Review of the VGB-based VEGF/VEGFR interaction.

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Abstract

VGB is a new, effective peptide that inhibits the binding of VEGF to VEGFR1 and VEGFR2. Thereby it inhibits the angiogenic and metastatic effect of the ligands binding to these receptors. In order to give an insight in how VGB binds to the receptors, the most important domain for binding to VEGFR1, $VEGFR1_{D2}$ (domain 2), can be produced. To get a higher concentration of $VEGFR1_{D2}$, the processes of expression, refolding and purification in the production of the protein must be optimized. In this review several methods about how to optimize these processes are discussed.

Keywords: VGB, $VEGFR1_{D2}$, Ni^{2+}-NTA, size exclusion chromatography, refolding

Angiogenesis

Angiogenesis is the important process of inducing the growth of new blood vessels out of a pre-existing vascular network. This process in adults is rarely present, however it is very important in the early embryogenesis. (1) Normally the process is controlled with different factors. However in certain diseases there is an imbalance in these controlling factors. One of the diseases for which this is the case is cancer. Here the anti-angiogenic factors are downregulated and/or the pro-angiogenic factors are upregulated. (2) An important angiogenic factor in tumour growth is the vascular endothelial growth factor (VEGF) family with its receptors. (2,3) VEGF causes the endothelial cells to be stimulated and thereby adopting a pro-migratory phenotype. (1)

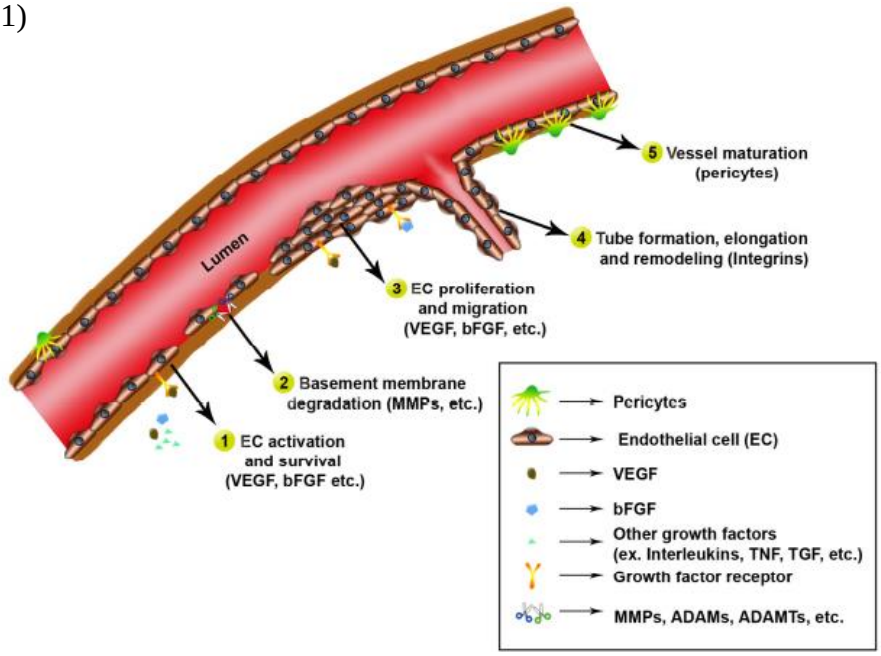

Figure 1: The process of angiogenesis depicted schematically, with the importance of the VEGF family depicted. (4)

As shown in the figure above (figure 1), the process of angiogenesis consists of multiple steps. First the endothelial cells will be activated by multiple factors, such as VEGF and bFGF. After the activation of the endothelial cells, the basement membrane will be degraded. This is done by MMPs, ADAMs, etc. Then the endothelial cells will proliferate and also migrate, which will lead to the tube formation, elongation and remodelling. This proliferation is also mediated by factors such as VEGF and bFGF. Finally the newly formed vessel will mature. Each of these steps are controlled by multiple factors which is also indicated in the figure. From this figure it can also be concluded that VEGF is an important factor in multiple steps in the process of angiogenesis. VEGF is one of the factors that causes endothelial cell activation and survival, as well as the proliferation and migration of these cells in the process of angiogenesis. (4)

VEGF

The induction of angiogenesis via VEGF in tumours is initiated when the tumour experiences hypoxic stress. The induction of the process takes place as a result of the induction of the VEGF gene by activation of the HIF transcription factor. (5) The induction of VEGF production will in turn lead to the formation of new blood vessels via angiogenesis. In tumours angiogenesis will lead to blood vessels with dead ends, an increased permeability and a disorganized vasculature (6), as shown in figure 2. Here the green area depicts an area of hypoxic stress. (1)

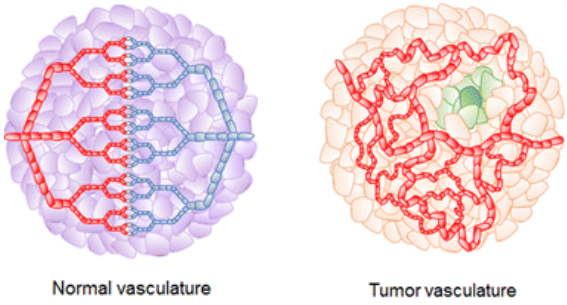

Figure 2: The difference between normal and tumour vasculature. With on the left the organized normal vasculature and on the right the vasculature in tumours, with dead ends and a disorganized structure. (1)

The formation of these kinds of blood vessels in tumours is due to the high secretion levels of VEGF in tumour cells cause the formation of immature vessels. These immature vessels are not sufficiently covered in pericytes. (1) Together the formation of the new blood vessels with dead ends, an increased permeability and a disorganized vasculature causes a disordered blood flow within the vessels. Therefore the problem of hypoxic stress will occur again, which will increase the VEGF production even further. (6)

The processes described above are initiated by different members of the VEGF family. In mammals the VEGF family consists of five members, namely VEGF-A, VEGF-B, VEGF-C, VEGF-D and the platelet growth factor (PlGF). These members can each bind to one or more of the tyrosine kinase receptors: VEGFR-1, VEGFR-2 and VEGFR-3. (7) VEGFR-1 is an important receptor for the process of metastasis of tumours, by controlling the migration of monocytes and macrophages. VEGFR-2 is important for both the survival and development of blood vessels. Finally VEGFR-3 mainly acts as an important receptor for the development of the cardiovascular system as well as for the lymphangiogenesis. This receptor can be activated by VEGF-C and VEGF-D. (8) For the process of angiogenesis VEGF-A is deemed to be the

most important, since it is the most potent pro-angiogenic member of the VEGF family by binding to both VEGFR-1 and VEGFR-2. (7)

It is important to note that VEGF-A has a higher affinity to VEGFR-1 than to VEGFR-2. Therefore it is most likely to bind to VEGFR-1, unless there is a high concentration of VEGF-B present which will occupy VEGFR-1. Due to competition for the receptor, this will lead to a higher binding of VEGF-A to VEGFR2. By this process the VEGF-A signalling in endothelial cells can be regulated, since VEGFR-1 has a weak kinase activity. Also the pro-migratory phenotype that the endothelial cells adopt when activated by VEGF, is mainly caused by VEGF-A binding to VEGFR2. (1)

More recently also the important role of VEGF-B has become more clear. VEGF-B itself does not stimulate the angiogenesis process in tumours, however it has an important role in the metastasis of cancer. As described above, VEGF causes the blood vessels that are formed in the tumours to be more permeable. This process is most likely the effect of VEGF-B binding to VEGFR1. (9)

Another factor from the VEGF family that binds to VEGFR-1 is PlGF. This factor is mainly important on other cells than the endothelial cells, such as mural cells and bone marrow-derived cells. The factor attracts these cells in the neo-angiogenic sites. Therefore this factor is also important in the processes around angiogenesis. (7)

The binding of each of these factors to the receptors and there effect are also depicted in the figure below (figure 3).

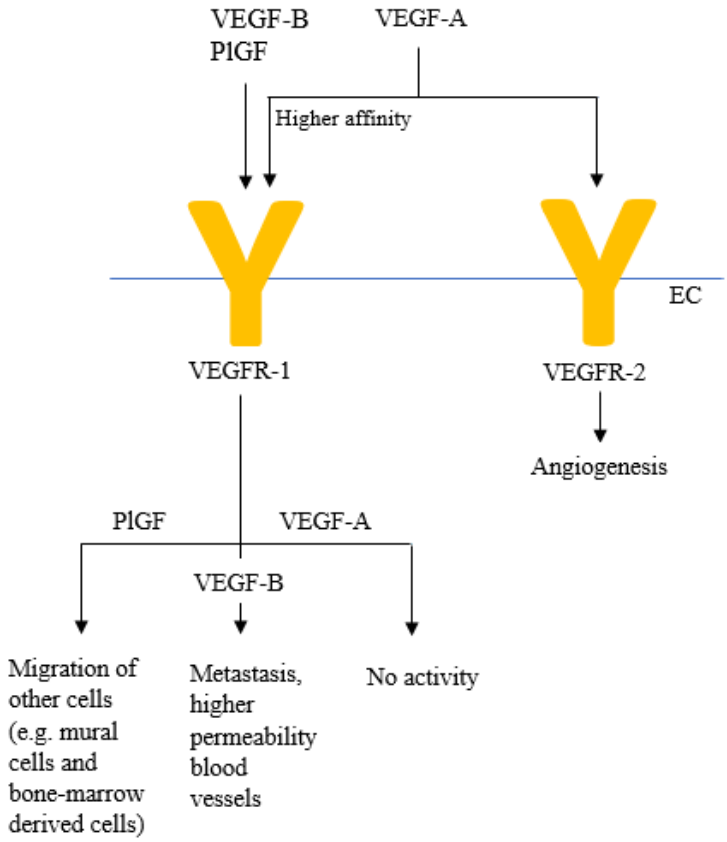

Figure 3: Graphical depiction of the different factors from the VEGF family on the receptors that are important in the process of angiogenesis.

VEGF receptors

As it becomes more clear that both VEGF-A as well as VEGF-B are important factors in tumour growth, the receptors of these factors have become a target of interest as well as their downstream signalling pathway. (1) When the VEGF receptors get activated by the binding of their specific ligands, the downstream signalling pathway is activated. For VEGF receptors, this signalling pathway consists of different molecules. The pathways that can be activated consist of PI3K-Akt, Cdc2-p38 MAPK and PKC-MEK-MRK among others. Together these pathways contribute to the regulation of the angiogenesis process, as well as cell survival and the vascular permeability. (4) A pathway that is mainly important in the process of angiogenesis is the MAPK/ERK$_{1/2}$ pathway. This pathway is activated by the binding of VEGF-A to VEGFR-2. The activation of the downstream pathway via this receptor leads to the proliferation, migration, an increased permeability and the tube formation, which are all important parts of the angiogenesis process. (10) The different downstream pathways are also shown in the figure on the next page (figure 4).

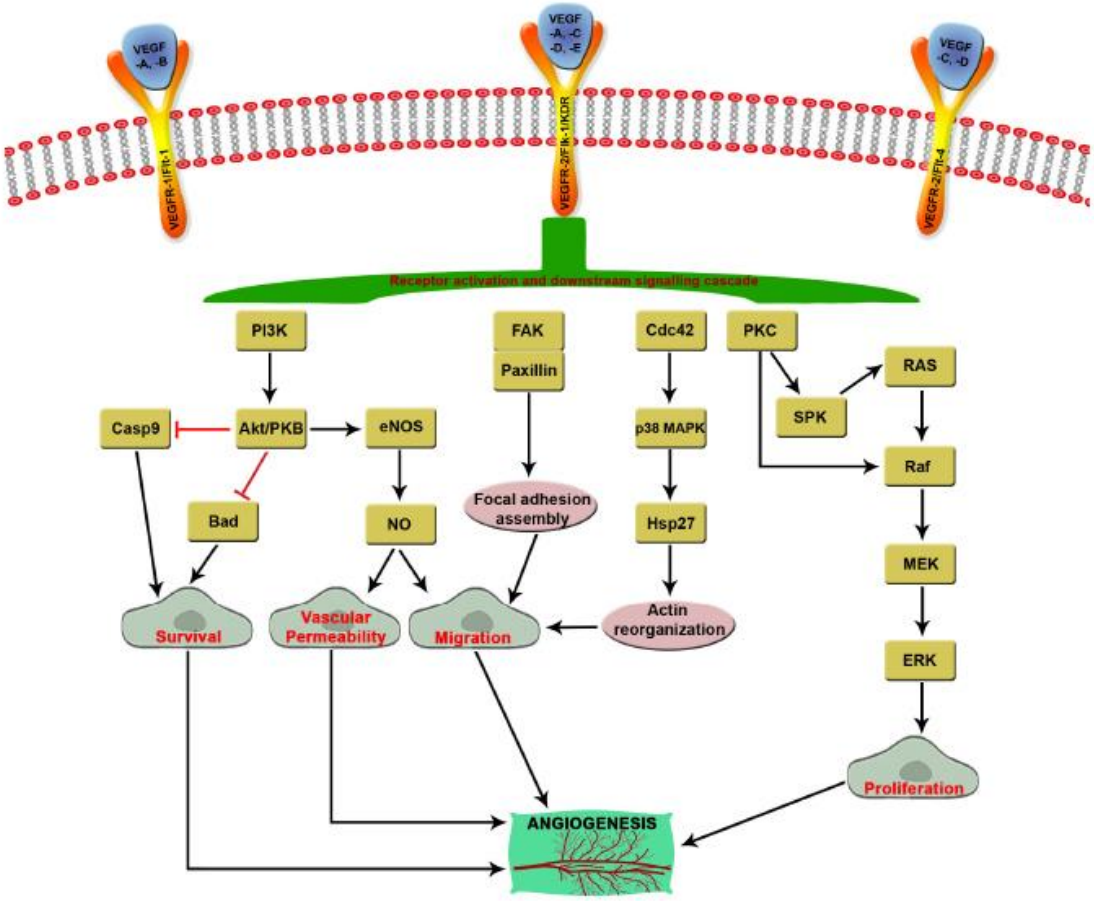

Figure 4: The downstream signalling pathways of the VEGF receptors, with their different effects in the process of angiogenesis. (4)

Before the activation of the downstream pathway it is necessary that the receptor first gets activated as described above. The activation of the receptor is started by the binding of a receptor ligand to the receptor binding site. For VEGFR-2, receptor domains 2 and 3 are important for the binding. While for VEGFR-1 this is domain 2 ($VEGFR1_{D2}$). (11) However this second domain is not the only important domain in the binding. In the binding of VEGF to VEGFR-1 domain 1, 2 and 3 are all important. However the second domain is the domain that is absolutely necessary to bind VEGF. This can also be seen by the fact that the second domain only binds VEGF with a 60 times weaker affinity than the receptor as a whole. (12)

When the receptor is activated by the binding of one of the ligands, first homo- and heterodimerization of the receptors as well as the phosphorylation of the tyrosine residues has to take place. (4) The dimerization of the receptor monomers is a necessary step in the activation of the receptor. This activation however is also depending on the orientation of the receptor monomers. (13) In heterodimerization one monomer from VEGFR-2 and one monomer from VEGFR-1 can form a dimer. Heterodimerization takes place when a cell expresses both VEGFR-1 as well as VEGFR-2. Since the VEGFR-2 receptor is more present on the endothelial cell surface than VEGFR-1, VEGFR-1 mainly exists in this heterodimer complex with VEGFR-2. This heterodimer complex therefore regulates angiogenesis by negatively regulating the VEGFR-2 homodimer. The homodimer is a pro-angiogenic complex, however the heterodimer does not show this characteristic. Therefore it is believed that the heterodimer complex causes the homeostatic function of VEGF-A. (14)

The expression of higher levels of VEGFR-1 and VEGFR-2 can also be used to predict survival in patients with cervical cancer. Higher expression of VEGFR-1 is correlated with a lower overall survival, the development of metastasis and with a lower progression free survival. A higher expression of VEGFR-2 on the other hand is correlated with a lower overall survival as well as the induction of angiogenesis in the tumours. Therefore the higher expression of VEGFR-2 was also associated with a larger tumour size. It is however important to note that no correlation between a higher expression level of VEGF-A/VEGF-B and the survival was found. This indicates that the receptors are an important target for drugs that are designed against tumour growth. (15)

VEGF acting compounds

In a study by Papadopoulos, N. et al. it also became clear that it is important to act on the activity of both VEGFR-1 and VEGFR-2. In this study a VEGF trap (aflibercept) was compared to other pharmaceuticals acting on VEGF: ranibizumab and bevacizumab. The VEGF trap, which acts as a decoy receptor, binds VEGF-A with a higher affinity and a faster association rate than the other two pharmaceuticals. Further the VEGF trap was able to bind VEGF-B and PlGF as well, which the other two pharmaceuticals are not able to, (16) since they are humanized anti-VEGF-A monoclonal antibodies. Bevacizumab was found to significantly improve the progression-free survival in combination with chemotherapy. (1) However the study comparing the three compounds found that the VEGF trap, interfering in multiple bindings to the VEGF receptors, had a higher potency to neutralize the VEGF receptor activation than the other two compounds. (16)

Other compounds that interfere with the VEGF/VEGFR binding, are inhibitors of the VEGFRs. These are compounds such as sunitinib. (1) This compound interferes with the downstream signals of the receptors and is thereby inhibiting the angiogenesis process, mainly that of VEGFR-2. (17) It has been found that the VEGF-receptor inhibition of sunitinib inhibits the endothelial differentiation *in vitro*, inhibits the induction of vasculogenesis *in vivo* and the endothelial differentiation that takes place as an effect of hypoxia caused by the reduction of the blood supply by the compound itself. These are all processes that were not inhibited by the VEGF blocker bevacizumab. (18) This is also shown in figure 5.

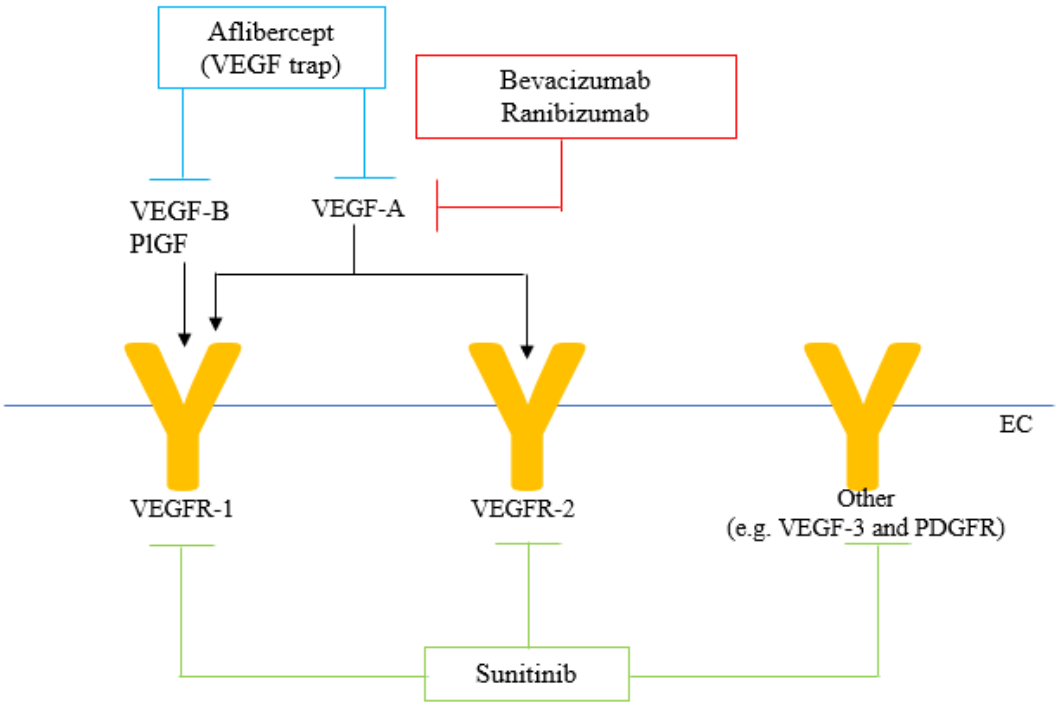

Figure 5: The effect of the different VEGF acting compounds that have been discussed.

The results of these studies show that the blocking of multiple VEGF receptor activation is necessary to give a more significant effect on the inhibition of angiogenesis in cancer.

VGB

A newer compound that was created lately is peptide $_2$HN-CIKPHQGQHICNDE-COOH, otherwise known as VGB. This peptide was created based on the interaction of VEGF-A with both receptors VEGFR-1 and VEGFR-2. Since the peptide consists of both interaction sites, it can bind to both receptors and thereby block these receptors. By the blocking of both receptors the downstream signalling pathway is also inhibited, which decreased the angiogenic process in tumours. Therefore VGB is deemed to be a promising new compound in the therapy of cancer. (10)

In vivo studies in mice have already indicated that the treatment with VGB is causing tumour growth inhibition. (10)

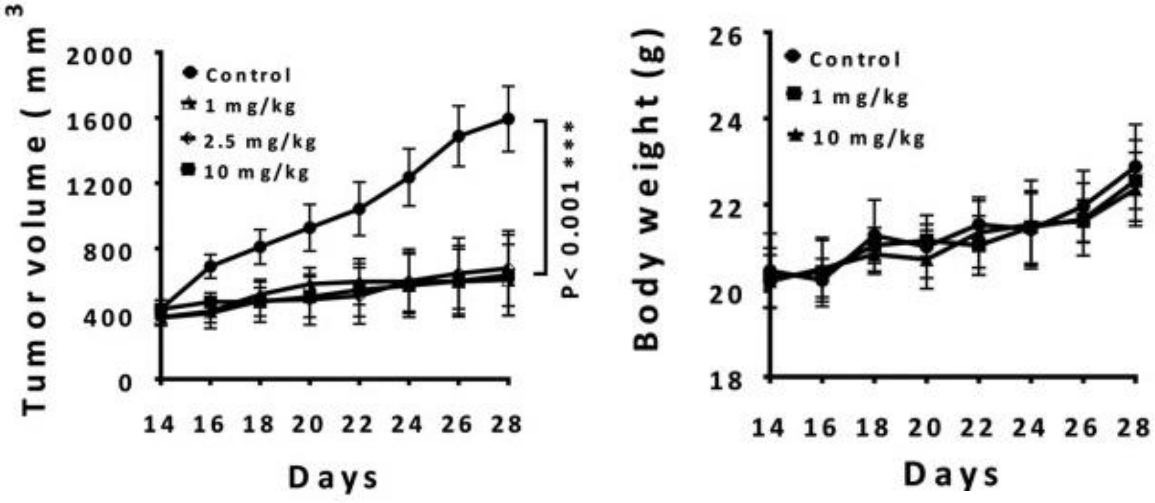

Figure 6: Results of the effect of VGB in different concentrations in mice. With on the left the tumour volume, which is determined as a part of the total body weight on the right. (10)

As shown in the figure above (figure 6) the tumour volume in the mice in the control increased over time. The tumour volume that was found in the mice after treatment with the VGB peptide, stayed around the same volume. This volume was determined as a part of the total body volume of the mice, which is shown in the right figure. As can be seen in this figure, the total body weight of the control mice in contrast with the mice treated with VGB, did not differ over time. This indicates that VGB indeed did inhibit the tumour growth in these mice. (10)

Apart from the fact that VGB decreased tumour growth, it is also found that VGB inhibits the downstream mediators of metastasis. Thereby VGB acts on multiple components of the tumour growth. (19) The downstream pathways of the MAPK/ERK$_{1/2}$ and PI3K/AKT are found to be suppressed by VGB binding to VEGFR2. Therefore the proliferation, migration, permeability and the tube formation is also inhibited by this binding of VGB. This is because these are the main effects of the activation of VEGFR2 by VEGF-A via the MAPK/ERK$_{1/2}$ pathway. (10)

When comparing the effects of VGB with other studies, VGB was studied in different circumstances. VGB was studied in the presence of higher concentrations of VEGF-A. VGB was found effective, even in these higher concentrations. Also the inhibition of the tumour growth of VGB was tested when the tumour already reached a higher volume than the compounds in the other studies. (10) When these situations of the research are taken into account, VGB seems to be a compound that inhibits the effects of VEGF/VEGFR on the angiogenesis and metastasis of tumours in an effective manner. Therefore further research of VGB is necessary, to be able to identify the binding actions of the peptide. This can be of great importance when trying to increase the affinity of the compound as well as for the production of other compounds that can inhibit the VEGF/VEGFR interaction more specifically than the compounds already on the market.

Cloning, expression and purification

To determine the binding of VGB to the receptors, $VEGFR1_{D2}$ has to be produced, since this extracellular domain is important in the binding of the VEGF family to the receptor. (11) This is done by a process of cloning, expression and purification of the protein. To do this, first the $VEGFR1_{D2}$ gene is amplified by PCR. Then digestion of the fraction takes place with restriction enzymes. After the fragment is cloned into the corresponding sites on the pETM11 expression vector, a ligation reaction is started by adding the insert DNA and the DNA ligase. This results in the recombinant plasmid that can be used for the transformation, growing of the culture and the concentration of the protein. (12)

When the recombinant plasmid is obtained, a transformation can be performed. For the transformation a specific strain of *E. coli* is used, *E. coli* BL21 *Codon Plus* (DE3) RIL. This strain was transformed with the pETM11- $VEGFR1_{D2}$ plasmid that was made earlier. The cells then are left to grow in the presence of 50 µg/mL kanamycin and 33 µg/mL chloramphenicol. The culture then is left to grow until an optical density (OD) of 0.7/0.8 is reached, when measured at a wavelength of 600 nm. When this OD is reached, 0.7 mM IPTG is added to the cells to cause an induction. After the IPTG is added the culture is incubated for 4 hours at 37 °C, after which the cells will be harvested by centrifugation. The pellet that is left after the centrifugation is then dissolved into 50 mM Tris-HCl, with a pH of 8 and protease inhibitors. This solution is sonicated after which the lysate is centrifuged. Both the pellet and the solution are analysed on a SDS-PAGE gel. (12)

Now the steps of the purification process can be started. First the recombinant His-tagged $VEGFR1_{D2}$ has to be solubilized from the inclusion bodies using 50 mM Tris-HCl, 10 mM imidazole, 8 M urea (pH = 8), after which the cleared lysate solution can be added on the Ni^{2+}-NTA resin with 300 mM NaCl. The 8 M urea is used to denature the protein, so that the aggregates of the protein are dissolved better. This causes the protein to bind the resin with a higher affinity. Also imidazole is added to prevent the untagged proteins that are in the sample from binding to the column. (12) This concentration could be changed, since the His-tagged protein might also not bind to the column if the concentration is too high. (20) The presence of Tris-HCl and NaCl is because this causes the protein to be stable. (21) After this step the protein is refolded with 50 mM Tris-HCl, 10 mM imidazole, 300 mM NaCl and a decreasing concentration of urea. The protein finally is eluted by increasing the concentration of the imidazole from 100 until 300 mM. Then the protein is dialyzed by leaving it overnight at 4°C in 50 mM Tris-HCl and 250 mM NaCl (pH = 7). After dialyzing the protein, glutathione is added with a concentration of 3 mM reduced and 0.3 mM oxidized glutathione. The product then is further purified using size exclusion chromatography, with a S75 column that was equilibrated with 50 mM Tris-HCl and 250 mM NaCl (pH = 7). After the purification with the size exclusion chromatography the protein will be further concentrated using the Amicon Ultra system (3000 MWCO, Millipore). (12)

After this new method for refolding and purification was produced by Rossella Di Stasi *et al.* (2009), to provide a relatively cheap way to produce the $VEGFR1_{D2}$ protein, it was also tested in several set ups. This was done in order to provide information on whether the protein was refolded correctly. To study this, the ability of the produced protein to bind to VEGF was studied, since it could only bind if it was refolded correctly. Also the activity of the protein on the VEGF induction of the HUVEC proliferation was analysed. In this study, it was found that $VEGFR1_{D2}$ did bind to VEGF with approximately the same binding constant as found in

literature. Also the protein had the ability to inhibit the HUVEC proliferation. Therefore it was concluded that the refolding of the protein was successful, with a final yield of 6 mg per liter of culture. (12) Since the method of the refolding in this study was successful it would be an efficient as well as relatively cheap method of producing the protein in future studies.

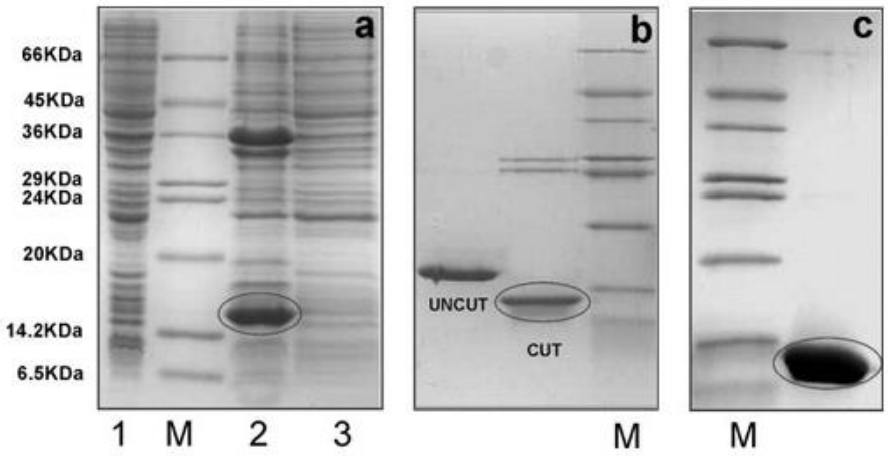

Figure 7: The SDS-PAGE analysis of multiple steps in the purification of VEGFR1$_{D2}$. With on the left the results (a) being the gel after the expression of the protein in E. coli. The middle figure (b) showing the gel after the TEV proteolytic reaction. And the right gel (c) being that after size exclusion chromatography. VEGFR1$_{D2}$ is encircled in all gels and the marker is indicated with M. (12)

The effect of the purification process that was described above also became clear, while studying the SDS-PAGE results. The gel that is shown on the left (a), gives an indication of the protein after the expression in the *E. coli* strain. As shown in the figure (figure 7) the sample consists of multiple proteins apart from the VEGFR1$_{D2}$ protein, that is encircled in each of the figures. By comparing this to the marker (indicated with M) and the corresponding weights of the marker proteins, the VEGFR1$_{D2}$ protein can be identified. This is because this protein has a molecular weight of 11851.7 Da. The second figure (b) indicates the presence of proteins after the TEV proteolytic reaction, so before the final purification step of the size exclusion chromatography. Lastly the most right figure (c) shows the concentrated protein after the final purification. (12) These figures show that at the end of the purification only the VEGFR1$_{D2}$ protein is left, as is indicated by the presence of only one band on the SDS-PAGE gel. Therefore the given process of purification also seems to be effective.

The process as describe above is also used again in a different study of the same group. During this study the procedure above was used to obtain VEGFR1$_{D2}$ to provide structural insights into the binding of a peptide, called MA, to the domain. However the determination of the bond between the peptide and the protein was not performed using crystals. (11) Since the study did not use crystallisation to determine the interaction with the peptide, it is not clear whether the concentration that was achieved with this process of expression, refolding and purification was sufficient for crystallisation.

To study the interaction of VEGFR1$_{D2}$ with the new peptide VGB, it is important that there is a possibility to crystallize the protein. Therefore a sufficient amount of VEGFR1$_{D2}$ should be produced in order to be able to perform the crystallisation. (10) To do this, the process of purification and refolding should be optimized. To optimize the processes it is important to investigate the different studies that have already been performed and the different outcomes of these studies.

Refolding

Since the foreign $VEGFR1_{D2}$ proteins are over expressed in the *E. coli* strain, they will be included in inclusion bodies. Therefore the denaturing and refolding of the proteins is necessary, since the protein will be inactive as long as it is in the inclusion bodies. (20) For the denaturation, urea can be used, as is done in the method described above. (12) However it is also possible to use other detergents, such as guanidine hydroxide or sarkosyl. Still after the refolding process, not all the formed protein will be refolded. This is because there will be aggregation of the proteins taking place, which will lead to the fact that not all proteins can be refolded. Sarkosyl is a mild detergent, which does work better when it comes to the aggregation of the proteins. To cause denaturation however, stronger detergents are a more effective option. (20)

A different method that was described when it comes to the refolding of His-tagged proteins is that by using SDS as the detergent. To do this the denaturation buffer consists of 50 mM Tris-HCl, 300 mM NaCl and 2% SDS (pH = 8). After sonication until the solution became clear, the excess SDS was removed at 4°C for 30 minutes to overnight. Then the solution was centrifuged at 10,400 g for 10 minutes. After the centrifugation 400 mM KCl (final concentration) was added to the supernatant. This KCl would cause a precipitation of an insoluble crystal with SDS overnight at 4°C. The next day the supernatant could be loaded on the Ni^{2+}-NTA. (20)

Ni^{2+}-NTA

In other papers the method of the expression, purification and refolding steps are somewhat similar to that described earlier. In one other papers however there are more possibilities given for the Ni^{2+}-NTA resin. It is suggested that the purification of His-tagged proteins can be performed with either nitrilotriatic acid (NTA) or iminodiacetic acid (IDA) as a chromatography matrix for the Ni^{2+} ions. NTA however contains four instead of three bonds to the metal-chelating sites which IDA has. This causes the NTA to bind the metal ions more stably. Because this causes a reduction in the ion leaching, the NTA is the preferred matrix to be using. (22)

The article also suggest using an increase of imidazole from 100 to 500 mM for the elution step of the protein. (22) while the other process description suggested an increase from 100 to 300 mM. (12) To gain an increase in the final protein concentration this is an option that should be considered, since protein that might still be on the resin could be able to get off with the higher concentration of imidazole.

Size exclusion chromatography

With size exclusion chromatography, different molecules in a solution can be separated. This separation depends on the size of the molecule, which will cause the molecules to have different retention times. The smaller molecules will typically elute at a higher retention time than the larger molecules, since the smaller molecules can enter the small pores in the stationary phase. The method is mainly a secondary purification step after another purification step. This is because there are too many proteins present in the sample if this is not purified once already. (21)

In order to do the chromatography the sample should be concentrated to approximately 90% pure product in a volume of 1 mL. This volume could be more, but should not exceed 3% of the volume of the total column, since the different molecules will not be separated enough when a larger volume is used. (21)

Another important factor in the size exclusion chromatography is the cut off size. In most cases this size is given as a molecular weight, however this is based on spherical molecules. (21) For $VEGFR1_{D2}$ this is an important factor as well, since the molecule is relatively flat as it consists mainly out of β-sheets. (12) In order to further optimize the purification of $VEGFR1_{D2}$ this is therefore also a factor to consider.

Discussion and conclusion

VGB is a new peptide which has the possibility to become successful as an addition in the treatment of cancer. VGB inhibits multiple pathways of VEGF that are important in the process of angiogenesis and metastasis of tumours. (10) As seen when comparing other compounds that act on the VEGF/VEGFR interaction, or the downstream pathways of the VEGFRs, the effect on the tumour progression was more significant when attacking multiple pathways of this interaction. (16,18) To study the exact binding of VGB to $VEGFR1_{D2}$ it is necessary to produce crystals from the protein $VEGFR1_{D2}$. This is of importance since $VEGFR1_{D2}$ is an important part of the binding site of VEGFR1. (11) In this way the binding interactions can be studied. To be able to produce the crystal structure, the protein should be obtained in a high concentration.

In the literature a method for this is described, however some changes could be made to optimize the expression, refolding and purification process. Starting with the refolding process, a different detergent or different concentration of the detergent could be used. One option here is the use of SDS as a detergent. (20) Another option is the increase in the urea concentration in the buffer. (12) This will lead to a stronger denaturing effect. The use of SDS as a detergent might be challenging with the protein, since irreversible aggregation of the protein might take place when the SDS is removed. (20)

A method for improving the purification process, is to change the concentration of imidazole in the buffers. Increasing the concentration of imidazole in the elution buffer could lead to better elution of the protein and therefore a higher final concentration. Another option that could be considered is using lower concentration of imidazole on the column. (12) A high concentration could lead to the His-tagged protein to not bind to the column. (20) However the binding of the protein to the column has not yet been found as a problem, since in all literature there was pure protein obtained as the final product. This indicates that at least part of the His-tagged protein did bind to the column. The increase of the imidazole concentration in the elution buffer is a possible way to increase the final concentration of the protein.

Finally it would also be an option to change the incubation periods, to allow more time for certain processes to take place.

Overall multiple options to try to optimize the expression, refolding and purification of $VEGFR1_{D2}$ have been considered. For the optimization of the purification, the change of concentrations of imidazol in the elution buffer seems the most promising. For the expression process, the incubation conditions could be changed. Lastly for the refolding, the concentration of the detergent could be altered to obtain a higher final concentration. Studying the effect of one or more of these changes on the final concentration that is obtained, will hopefully lead to crystallization of the product. The crystallized product can lead to new insights into the binding of VGB to $VEGFR1_{D2}$.

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