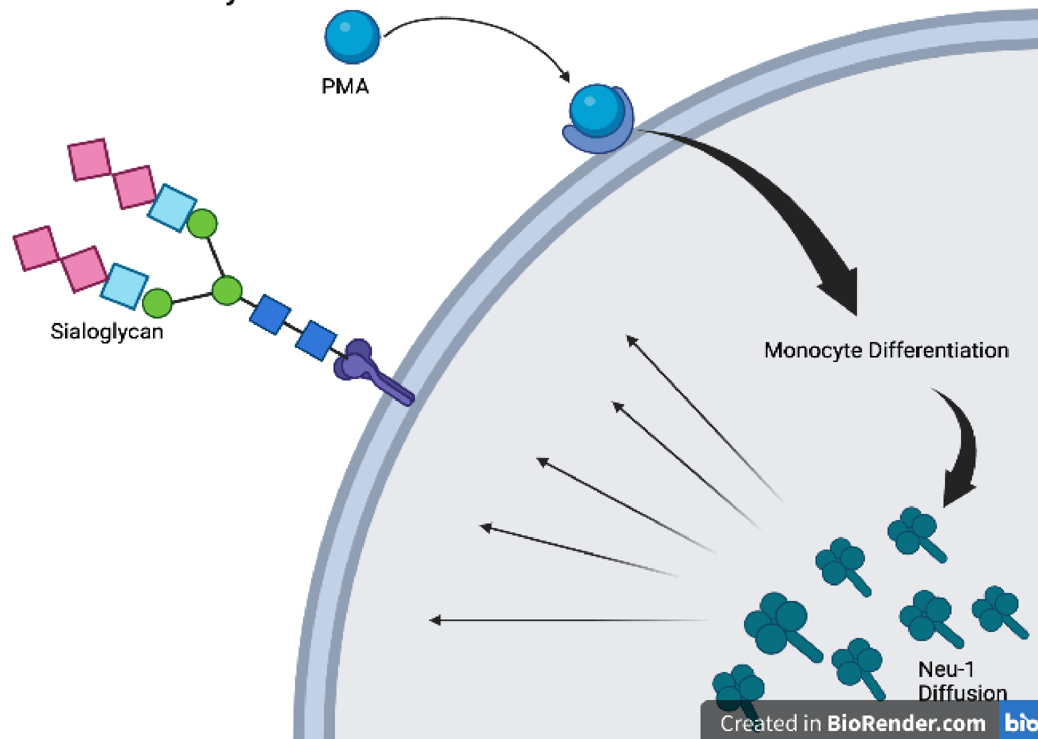

Figure 7. PMA inserted onto the THP-1 type human monocyte causing monocyte differentiation into a macrophage. This induces Neu-1 proliferation to occur causing it to appear more on the cell surface.

It has been tested and confirmed that "THP-1 cells transfected with Neu1 siRNA were differentiated in the presence of PMA only"(Liang et al. 2006). This molecule has been used to induce the differentiation from monocytes to macrophages "as a reliable in vitro model for studying immune cell processes"(Gatto et al. 2017). THP-1 cells have been transfected by the CAT reporter gene, which binds to regulatory proteins and produces an image when affected, and after 24 hours of the injection of PMA into THP-1, the reporter gene activity remarkably increased. CAT reporter gene activity especially increased in Neu-1 promoter sequences showing that when PMA is injected, the Neu-1 promoter is stimulated therefore more production of Neu-1. Some transcription factors at the Neu-1 promoter region are "promoter-selective transcription factor (Sp1), activator proteins 1 and 2 (AP-1 and AP-2), hematopoietic transcription factor (E2F-1), cAMP-response element-binding protein (CREB-2), and erythroid transcription factors (GATA-1 and NF-E2)"(Liang et al. 2006). It is safe to assume that these transcriptions may have some part of higher Neu-1 production. During the differentiation, many types of neuraminidase are present in the human macrophage, but only Neu-1 diffuses to the surface. Since more Neu-1 diffuses onto the surface, there is a higher chance of dimerization which increases the activity of Neu-1 even more. If PMA is injected into the body, it may potentially cause more macrophages to appear and bind to more copies of the virus variants that just bind to SA allowing more Neu-1 to take action to cleave SA residues for the pathogen to be engulfed. Viral variants that bind to SA slow down the cleansing process since human Neu-1 has to step in to cleave SA rather than initial cleavage from the viruses. If more pathogens are engulfed and broken down, the faster the body will be cleansed out of that pathogen. PMA may be the potential solution to hasten our immune system against SA binding mutants by stimulating Neu-1 production in our bodies by differentiating THP-1 monocytes into macrophages.

Can PMA Be Used as A Drug?

With the right concentration, total volume, and other molecules, PMA has the potential to be a future drug. If PMA is injected at a high amount, it may cause heart failure as it was found that "high NEU1 activity in cardiomyocytes as well as in invading inflammatory cells promote cardiac dysfunction"(Heimerl et al. 2020). This has the potential to transpire by injecting PMA on its own since an upregulation of Neu-1 due to an "observed increase of the endogeneous sialidase activity"(Liang et al. 2006). However, the presence of anti-Neu1 antibodies can counteract that. THP-1 monocytes have been tested with an injection of PMA, and "24 h after the induction with PMA, the intensity of anti-Neu1 staining was significantly increased"(Liang et al. 2006). Anti-Neu1 antibodies suppress the function of Neu1. Because anti-Neu1 increases with an increase of PMA, Neu-1 concentration can be controlled, potentially making it incapable of reaching cardiac dysfunction in the human body. Even if this is tested *in vitro*, it should still be capable of occurring *in vivo* since the molecules would be the same. Since this test included injected antibodies rather than natural ones, this drug can contain some type of anti-Neu1 antibody which has not been researched enough to give an exact amount or type of it. If a sufficient amount of research has been done testing this antibody in human cells, it may potentially inhibit host cell phagocytosis for viruses as well, calming down the upregulation of Neu-1 by PMA both which help the immune system do its job. The perfect concentration of PMA has been tested in monocytes *in vitro* revealing that "THP-1 cells were differentiated in vitro into macrophages by the administration of 50 ng/mL PMA for 3 days"(Gatto et al. 2017). Even though the right concentration of PMA needed to be delivered to monocytes has been discovered, the total amount of PMA that could be injected is not known with many unknown factors that still need to be researched like those related to the excretory and cardiovascular system. Since PMA has never been tested as a drug, there will be unknown side effects that can occur, emphasizing the necessity for further research on this molecule. PMA could be used as a drug but requires much more research to be done.

Conclusion

There are viral mutants out in the world that bind to SA on macrophages rather than cleave them causing a hindrance to phagocytosis in the immune system. The mutation that replaces D151 with D151G in the enzyme reduces its enzymatic activity shown kinetically and through its interactions causing its binding affinity to SA rather than its cleavage. SA is able to inhibit phagocytosis by PMA is a potential solution to this problem as it is able to bind to monocytes differentiating them into macrophages. This causes a higher production of Neu-1 and therefore a higher density in the cytosol allowing for diffusion to occur towards the plasma membrane. Since there is a higher concentration of Neu-1 present, it is able to cleave more SA residues on the sialoglycoconjugates to get ready for phagocytosis. The concentration needed to be delivered to monocytes is revealed, but there is not enough research on the total volume of PMA needed to be taken for the effects of the molecule not to be negligible or too much. This makes the article inconclusive, but if there is more research done on the molecule, it will make it a higher chance for PMA to be used as a drug to solve the neuraminidase-SA binding problem.

Acknowledgments

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