Abstract:
The invention relates to at least one molecular target for healing or treating wounds and, in particular, chronic, human wounds. The molecular target is PTPRK, or a protein 50% homologous therewith, and which retains the same activity as PTPRK protein. Further, the invention concerns a novel therapeutic for treating said wounds and a novel gene therapy approach, involving said molecular target, for treating said wounds.
Molecular Targets for Healing or Treating Wounds

Field of the Invention
The present invention relates to at least one molecular target for healing or treating wounds and, in particular, human wounds. More particularly still, the molecular target has application in the treatment of chronic wounds. Further, the invention concerns a novel therapeutic for treating said wounds and a novel gene therapy approach, involving said molecular target, for treating said wounds. Additionally, the invention concerns a method for treating wounds using said therapeutic or said gene therapy.

Background of the Invention
In one form or another, chronic and poorly healing wounds constitute a major burden on the UK health system. Moreover, in certain member countries of the EU health expenses relating to wound healing are already approaching the third most expensive drain on health care funding.

A Chronic wound is herein defined as one exhibiting delayed or defective healing which does not progress through the predictable stages of the healing process (as described below). Commonly, chronic wounds are classified into three broad categories: venous ulcers, diabetic, and pressure ulcers. Long-term venous insufficiency accounts for 70% to 90% of chronic wounds and commonly affects the elderly. Venous insufficiency results in venous hypertension, in which blood flow is abrogated resulting in subsequent ischaemia. Venous insufficiency can occur as a result of obstructions to venous outflow or reflux due to valve damage. Following a period of ischaemia, tissue reperfusion can result in reperfusion injury, causing the tissue damage that leads to wound formation.

Chronic foot ulcers are a major complication of diabetes, accounting for up to 25% of all hospital admissions involving diabetes, and at a cost to the UK National Health Service of £250M annually. Chronic foot ulcers cause substantial morbidity, impair the quality of life, and are the major cause of lower
limb amputation. Despite careful attention to foot care, as many as 25% of diabetics develop foot ulcers in their lifetimes. The causes of lower limb ulceration are the same in diabetics as in non-diabetics, namely neuropathy, ischaemia and trauma. However, this "pathogenic triad" predisposes wounds to infection, which can also contribute to the non-healing nature of the wounds.

Pressure wounds are another major resource health cost. They are typically caused by failure to provide routine nursing or medical care. In the UK 412,000 people are affected annually by this sort of wound at a cost of £1.4-2.1 billion.

Furthermore, chronic wounds can also be categorised by whether they are caused by surgery, burns, dermatitis, vasculitis or radiation.

Current wound treatment strategies involve removing pressure from the area, debridement, wound dressing and management of infection: surgical resection and vascular reconstruction may be required in more advanced disease, which ultimately may necessitate amputation. These strategies commonly seek to address problems that are associated with chronic wounds, such as bacterial load, ischaemia, and imbalance of proteases, all of which can further affect the wound healing process.

The healing of a wound is controlled by complex biological processes that involve a diverse number of cell types; complex interactions between cells and tissues; the activation of the immune system and the activation of the angiogenic process. Moreover, all of these processes involve a large number of molecules.

A typical healing process can be divided into 5 distinct, but closely related, stages: clotting stage, acute inflammation stage, matrix deposition stage, capillary formation stage and re-epithelialisation stage. A diverse number of factors are involved in and control each of these stages. Deficiencies in any aspect of the process may result in defective wound healing. Thus, a 'normal' healing process may be defective as a result of either intrinsic or external
factors, which manifest as 'abnormal non-healing' or 'chronic' wounds. It is these chronic or 'non-healing' wounds that present the greatest challenge to the quality of a patient's life and mounting expenses to the healthcare system.

A chronic wound often arises from failure to progress through the normal stages of wound healing, whereby an initial injury resulting in a wound cannot subsequently be repaired. Changes occur within the molecular environment of a chronic wound, such as high levels of inflammatory cytokines or proteases, and low levels of growth factors, these changes detain or terminate the healing process and increase the potential for septic infections. By enhancing or manipulating factors that contribute to wound healing it may therefore be possible to correct the process, thereby reducing the likely occurrence of a chronic wound, or accelerate its subsequent repair.

PTPRK

Protein tyrosine phosphatase receptor type K, PTPRK, is also known as DKFZp686C2268, DKFZp779N1045 and R-PTP-kappa. It is a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signalling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation. PTPRK possesses an extracellular region, a single transmembrane region, and two tandem catalytic domains, and thus represents a receptor-type PTP. The extracellular region contains a meprin-A5 antigen-PTP mu (MAM) domain, an Ig-like domain and four fibronectin type III-like repeats. Moreover, PTPRK has been shown to mediate homophilic intercellular interaction, possibly through the interaction with beta- and gamma-catenin at adherens junctions. Expression of the PTPRK gene has been found to be stimulated by TGF-beta 1, which may be important for the inhibition of keratinocyte proliferation. In cancer, PTPRK has been found to be suppressed in aggressive tumours as shown by our recent study in breast cancer (Sun et al, SABCS, Cancer Res 2010).
While the biochemical functions of the PTP family is known to some degree, the therapeutic implication of the PTPRK enzyme has rarely been explored, particularly in relation to wound healing.

We, therefore, have surprisingly discovered that PTPRK has a role to play in wound healing. Indeed, we have discovered that the expression of this protein impedes the wound healing process. Moreover, the inhibition of PTPRK promotes wound healing.

Inhibitors of PTPRK are known. The most readily available is a salt of stibogluconate. Sodium stibogluconate is a medicine used to treat leishmaniasis, a disease resulting from infection by one of over 20 different species of the *Leishmania* species of parasite. Sodium Stibogluconate belongs to the class of medicines known as the pentavalent antimonials. Whilst its exact paracidal effect on the Leishmania parasite is unknown it is thought that the parasite is killed by inhibition of glucose catabolism resulting in reduced ATP synthesis, thereby decreasing subsequent macromolecular synthesis and preventing replication.

Sodium stibogluconate is sold in the United Kingdom as Pentostam™ (manufactured by GlaxoSmithKline) and is currently only available for administration by injection. Unfortunately, widespread resistance to this medicine has limited the utility of sodium stibogluconate, and in many parts of the world, amphotericin or miltefosine is used instead to treat leishmaniasis.

In summary, we have identified at least one molecular target for treating wounds and in particular human wounds. More particularly, but not exclusively, said molecular target has application in the treatment of chronic wounds. The molecular target is PTPRK and therefore the invention relates to a novel therapeutic comprising an inhibitor of either, or both, PTPRK expression or PTPRK activity. In the former instance, the invention involves a novel gene therapy approach and in the latter instance a novel protein therapy approach. Accordingly, the invention also relates to a novel therapeutic comprising an inhibitor of either, or both, PTPRK expression or PTPRK activity. In the former
Reference herein to PTPRK, is reference to a gene or protein whose identity is shown in Figure 16.

Our invention can improve the quality of a patient's life by ensuring that new wounds do not deteriorate into a chronic state and existing chronic wounds can be treated in a way that actively promotes healing.

Statements of Invention
Accordingly, in one aspect of the invention there is provided a therapeutic comprising an inhibitor of either, or both, PTPRK gene expression or PTPRK protein activity for use in the treatment of wounds.

In the former instance, the invention involves a novel gene therapy approach and in the latter instance a novel protein therapy approach. Thus, in one embodiment the novel therapeutic comprises an inhibitor of PTPRK gene expression, this inhibitor can be either anti-sense DNA or RNA, siRNA, or ribozymes, either naked or in the form of plasmid and viral vectors. Those skilled in the art are aware of the aforementioned inhibitory molecules and so would be able to work the invention once they knew that expression of PTPRK contributed to the chronic wound phenotype. However, in another embodiment the novel therapeutic comprises an inhibitor of PTPRK protein function, this inhibitor can be either a PTPRK binding agent that binds, either reversibly or irreversibly, to inhibit protein function such as an antibody or a known, or synthesized, PTPRK antagonist; or an agent that works upstream or downstream of the PTPRK signalling mechanism to inhibit PTPRK signalling and so negate the effects of expression of PTPRK protein in wound tissue. Those skilled in the art are aware of the aforementioned inhibitory molecules and so would be able to work the invention once they knew that expression of PTPRK contributed to the chronic wound phenotype.
In a preferred embodiment of the invention the therapeutic comprises a PTPRK gene inhibitor such as transgene 1 or transgene 2 or transgene 3 described herein. These molecules are termed anti-PTPRK ribozyme/RNA transgenes.

Transgene 1 is produced by transcription of the PTPRK gene using the following short oligos:

**Anti-PTPRK transgenel F**

Ctgcgagtgtgatcctacagctgagctcggagga

**Anti-PTPRK transgenel R**

Actagtgacacaatgacctacatggtgatcggagga

Transgene 2 is produced by transcription of the PTPRK gene using the following short oligos:

**Anti-PTPRK transgene2F**

Ctgcgagtgtgatcctacagctgagctcggagga

**Anti-PTPRK transgene2R**

Actagtgacacaatgacctacatggtgatcggagga

Transgene 3 is produced by transcription of the PTPRK gene using the following short oligos:

**Anti-PTPRK transgene3F**

Ctgcgagtgtgatcctacagctgagctcggagga

**Anti-PTPRK transgene3R**

Actagtgacacaatgacctacatggtgatcggagga

These products are antisense-hammerhead ribozyme also known as antisense-hammerhead RNA, ideally they are flanked by selected restriction sites such as pstI and Spel and more ideally still they are cloned into a cloning vector such as pEF6A/5His-TOPO vector (Invitrogen).

The sequence structure of transgene 1 is:

5’ Ctgcgagtgtgatcctacagctgagctcggagga tacaaatcctgttactttg t'3
The sequence structure of transgene 2 is:

5’Ctgaggtgataggccatcgtggagttgggcatctgttgagactagt’3

The sequence structure of transgene 3 is:

Ctgaggtgataggccatcgtggagttgggcatctgttgagactagt’3

In a preferred embodiment of the invention the therapeutic comprises a commercially available PTPRK protein inhibitor such as, without limitation, Stibogluconate (GSK) or (Santa Cruz Biotechnologies Inc., Tocris and Sigma-Aldrich).

In a further preferred embodiment of the invention the therapeutic comprises a commercially available PTPRK protein inhibitor, such as Pentostam™ (GlaxoSmithKline).

The therapeutic of the invention is for use in treating, ideally, mammalian wounds, more ideally chronic mammalian wounds, and, more ideally still, chronic human wounds. Chronic wounds that are preferably treated using the invention are venous ulcers, diabetic ulcers, and pressure ulcers. Preferably, the wounds to be treated are non-parasitic i.e. not caused by or occupied by parasites.

An antibody for use in the invention is most ideally a monoclonal antibody or a humanised antibody.

In the above aspects and embodiments of the invention the therapeutic is formulated for topical application.

Alternatively, in the above aspects and embodiments of the invention the
therapeutic is formulated for oral application.

Alternatively again, in the above aspects and embodiments of the invention the therapeutic is formulated for application to a dressing or impregnation in a dressing.

The therapeutic of the invention may be administered in combination with an antibiotic or antibacterial agent. Numerous such agents are known and suitable choices will be familiar to skilled practitioners.

In yet another aspect of the invention, there is provided a pharmaceutical composition for use in treating wounds comprising a therapeutic of the invention together with a pharmaceutically acceptable carrier.

Other active materials may also be present in the pharmaceutical composition, as may be considered appropriate or advisable for the wound being treated. For example, the composition may also contain an emollient, or the like.

The carrier, or, if more than one be present, each of the carriers, must be acceptable in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient.

The formulations include those suitable for topical (including eye drops), oral (including buccal and sublingual), rectal, nasal or vaginal administration and may be prepared by any methods well known in the art of pharmacy.

The composition may be prepared by bringing into association the therapeutic of the invention and the carrier. In general, the formulations are prepared by uniformly and intimately bringing into association the active agent with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product. The invention extends to methods for preparing a pharmaceutical composition comprising bringing a therapeutic of the invention in conjunction or
association with a pharmaceutically or veterinarily acceptable carrier or vehicle.

For topical application to the skin, compounds of conventional use may be made up into a cream, ointment, jelly, solution or suspension etc. Cream or ointment formulations that may be used for the composition are conventional formulations well known in the art, for example, as described in standard text books of pharmaceutics such as the British Pharmacopoeia.

Formulations for oral administration in the present invention may be presented as: discrete units such as capsules, sachets or tablets each containing a predetermined amount of the active agent; as a powder or granules; as a solution or a suspension of the active agent in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water in oil liquid emulsion; or as a bolus etc.

For compositions for oral administration (e.g. tablets and capsules), the term "acceptable carrier" includes vehicles such as common excipients e.g. binding agents, for example syrup, acacia, gelatin, sorbitol, tragacanth, polyvinylpyrrolidone (Povidone), methylcellulose, ethylcellulose, sodium carboxymethylcellulose, hydroxypropylmethylcellulose, sucrose and starch; fillers and carriers, for example corn starch, gelatin, lactose, sucrose, microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, sodium chloride and alginic acid; and lubricants such as magnesium stearate, sodium stearate and other metallic stearates, glycerol stearate stearic acid, silicone fluid, talc waxes, oils and colloidal silica. Flavouring agents such as peppermint, oil of wintergreen, cherry flavouring and the like can also be used. It may be desirable to add a colouring agent to make the dosage form readily identifiable. Tablets may also be coated by methods well known in the art.

Other formulations suitable for oral administration include lozenges comprising the active agent in a flavoured or inert base base and mouthwashes comprising the active agent in a suitable liquid carrier.
In a further aspect of the invention there is provided a method for treating a mammalian wound, typically a chronic wound, which method comprises: administering to said wound a therapeutic that inhibits either, or both of, PTPRK gene expression or PTPRK protein activity.

Additionally, or alternatively, the further aspect of the invention also, or alternatively, comprises a novel method for treating a mammalian wound, typically a chronic wound, which method comprises: administering to said wound a therapeutic that inhibits either, or both of, PTPRK gene expression or PTPRK protein activity.

According to yet a further aspect of the invention there is provided a kit for treating a wound, preferably a chronic wound, wherein said kit comprises:

(a) at least one therapeutic as above described; and
(b) at least one dressing for applying to said wound.

According to a yet further aspect of the invention there is provided a combination therapeutic for treating a wound comprising an inhibitor of PTPRK gene expression and an inhibitor of PTPRK protein activity.

According to a further aspect of the invention there is provided a therapeutic for treating a wound comprising an inhibitor of PTPRK protein, or a homologoue thereof.

According to a further aspect of the invention there is provided use of an inhibitor of PTPRK protein, or a homologoue thereof, in the manufacture of a medicament for treating a wound.

According to a further aspect of the invention there is provided use of an inhibitor of PTPRK, or a homologue thereof, for treating a wound.
The term "homologue" as used herein refers to amino acid sequences which have a sequence at least 50% homologous to the amino acid sequence of PTPRK and which retain the biological activity of the PTPRK sequence. It is preferred that homologues are at least 75% homologous to the PTPRK peptide sequence and, in increasing order of preference, at least 80%, 85%, 90%, 95% or 99% homologous to the PTPRK peptide sequence.

Treatment of a wound described herein includes reference to human or veterinary use.

In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprises", or variations such as "comprises" or "comprising" is used in an inclusive sense i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

Preferred features of each aspect of the invention may be as described in connection with any of the other aspects.

Other features of the present invention will become apparent from the following examples. Generally speaking, the invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including the accompanying claims and drawings). Thus, features, integers, characteristics, compounds or chemical moieties described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein, unless incompatible therewith.

Moreover, unless stated otherwise, any feature disclosed herein may be replaced by an alternative feature serving the same or a similar purpose.

The present invention will now be described by way of the following examples
with particular reference to Figures 1-21 wherein:

Figure 1. Shows the secondary structure of human PTPRK mRNA;

Figure 2. Shows HaCaT cells after lost PTPRK by way of anti-PTPRK transgenes showed an increase in cell adhesion. Shown are traces (Top two panels) at 4000Hz and 32,000 Hz and 3D modelling at 4,000Hz and 500Hz (bottom two panels);

Figure 3. Shows effects of knocking down PTPRK in endothelial cells on the adhesion of the cells and their response to PTPRK inhibitor, stibogluconate. Left: traces of cells response in ECIS assays. Right: A: HECV WT, B: HECV/PTPRKrib; C: HECV wt plus stibogluconate; and D: HECV/PTPRKrib plus stibogluconate;

Figure 4. Shows effects of knocking down PTPRK in endothelial cells on cellular migration s and their response to PTPRK inhibitor, stibogluconate. Left: traces of cells response in ECIS assays. Right: A: HECV WT, B: HECV/PTPRKrib; C: HECV wt plus stibogluconate; and D: cHECV/PTPRKrib plus stibogluconate. Cell were wounded at 6v for 30 seconds and traced immediately after wounding;

Figure 5. Shows Traces (in triplicate) of HaCaT (WT) response to stibogluconate over an arrange of concentrations;

Figure 6. Shows 3D modelling of HaCaT (WT) adhesion response to stibogluconate over an arrange of concentrations;

Figure 7. Shows traces (in duplicate) of HaCaT (WT) response to stibogluconate over an arrange of concentrations. Shown are traces at 100hHz;

Figure 8. Shows 3D modelling of HaCaT (WT) migration response to stibogluconate over an arrange of concentrations. Shown at at 1000Hz;

Figure 9. Shows Using Rb modelling methods, a concentration dependent stimulation of cellular migration was also demonstrated. Shown are a 5-hour wounding assay, with mean plus SD displayed in the graph;

Figure 10. Shows the concentration related effect of Pentostam™ (GlaxoSmithKline), a commercially available form of stibogluconate on the migration of the cells;
Figure 11. Shows the effect of systemic administration of sodium stibogluconate, via the I.P. route, on the rate of wound healing.
Figure 12 Shows effects of stibogluconate on wound healing in the db/sb model. The compound was given topically every other day;
Figure 13. Shows the effect of weekly delivery of Stibogluconate on the rate of wound healing;
Figure 14. Shows the effect of twice weekly delivery of Stibogluconate on the rate of wound healing;
Figure 15. Shows a scatter plot of stibogluconate concentration vs size of the wounds after two weeks of treatment (weekly);
Figure 16. shows the amino acid and cDNA sequence structure of PTPRK;
Figure 17 shows the effect of removing treatment between the third and fourth week in either a weekly dosage regimen or a twice weekly dosage regimen; and Table 1. shows the primers and oligonucleotides used for the Construction and verification of ant-human PTPRK ribozyme transgenes described herein.

MATERIALS AND PROCEDURE

Cells
HaCaT, a human keratinocyte cell line was purchased from the German Cancer Centre, HECV, a human vascular endothelial cells from Interlab, Milan, Italy, DB/DB mice from Harlan UK.

Construction of ant-human PTPRK ribozyme transgenes
The transgenes were based on the human PTPRK mRNA secondary structure (Figure 1). Three transgenes were generated, targeting ATC and GTC sites, using respective oligos listed in Table 1. Ribozymes were generated by way of touchdown PCR, followed by verification using 2% agarose gels. The correct ribozymes were ligated into a pEF6A/5His-TOPO vector (Invitrogen), followed by transformation of the ligated product to Top10 E.Coli. After heat shock for 30 seconds and recover over ice for 2 minutes, the bacteria was resuspended in SOC medium and allow to grow on a shaker (200rpm) for 1 hour. The
transformed bacteria were then plated over LB agar dishes which contained 100ug/ml Ampicillin. After incubating the plate at 37°C overnight, discreet colonies were identified and screened for the presence of the ribozyme and the orientation of the insert, by using orientation specific PCR, using T7F primers vs RBBMR and RBTPF primers. Correct colonies were picked, grew up in LB medium in the presence of Ampicillin. Plasmids were extracted, purified and further verified by direction specific PCR (using RBTOP vs T7F and RBBMR).

Generation of sublines of human keratinocytes and endothelial cells with differential expression of PTPRK

HaCaT and HECV cells, which were positive for PTPRK, were transfected with anti-PTPRK transgenes by way of electroporation (270v). After selection with a selection medium (DMEM with 10ug/ml blasticidin) for 10 days, clones of selected cells were pooled and used for subsequent analysis.

In vivo tolerance test

The first tolerance test was conducted on the CD-1 athymic (Charles River Laboratories). Briefly, CD-1 of 4-6 weeks old, 20g in weight, were housed in filter topped cages. Sodium stibogluconate a known PTPRK inhibitor was injected, via the intraperitoneal route, on a daily basis. The compound was given at 100 final concentration (equivalent to ~10mg/kg body weight) in 100ul in volume. CD-1 were observed daily, weighed twice weekly. An additional tolerance and efficacy test was carried out using the db/db strain.

In vivo efficacy test and wound healing

The diabetic strain of db/db was obtained from Harlan. 4-6 weeks old with body weight at 20g were used. Creation of a wound was according to a recently described method. Briefly, after being housed for a week, the db/db were first ear-pieced using a puncher, in order to create a wound (hole) of 1mm in diameter. The following day after wound creation, all the db/db were weighed and the wound was photographed using a digital camera. Treatment was given systemically (by IP injection) or topically (by manually applying the compound in
gel into the wound area). Both treatments were given every other day, twice weekly or weekly. Images were obtained weekly. The size of the wounds was determined using an image analysis software and is shown here as the area in pixels.

5

**Effects of knocking down PTPRK on the function of cells**

Three models of ECIS instrument were used: ECIS 9600 for screening and ECIS1 600R and ECIS ΖΘ for modelling. In all systems, 8W10 arrays were used (Applied Biophysics Inc., Troy, NY, USA) (Giaever and Keese 1991, Kees et al 2004). Following treating the array surface with a Cysteine solution (or array stabilization procedure for ECIS ΖΘ), the arrays were incubated with complete medium for 1 hr Electric changes were continuously monitored for up to 24 hrs. In the 9600 system, the monitoring was at fixed 30Hz. In the 1600R and ECIS ΖΘ systems, cells were monitored at 62.5, 125, 250, 500, 1,000, 2,000, 4,000, 8,000, 16,000, 32,000 and 64,000Hz. The adhesion was analysed by the integrated Rb modelling method.

**RESULTS**

*Knocking down PTPRK from HaCaT and endothelial cells resulted in an acceleration of cell adhesion and migration*

It was found that after knocking down PTPRK in HaCaT cells, there was a rapid increase in cell adhesion, Figure 2. Endothelial cells, after loss of PTPRK, showed a high rate of adhesion using an ECIS assay. Likewise, HECV/WT when treated with stibogluconate, also showed a rapid adhesion to the surface of the electrode. It is interesting to observe that HECV/PTPRKrib cells' response to stibogluconate was markedly reduced compared with that of HECV/WT. The similar changes in cellular migration were seen using the electric wounding assay of the endothelial cell model, Figure 3 and Figure 4.

*Human keratinocytes showed a dose dependent response to PTPRK inhibitor stibogluconate*
Using the ECIS Theta96 model, we tested the response of cells to stibogluconate over a range of concentrations. HaCaT cells responded over the range of concentrations tested in that there was an increase in cell adhesion between 0.16 - 20uM with 20uM showing the maximum effects, Figures 5 & 6. Likewise, the cells also responded to stibogluconate by increasing their migration from concentrations as low as 160nM to 100uM, Figures 7, 8 & 9.

We have also tested the concentration related effect of Pentostam™ (GlaxoSmithKline), a commercially available form of stibogluconate, on the migration of the cells, Figure 10.

**Stibogluconate was well tolerated in vivo**

The first tolerance test was conducted the CD-1 athymic (Charles River Laboratories). Briefly, CD-1 of 4-6 weeks old, 20g in weight, were housed in filter topped cages. Sodium stibogluconate was injected, via the intraperitoneal route, on a daily basis. The compound was given at 100 final concentration (equivalent to 10 mg/kg body weight) in 10Oul in volume. CD-1 were observed daily, weighed twice weekly. An additional tolerance and efficacy test was carried out using the db/db strain.

**Stibogluconate accelerates wound healing in vivo.**

*Formulation of the compounds.*

1. **For systemic application.** Sodium stibogluconate was dissolved in BSS and diluted in the same for the required concentration. The solutions were prepared that each 10Oul contained the correct amount of compounds and was aliquotted and stored as such at -20°C until used. The compound was injected every other day by the IP route.

2. **For topical application,** we used two carrier gels that are currently used in wound care, namely Bactroban and Aquagel. From the concentrated master stock of Sodium stibogluconate, 10Oul of the stock solution was mixed with 2 grams of the respective gels, followed by low speed
homogenisation using a hand held homogeniser, for 2 minutes. The newly formulated gels which showed no signed changes of the strength and consistency, were stored at 4°C until use. For use, small amount (150ul) of the gel was applied to the wound area and gently rubbed in using fingers.

3. Sodium stibogluconate was well tolerated

We have delivered the compounds systemically every other day, for a two week period in db/db. Throughout the study, we did not observe any side effects. There was no weight loss in any of the groups.

4. Sodium stibogluconate increased the rate of wound healing without producing any side effects.

Sodium stibogluconate was given systemically, at 100uM. After one week, wounds in the treated were smaller than the control group as shown in Figure-1 (p=0.0927 vs control).

However, topical application of Sodium stibogluconate showed no significant effect after one week, both in Bactroban and in Aquagel (figure-2 and 3).

In vivo test on the dosing effect and exploration of the optimal way of applying the stibogluconate

Using the same db/db mice, we further tested the possible dose response by applying stibogluconate at 2mg/ml. 20mg/ml and 100mg/ml, using topical applications. At the same time, we tested two treatment methods: applying the agent on a weekly basis or twice weekly basis. We determined the size of the wound on a weekly basis. It was clear that both weekly and twice weekly application resulted in a rapid rate of wound healing. It was also clear that the therapeutic effects of stibogluconate is dependent on the dosage, in that the highest concentration used, namely 100mg/ml appear to be most effective of all the concentrations using in the present study. Using a Two-way ANOVA (Holm-
Sidak model), it was shown that in both treatment regimes, there was a highly significant difference between the treatment group and control group, \( p=0.013, 0.10 \) and 0.009, control vs 2mg/ml, 20mg/ml and 100mg respectively, for the twice weekly treatment, and \( p=0.05, 0.013, 0.009 \) for the weekly treatment group.

Using Spearman correlation coefficient, we have found that after two weeks treatment, the size of the wounds was significantly correlated with the concentration (\( p=0.049, r=-0.950 \)).

Further, we have also shown that interrupting treatment, in either a weekly or twice weekly dosing regimen, prior to complete healing had a significant effect on the healing process, resulting a noticeable reduction in wound closure (figure 17).

**Summary**

The main findings of the present study can be summarised as follows:

In wound tissue PTPRK is an important regulator of the migration of keratinocytes. PTPRK responds to a PTPRK inhibitor, stibogluconate, by way of increasing the adhesion and in particular migration of keratinocytes and also the migration of vascular endothelial cells. Moreover, Stibogluconate has a concentration dependent effect on the migration of keratinocytes. *In vivo*, both topical and systemic administration of stibogluconate increased the rate of wound healing, without noticeable side effects. The effect of stibogluconate on wound healing *in vivo* appears to be dose dependent. Both weekly and twice weekly administration of stibogluconate significantly increased the rate of wound healing, although twice weekly appears to be marginally more effective.

Interrupting the treatment regimen adversely affects the healing process.

These findings collectively show that PTPRK is critical in controlling the migration and healing of wounds. Thus, both *in vitro* and clinical data point to PTPRK being an important therapeutic target in wounds.
Table 1. Primer and oligo sequences used in the present study.

<table>
<thead>
<tr>
<th>Primer names</th>
<th>Sense primers</th>
<th>Anti-sense primers</th>
</tr>
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<tbody>
<tr>
<td>PTPRK pair F11/R11</td>
<td>aattacaattgatggggaga</td>
<td>Ccacttttccacctgaagta</td>
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<tr>
<td>PTPRK pair ZF11/ZR11</td>
<td>aattacaattgatggggaga</td>
<td>actgaacctgaccgtacacatatttggtgacgatgaaac</td>
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<td>PTPRK pair F12/R12</td>
<td>Gcgagtcaagtataaacc</td>
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<td>PTPRK pair ZF12/ZR12</td>
<td>gcaggtcagttataaacc</td>
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Claims

1. A therapeutic comprising an inhibitor of either, or both, PTPRK gene expression or PTPRK protein activity; or an inhibitor of a protein that is at least 50% homologous to PTPRK for use in the treatment of wounds.

2. A therapeutic according to claim 1 wherein said inhibitor is an inhibitor of PTPRK gene expression.

3. A therapeutic according to claim 2 wherein said inhibitor is selected from the group consisting of: anti-sense DNA or RNA, siRNA, or ribozymes, either naked or in the form of plasmid or viral vectors.

4. A therapeutic according to claim 3 wherein said inhibitor is anti-PTPRK ribozyme/RNA transgene selected from the group comprising:

transgene 1 5'Ctgcagagtgagttacacagcctgatgagtccgtgaggacgaaa
tacaatctgtgccagttttgtt actagt'3;

transgene 2 5'Ctgcaggatgataggaccatcgccaatctgatgagtccgtgaggacgaaa
tcgagtggcattttagtggatcactagt'3; and

transgene 3 Ctgaggtttgcttttttacaattaatatctgtgagtccgtgaggacgaaa
caatgggaggagaggatgaactagt'3.

5. A therapeutic according to claim 1 wherein said inhibitor is an inhibitor of PTPRK protein function.

6. A therapeutic according to claim 5 wherein said inhibitor is selected from the group consisting of: a PTPRK binding agent that binds, either reversibly or irreversibly, to inhibit protein function such as an antibody or a known, or synthesized, PTPRK antagonist; or an agent that works upstream or downstream of the PTPRK signalling mechanism to inhibit PTPRK function.

7. A therapeutic according to claim 6 wherein said inhibitor is Stibogluconate, or a salt thereof, or Pentostam™ (GlaxoSmithKline).

8. A therapeutic according to any preceding claim wherein the therapeutic is
formulated for use in treating mammalian wounds.

9. A therapeutic according to any preceding claim wherein the therapeutic is formulated for use in treating chronic wounds comprising venous ulcers, diabetic ulcers, and pressure ulcers.

10. A therapeutic according to any preceding claim wherein the therapeutic is formulated for use in treating human wounds.

11. A therapeutic according to any preceding claim wherein the therapeutic is formulated for topical application.

12. A therapeutic according to any preceding claim wherein the therapeutic is formulated for application to a dressing or impregnation in a dressing.

13. A pharmaceutical composition comprising a therapeutic according to any preceding claim together with a pharmaceutically acceptable carrier.

14. A method for preparing a pharmaceutical composition according to claim 13 comprising bringing said therapeutic in conjunction or association with a pharmaceutically or veterinarily acceptable carrier or vehicle.

15. A method for treating a mammalian wound which method comprises: administering to said wound a therapeutic that inhibits either, or both of, PTPRK gene expression or PTPRK protein activity.

16. A kit for treating a wound wherein said kit comprises:
(a) at least one therapeutic according to claims 1-12 or a composition according to claim 13; and
(b) at least one dressing for applying to said wound.

17. A combination therapeutic for treating a wound comprising an inhibitor of PTPRK gene expression and an inhibitor of PTPRK protein activity.

18. A combination therapeutic for treating a wound comprising:
a) either an inhibitor of PTPRK gene expression or an inhibitor of PTPRK protein activity; and
b) at least one further therapeutic.

19. Use of an inhibitor of either, or both, PTPRK gene expression or PTPRK protein activity; or an inhibitor of a protein that is at least 50% homologous to PTPRK and that modulates cell adhesion or cell migration for treating a wound.
Figure 1 Secondary structure of human PTPRK mRNA

dG = -1251.38
Figure 2A HaCaT cells after lost PTPRK by way of anti-PTPRK transgenes showed an increase in cell adhesion. Shown are traces at 4000Hz and 3D modeling at 4,000Hz and 500Hz.
Figure 2B HaCaT cells after lost PTPRK by way of anti-PTPRK transgenes showed an increase in cell adhesion. Shown are traces at 32,000 Hz and 3D modelling at 4,000Hz and 500Hz.
Figure 3

Effects of knocking down PTPRK in endothelial cells on the adhesion of the cells and their response to PTPRK inhibitor, stibogluconate. Left: traces of cells response in EICS assays. Right: A: HECV WT; B: HECV+PTPKRab; C: HECV wt plus stibogluconate, and D: HECV+PTPKRab plus stibogluconate.
Figure 4

Effects of knocking down PTPRK in endothelial cells on cellular migration s and their response to PTPRK inhibitor, stibogluconate. Left: traces of cells response in ECIS assays. Right: A: HECV WT, B: HECV/PTPRKnib; C: HECV wt plus stibogluconate; and D: cHECV/PTPRKnib plus stibogluconate. Cell were wounded at 6v for 30 seconds and traced immediately after wounding.
Figure 5

Traces (in triplicate) of HaCaT (WT) response to stibogluconate over an arrange of concentrations.
Figure 6 3D modelling of HaCaT (WT) adhesion response to stibogluconate over an arrange of concentrations.
Figure 7 Traces (in duplicate) of HaCaT (WT) response to stibogluconate over an array of concentrations. Shown are traces at 100Hz.
Control, 0.8 μM  20 μM
Cell free

1,000Hz, 12 hours,
Ztheta96

Figure 8 3D modelling of HaCaT (WT) migration response to stibogluconate over an arrange of concentrations. Shown at at 1000Hz
Figure 9

Using Rb modelling methods, a concentration dependent stimulation of cellular migration was also demonstrated. Shown are a 5-hour wounding assay, with mean plus SD displayed in the graph.
Figure 10 Shows the concentration related effect of Pentostam\textsuperscript{TM} (GlaxoSmithKline), a commercially available form of stibogluconate on the migration of the cells.
Figure 11  The effect of systemic administration of sodium stigbogluconate, via the I.P. route, on the rate of wound healing.
Figure 12
Effects of stibogluconate on wound healing in the db/sb model. The compound was given topically every other day.
Figure 13  The effect of weekly delivery of Stibogluconate on the rate of wound healing.
Figure 14  The effect of twice weekly delivery of Stibogluconate on the rate of wound healing.
Figure 15 Scatter plot of stibogluconate concentration vs size of the wounds after two weeks of treatment (weekly).
cDNA sequence of human PTPRK
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121 ggtcctcaggg gctgtgatta ccaccccgag atgtcgcccc aagtttcctta tatgatatgt
181 agtgctcaag acgccttatta ttcaccaccc gagaatgc cag cag cag cag cag cag aatgaagagg
241 gactcttcag atcagccct cggagaaaaa ggccagcttc acgtgccttc aatgaagagg
301 aacgacactc actgcatttg tgtaccttac ctatttata gccagaaagg actgaatcct
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421 ggtactttga tcacgggtag agattgctt cggcgtgagc tagcagtzg ctcctttggg
481 cccaatgtaa atcagtttaat attgaagct gaaagttccag gaggagaag ttggttattt
541 gcctgtgtag actacccaa tcaccagttct ccttgtgata aatcctctca ttctcttggagt
601 ctaggggtag tggagttgaa tcgacggcggc aacgctcata gtcagttgcat tgcacccaggg
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1021 catgcgtcag atgcgtcagc caataatcag ttgcaatgta atccagatac gaaatgag
1081 atcaggcttc atcactacag acctggtgaa gttggaaacgg ggtttcctcag acctccacta
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Figure 16
SUBSTITUTE SHEET (RULE 26)
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1981 gcccaggttg gggggcacc gattactttgt gtcgacaaac tacccccgggg aaacctaccct
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Figure 16 (continued)
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Figure 16 (continued)

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Figure 16 (continued)
Protein sequence of human PTPRK

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Figure 16 (continued)
Figure 17
A. CLASSIFICATION OF SUBJECT MATTER

 According to International Patent Classification (IPC) into both national classification and IPC

 A61K31/29

 B. FIELDS SEARCHED

 Minimum documentation searched (classification system followed by classification symbols)

 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, MEDLINE, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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**S** document member of the same patent family

Date of the actual completion of the international search

9 May 2012

Date of mailing of the international search report

24/05/2012

Name and mailing address of the ISA/
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Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016

Authorized officer

Sindler, Mark-Peter
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