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(54) Title: USE OF CYCLOPAMINE IN THE TREATMENT OF BASAL CELL CARCINOMA AND OTHER TUMORS

(57) Abstract: This invention concerns the use of cyclopamine in vivo on basal cell carcinomas to achieve therapeutic effect by causing differentiation of the tumor cells and, at the same time, highly efficient apoptotic death and removal of these tumor cells while preserving the normal tissue cells, including the undifferentiated cells of the normal epidermal basal layer and hair follicles. Causation of apoptosis by cyclopamine is by a non-genotoxic mechanism. These effects make the use of cyclopamine highly desirable in the treatment of basal cell carcinomas and other tumors that use the hedgehog/smoothened signal transduction pathway for proliferation and prevention of apoptosis.

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Use of Cyclopamine in the Treatment of Basal Cell Carcinoma and Other Tumors

The invention concerns use of cyclopamine, an inducer of the differentiation and apoptosis of the basal cell carcinoma (BCC) cells, in the treatment of BCC's and other tumors that use the hedgehog/smoothened pathway for proliferation and prevention of apoptosis.

This invention concerns the use of cyclopamine *in vivo* on basal cell carcinomas (BCC's) to achieve therapeutic effect by causing differentiation of the tumor cells and, at the same time, apoptotic death and removal of these tumor cells while preserving the normal tissue cells, including the undifferentiated cells of the normal epidermal basal layer and hair follicles. Causation of apoptosis by cyclopamine is by a non-genotoxic mechanism and thus unlike the radiation therapy and most of the currently used cancer chemotherapeutics which act by causing DNA-damage. These novel effects, previously unachieved by a cancer chemotherapeutic, make the use of cyclopamine highly desirable in cancer therapy, in the treatment of BCC's and other tumors that use the *hedgehog/smoothened* signal transduction pathway for proliferation and prevention of apoptosis.

According to a first aspect, the present invention provides use of cyclopamine or a pharmaceutically acceptable salt or derivative thereof that is capable of inducing apoptosis and differentiation of the tumor cells that employ Hedgehog/Smoothened signaling for prevention of these processes, for the manufacture of a medicament, wherein said medicament is to be administered in an amount which causes decrease of size or disappearance of a tumor that employs Hedgehog/Smoothened signaling for prevention of said processes in tumor cells.

According to a second aspect, the present invention provides a method for inducing apoptosis and differentiation of tumor cells, comprising identification of the tumor cells that use Hedgehog/Smoothened signaling for prevention of these processes and contacting said tumor cells with a sufficient amount of cyclopamine or a pharmaceutically acceptable salt or derivative thereof that is capable of inducing apoptosis and differentiation of said tumor cells.

According to a third aspect, the present invention provides cyclopamine or a pharmaceutically acceptable salt or derivative thereof that is capable of inducing apoptosis and differentiation of the tumor cells that employ Hedgehog/Smoothened signaling for prevention of these processes when used for inducing apoptosis and

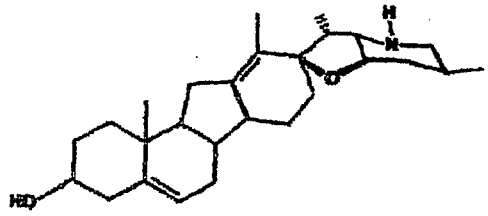
differentiation of tumor cells by causing decrease of size or disappearance of a tumor that employs Hedgehog/Smoothened signaling for prevention of said processes in tumor cells.

Basal cell carcinoma is a common epithelial tumor. Its incidence increases with increasing age. Current treatments for BCC's include the surgical excision of the tumor together with a margin of normal tissue and, when surgery is not feasible or desirable, destruction of the tumor cells by ionizing radiation or other means. Although scarring and disfigurement are potential side effects, surgical excisions that do not leave neoplastic cells behind can provide cure. Radiation therapy acts by causing irreparably high quantity of DNA-damage which, in turn, triggers apoptotic death of the tumor cells. This mode of action of radiation-therapy, i.e. apoptosis secondary to DNA-damage, is similar to those of many chemotherapeutic agents that are currently used in the treatment of cancers. However both radiation therapy and the cytotoxic cancer chemotherapeutics are capable of causing DNA-damage in the normal cells of patients in addition to the tumor cells. As a result their effectivity and usefulness in cancer therapy are seriously limited. A further dilemma with the use of radiation and genotoxic cancer chemotherapeutics is the disturbing fact that, even when cure of the primary tumor is achieved, patients have markedly increased risk of developing new cancers because of the DNA-damage and the resulting mutations they have undergone during the treatment of primary tumor. Induction of apoptosis selectively in tumor cells by non-genotoxic means would

therefore be most desirable in the field of cancer therapy.

BCC's frequently show inactivating mutations of the gene *patched* which encodes a transmembrane protein acting as a receptor for the *hedgehog* proteins identified first by their effect on the patterning of tissues during development. When not liganded by *hedgehog*, the *patched* protein acts to inhibit intracellular signal transduction by another transmembrane protein, *smoothened*. Binding of *hedgehog* to the *patched* causes relieving of this inhibition. Intracellular signal transduction by the relieved *smoothened* then initiates a series of cellular events resulting ultimately in alterations of the expressions of the *hedgehog* target genes and of cellular behaviour. General features of this *hedgehog/smoothened* pathway of signal transduction, first identified in Drosophila, are conserved in diverse living organisms from Drosophila to Human. However the pathway gets more complex in more advanced organisms (e.g. presence in human of more than one genes that display significant similarity to the single *patched* gene of Drosophila). Inactivating mutations of the *patched* have been found to cause constitutive (ligand-free) signalling through the *hedgehog/smoothened* pathway. The *hedgehog/smoothened* pathway overactivity, resulting from mutations of the *patched* and/or further downstream pathway elements, is found in all BCC's. The nevoid basal cell carcinoma syndrome (NBCCS) results from *patched* haploinsufficiency. Patients with the NBCCS, because of an already mutant *patched* in all cells, develop multiple BCC's as they grow older.

Cyclopamine, a steroid alkaloid, has the chemical formula shown below.



It is found naturally in the lily *Veratrum californicum* and can be obtained by purification from this and other sources. Inhibition of the *hedgehog/smoothened* pathway by cyclopamine has been found in chicken embryos and in cultured cells of mouse.

For topical applications, cyclopamine can be dissolved in ethanol or another suitable solvent and mixed with a suitable base cream, ointment or gel. Cyclopamine may also be entrapped in hydrogels or in other pharmaceutical forms enabling controlled release and may be adsorbed onto dermal patches. The effects

shown in figures Fig 1A to Fig 1D, Fig 2A to Fig 2F, Fig 3A to Fig 3G and Fig 4A to Fig 4D have been obtained by a cream preparation obtained by mixing a solution of cyclopamine in ethanol with a base cream so as to get a final concentration of 18 mM cyclopamine in cream. The base cream used is made predominantly of heavy paraffin oil(10% w/w), vaseline (10% w/w), stearyl alcohol (8% w/w), polyoxyistearеth-40 (3% w/w) and water (68% w/w) but another suitably formulated base cream is also possible. Optimal concentration of cyclopamine in a pharmaceutical form as well as the optimal dosing and application schedules can obviously be affected by such factors as the particular pharmaceutical form, the localisation and characteristics of the skin containing the tumor (e.g. thickness of the epidermis) and the tumor size; however these can be determined by following well known published optimisation methods. The dosing and the application schedules followed for the tumors shown in Fig 1A (BCC on the nasolabial fold, ~ 4x5 mm on surface) and Fig 1C (BCC on the forehead, ~ 4x4 mm on surface) are as follows: 10 ± 2 µl cream applied directly onto the BCC's with the aid of a steel spatula four times Per day starting ~ 9.00 a.m. with ~ 3½ hours in between. Night-time applications, avoided in this schedule because of possible loss of cream from the patient skin to linens during sleep, can be performed by suitable dermal patches. Preservation of the undifferentiated cells in the normal epidermis and in hair follicles following exposure to cyclopamine, as described in this invention, provide information about the tolerable doses in other possible modes of administration as well; e.g. direct intratumoral injection of an aqueous solution or systemic administration of the same or of cyclopamine entrapped in liposomes.

Fig 1A, Fig 1B, Fig 1C and Fig 1D show rapid clinical regressions of the BCC's following exposure to cyclopamine. Besides the visual disappearence of several tumor areas within less than a week of cyclopamine exposure, there is a loss in the typically translucent appearance of the BCC's as seen by the comparison of Fig 1B to Fig 1A and of Fig 1D to Fig 1C.

Fig 2A to Fig 2F show microscopic appearences of the tumor areas subjected to surgical excisions together with a margin of normal tissue on the fifth and sixth days of cyclopamine applications when the BCC's had lost most of their pre-treatment areas but still possessed few regions that, although markedly decreased in height, had not yet completely disappeared and therefore had residual tumor cells for microscopic analyses.

Fig 2A and Fig 2B show, on tissue sections, the skin areas corresponding to the visually disappeared tumor nodules. The tumors are seen to have disappeared to leave behind large cystic structures containing little material inside and no detectable tumor cells.

Fig 2C shows microscopic appearance of a skin area that contained still visible BCC in vivo. These regions are seen to contain residual BCC's displaying large cysts in the tumor center and smaller cystic structures of various sizes located among the residual BCC cells towards the periphery.

Fig 2D and Fig 2E show 1000X magnified appearances from the interior and palisading peripheral regions of these residual BCC's and show the presence of massive apoptotic activity among the residual BCC cells regardless of the tumor region. These high magnifications show greatly increased frequency of the BCC cells displaying apoptotic morphology and formation of the cystic structures by the apoptotic removal of cells as exemplified on Fig 2D by the imminent joining together of the three smaller cysts into a larger one upon removal of the apoptotic septal cells.

Fig 2F shows that the BCC's treated with the placebo cream (i.e. the cream preparation identical to the cyclopamine cream except for the absence of cyclopamine in placebo) show, by contrast, the typical neoplastic BCC cells and no detectable apoptotic activity.

Cells undergoing apoptosis are known to be removed by macrophages and by nearby cells in normal tissues and the quantitation of apoptotic activity by morphological criteria on hematoxylene-eosine stained sections is known to provide an underestimate. Despite these, the quantitative data shown in Table I show greatly increased apoptotic activity caused by cyclopamine among the residual BCC cells.

The loss of translucency in the cyclopamine-treated BCC's raises the intriguing possibility of differentiation of BCC's under the influence of cyclopamine. This possibility, which can be tested by immunohistochemical analyses of the BCC's, is found to be the case in this invention. In normal epidermis, differentiation of basal layer cells to the upper layer cells is accompanied by a loss of labelling with the monoclonal antibody Ber-Ep4. Ber-Ep4 labels also the BCC cells and is a known marker for these neoplasms. Fig 3A, Fig 3B and the quantitative data on Table I show that, while Ber-Ep4 strongly labels all

peripheral palisading cells and over 90% of the interior cells of the placebo-treated BCC's, none of the residual peripheral or interior cells of the cyclopamine-treated BCC's are labelled by Ber-Ep4. Differentiation of the BCC's under the influence of cyclopamine, hitherto unknown by any other means and highly unusual because of achievement of it in vivo and in all cells by immunohistochemical criteria, has independent value in the treatment of cancer.

Another differentiation marker, *Ulex Europaeus* lectin type 1, normally does not label the BCC's or the basal layer cells of normal epidermis but labels the differentiated upper layer cells. Fig 3C, showing the heterogenous labelling of the residual cells of cyclopamine-treated BCC's with this lectin, shows differentiation of some of the BCC cells beyond the differentiation step detected by Ber-Ep4 all the way to the step detected by *Ulex Europaeus* lectin type 1.

The p53 is a master regulator of the cellular response to DNA-damage. Amount of this protein is known to increase in the cell nucleus following exposure of cells to genotoxic agents. When the DNA-damage is increased beyond a threshold, p53 serves for the apoptotic death of cells. Radiation therapy of cancer and the genotoxic cancer chemotherapeutics that are currently common, act largely by this mechanism, i.e. by causation of apoptosis secondary to the damaging of DNA. The monoclonal antibody DO-7 can bind both normal and missense mutant (i.e., nonfunctional) forms of p53 and is known to be capable of detecting the increase of p53 in the cells following exposure to DNA-damaging agents.

Fig 3D, Fig 3E and the quantitative data in Table I show that both the DO-7 labelling intensity and the frequency of labelled cells are markedly decreased in cyclopamine-treated BCC's in comparison to the placebo-treated BCC's. Thus cyclopamine causes, not an increase, but rather a decrease of p53 in the nuclei of cyclopamine-treated BCC cells. Since expression of p53 is known to decrease in epidermal cells upon differentiation, the decreased DO-7 labelling of the cyclopamine-treated BCC's is likely to be secondary to the cyclopamine-induced differentiation of the BCC cells. In any case, massive apoptotic activity in the cyclopamine-treated BCC's despite markedly decreased p53 expression means that the cyclopamine-induced apoptosis of these tumor cells is by a non-genotoxic mechanism.

Arrest of the proliferation of BCC's is known to be associated with their retraction from stroma. Although retraction from stroma can also be caused artefactually by improper fixation and processing of the tissues, adherence to published technical details ensures avoidance of such artefacts. As shown in Fig 3F and Fig 3G, cyclopamine-treated, but not placebo-treated, BCC's are consistently retracted from stroma. Exposure BCC's to cyclopamine thus appears to be associated also with an arrest of proliferation.

Fig 4A to Fig 4D show Ber-Ep4 labelling of the normal skin tissue found on and around the cyclopamine-treated BCC's. Different epidermal areas that were treated with cyclopamine are seen in Fig 4A, Fig 4B and Fig 4C to display normal pattern of labelling with Ber-Ep4, i.e. labelling of the basal layer cells. Similarly Fig 4D shows normal Ber-Ep4 labelling of a hair follicle exposed to cyclopamine. Thus the undifferentiated cells of normal epidermis and of hair follicles are preserved despite being exposed to the same schedule and doses of cyclopamine as the BCC's.

Causation of highly efficient differentiation and apoptosis of the tumor cells in vivo by cyclopamine at doses that preserve the undifferentiated tissue cells are hitherto unknown achievements that, together with the non-genotoxic mode of action of cyclopamine, support the use of cyclopamine not only on BCC's but also on those internal tumors that utilize the hedgehog/smoothened pathway for proliferation and for prevention of apoptosis and/or differentiation.

It is specifically contemplated that molecules can be derived from cyclopamine or synthesised in such a way that they possess structural features to exert similar receptor binding properties and biological/therapeutic effects as cyclopamine. Such a molecule is called here as a "derivative of cyclopamine" and defined as follows: A molecule that contains the group of atoms of the cyclopamine molecule required for the binding of cyclopamine to its biological target but contains also modifications of the parent cyclopamine molecule in such ways that the newly derived molecule continues to be able to bind specifically to the same biological target to exert the biological effects of cyclopamine disclosed herein. Such modifications of cyclopamine may include one or more permissible replacement of or deletion of a molecular group in the cyclopamine molecule or addition of a molecular group (particularly a small molecular group such as the methyl group) to the cyclopamine molecule provided that the resultant molecule is stable and possesses the capability of specific binding to the same biological target as cyclopamine to exert the biological effects described herein. Derivation of such new molecules from cyclopamine can be readily achieved by those skilled in the art and the possession or lack of the biological effects of cyclopamine in the newly derived molecule can also be readily determined by those skilled in the art by testing for the biological effects disclosed herein.

Further Examples

Fig 5A shows a large ulcerated BCC on the upper nasal region of a 68 years old man prior to treatment. Cyclopamine cream (18 mM cyclopamine in the base cream described above) was applied to the lower half of the BCC shown in Fig 5A. Every third hour, ~ 20 µl cream was applied directly onto the lower half and the upper half was left untreated. Thus the tumor cells in the uppermost part (Fig 5A) are least likely to receive cyclopamine by possible diffusion from the directly applied region and will be exposed to relatively much lower concentrations of cyclopamine, if any. Fig 5B shows the tumor on the 54th hour of treatment just prior to surgical excision for investigation. While rapid regression of the tumor is evident in the cyclopamine-applied lower half, the region of the tumor furthest away from the directly applied half is seen to be relatively unaltered (Fig 5B; the region towards the upper right corner of figure). Fig 5C shows a hematoxylene-eosine stained section from the lower (cyclopamine-treated) part of the excised tissue. Numerous apoptotic cells are seen together with variously sized cysts that are forming as a result of the death and removal of the tumor cells (Fig 5C). In contrast, the non-treated region of the same tumor furthest away from the cyclopamine-applied half shows a solid tumor tissue with mitotic figures and no detectable apoptotic cells (Fig 5D). Fig 5E and Fig 5F show the immunohistochemically stained tissue sections from the cyclopamine-treated and non-treated regions, respectively, of the tumor using the monoclonal antibody Ki-S5 (Dako A/S, Glostrup, Denmark) against the Ki-67 antigen. The Ki-67 antigen, which is a known marker of the proliferating cells, is no longer expressed in the cyclopamine-treated region of the tumor (Fig 5E) while the tumor cells furthest away from the cyclopamine-applied region clearly display proliferative activity (Fig 5F). Thus staining of the tissue sections with an antibody against the Ki-67 antigen shows again arrest of tumor cell proliferation by cyclopamine under the conditions described.

Trichoepithelioma is another tumor associated with genetic changes that cause increased hedgehog-smoothened signalling (Vorechovsky I. et al. (1997) Cancer Res. 57:4677-4681; Nilsson M. et al. (2000) Proc. Natl. Acad. Sci. USA 97:3438-3443). Fig 6A shows a trichoepithelioma on the cheek of a 82 years old man prior to treatment and Fig 6B shows the same skin area after only 24 hours of exposure to the cyclopamine cream (18 mM cyclopamine in the base cream ; ~25 µl cream was applied every third hour directly onto the tumor). Because of the rapid regression, treatment was discontinued on the 24th hour and the entire skin area corresponding to the original tumor was excised for investigation. Fig 6C and Fig 6D show the tissue regions that contained residual tumor cells on the 24th hour and reveal marked apoptotic activity among these residual tumor cells. Cystic spaces resulting from the apoptotic removal of tumor cells (Fig 6C, Fig 6D) as well as the mononuclear cellular infiltration of tumor (Fig 6D) are seen. Another noteworthy finding in this patient was the decreased size and pigmentation of a mole located nearby the treated tumor on the 24th hour of treatment (Fig 6B versus Fig 6A). As cyclopamine could have diffused from the adjacent area of

application, the mole (a benign melanocytic tumor) appears to be sensitive to relatively low concentrations of cyclopamine.

Fig 7A shows a pigmented BCC on the lower eyelid of a 59 years old man prior to treatment. Cyclopamine cream (18 mM cyclopamine in the base cream) was applied in this patient onto all of the nodules except for the one marked by the arrow. This nodule, which could have received cyclopamine only by diffusion from the adjacent treated region, would be exposed to a relatively lower concentration of cyclopamine. As the pigmented nature of this tumor facilitated clinical follow-up, treatment (application of ~20 µl cyclopamine cream on every fourth hour) was discontinued on the third day when the tumor in the treated region had largely regressed but still contained visible parts (Fig 7B). The tumor was then followed up without treatment for a study of the possible late effects. A clear further clinical regression was not observed in the absence of treatment and the skin area corresponding to the original tumor was excised on the sixth day of follow up (ninth day from the start of treatment). Hematoxylene-eosine stained sections from the treated region of tumor revealed many cystic spaces that lacked tumor cells (Fig 7C). The absence of an epithelium lining these cysts (Fig 7C) is consistent with the representation by these cysts of the tissue areas that were formerly occupied by the tumor cells. At this time point (the sixth day of non-treated follow up), tissue sections displayed a relative paucity of the apoptotic cells (Fig 7C) consistent with the known rapidity of the clearence of apoptotic cells from live tissues. On the other hand the residual tumor cells, particularly near the edges of cysts, showed unusually high frequencies of cells displaying features of spinous differentiation (e.g. the area towards the lower left of Fig 7C; seen more clearly on higher magnification as exemplified from another area in Fig 7D). Similar areas of differentiation or cysts were absent in the punch biopsy material obtained from the same tumor prior to the initiation of treatment (Fig 7E). The tumor nodule that received relatively lower concentration of cyclopamine (marked by arrow in Fig 7A) had a large cystic center on the sixth day of follow up (Fig 7F). The residual periphery of the nodule, however, continued to have cells with typical BCC morphology although frequency of the cells with features of differentiation (e.g. with enlarged and more eosinophilic cytoplasm) was again increased and smaller cysts existed within this periphery (Fig 7F). Thus, while the tumor response to optimal concentrations of cyclopamine was relatively rapid, exposure to suboptimal concentrations left behind tumor cells that persisted during the period of follow up.

These further examples demonstrate effectiveness of the described treatment in obtaining rapid clinical regression of the BCC's and other tumors that display increased hedgehog-smoothened signalling (e.g. trichoepithelioma). The clinical regressions are seen to be associated with the cyclopamine-induced differentiation and apoptosis of the tumor cells. In addition, tumor cell proliferation is also prevented. Effectiveness on several independent tumors in unrelated patients with differing genotypes is consistent with the general utility of the described treatment.

Cyclopamine was discovered as a teratogenic component of Veratrum plants (Keeler R.F. (1969) Phytochemistry 8:223-225). It has been reported to inhibit differentiation of the precursors of the ventral cells in the developing brain (Incardona J.P. et al. (1998) Development 125:3553-3562; Cooper M.K. et al. (1998) Science 280:1603-1607). Inhibition of cellular differentiation by cyclopamine has been reported in other systems as well, including the differentiation of bone marrow cells to erythroid cells (Detmer K et al (2000) Dev. Biol. 222:242) and the differentiation of urogenital sinus to prostate (Berman D.M. et al (2000) J. Urol. 163:204). However the opposite was found to be true in this invention with the tumor cells exposed to cyclopamine. Along with the cyclopamine-induced differentiation of tumor cells, apoptosis of tumor cells was also induced. Induction of tumor cell apoptosis by cyclopamine, again previously undescribed, is shown to be highly efficient. Furthermore induction of apoptosis by cyclopamine was not secondary to a genotoxic effect and had extreme specificity; even the outer root sheath cells of hair follicles and normal epidermis basal cells that were adjacent to the tumor cells were well preserved while the tumor cells had differentiated and were undergoing apoptosis. Lack of adverse effects of the described treatment is confirmed also by the presence of clinically normal-looking healthy skin at the sites of cyclopamine application in patients (longest duration of follow up of a human subject is over 15 months at the time of this writing and shows safety of the treatment also in the long term). Above summarised features of the treatment described in this invention make it highly desirable in cancer therapy and provide solutions to the long-standing problems of cancer therapy.

Description Of The Figures

Fig 1A, 1B, 1C, 1D : Rapid regressions of the cyclopamine-treated BCC's as indicated by disappeared tumor regions (exemplified by arrows), markedly decreased height from skin surface and by a loss of translucency in less than a week. 1A : BCC, located on left nasolabial fold, prior to treatment. 1B : Same BCC on the fifth day of topical cyclopamine treatment. 1C : BCC, located on forehead, prior to tretment. 1D : Same BCC on the sixth day of topical cyclopamine treatment.

Fig 2A, 2B, 2C, 2D, 2E, 2F : Microscopic appearences of the cyclopamine- and placebo-treated BCC's, showing the cyclopamine-induced massive apoptotic death and removal of the tumor cells and the disappearence of tumor nodules to leave behind cystic spaces with no tumor cells. Skin areas corresponding to the pre-treatment positions of the BCC's were excised surgically on the fifth and sixth days of cyclopamine exposure with a margin of normal tissue and subjected to conventional fixation, sectioning and hematoxylene-eosine staining for microscopic analyses. 2A : Large cyst in the dermis corresponding to the position of a disappeared tumor nodule showing no residual tumor cells. 2B : Similar cysts in another dermal area that contained BCC prior to, but not after, treatment with cyclopamine. 2C : Low power view of an area of the BCC shown on Fig 1D showing residual cells and formation of a large cyst by the joining together of the numerous smaller cysts in between these cells. 2D : High power view from an interior region of the same residual BCC as in Fig 2C showing greatly increased frequency of the apoptotic cells and the formation as well as enlargement of the cysts by the apoptotic removal of the BCC cells. 2E : High power view from from a peripheral region of the same residual BCC as in Fig 2C also showing greatly increased frequency of the apoptotic cells and the formation of cysts by the apoptotic removal of BCC cells. 2F : High power view from an internal area of a placebo-treated BCC showing typical neoplastic cells of this tumor and the absence of apoptosis. Original magnifications are 100X for 2A, 2B, 2C and 1000X for 2D, 2E, 2F.

Fig 3A, 3B, 3C, 3D, 3E, 3F, 3G : Immunohistochemical analyses of the cyclopamine- and placebo-treated BCC's showing differentiation of all residual BCC cells under the influence of cyclopamine and the decrease of p53 expression in BCC's following exposure to cyclopamine. 3A and 3B : Absence of staining with the monoclonal antibody Ber-Ep4 in all residual cells of cyclopamine-treated BCC (3A) contrasted with

the strong staining in placebo-treated BCC (3B) showing that all residual cells in the cyclopamine-treated BCC's are differentiated to or beyond a step detected by Ber-Ep4. Ber-Ep4 is a known differentiation marker that stains the BCC cells as well as the undifferentiated cells of the normal epidermis basal layer and of hair follicles but not the differentiated upper layer cells of normal epidermis. 3C : Heterogenous labelling of the residual cells of a cyclopamine-treated BCC with the *Ulex Europaeus* lectin type 1 showing differentiation of some of the BCC cells all the way to the step detected by this lectin which normally does not label the BCC's or the basal layer cells of the normal epidermis but labels the differentiated upper layer cells. 3D and 3E : Decreased expression of p53 as detected by the monoclonal antibody DO-7 in cyclopamine-treated BCC's (3D) in comparison to the placebo-treated BCC's (3E). Expression of p53 is known to decrease upon differentiation of the epidermal basal cells and upon differentiation of cultured keratinocytes. It is also well known that the amount of p53, detectable by DO-7, increases in cells when they are exposed to DNA-damaging agents. 3F and 3G : Consistent retraction of BCC's from stroma, which is a feature known to be associated with the arrest of tumor cell proliferation, seen in cyclopamine-treated (3F, arrow shows the retraction space) but not in placebo-treated (3G) tumors (difference of the cyclopamine- and placebo-treated BCC's in terms of retraction from stroma is seen also in 3D, 2C vs 3B, 3E). Original magnifications are 400X for 3A, 3B, 3D, 3E, 1000X for 3C and 100X for 3F, 3G. All immunohistochemical labellings are with peroxidase-conjugated streptavidin binding to biotinylated secondary antibody; labelling is indicated by the brown-colored staining. Sections shown in 3F and 3G are stained with Periodic Acid-Schiff and Alcian blue.

Fig4A, 4B, 4C, 4D: Normal pattern of labelling of the cyclopamine-treated normal skin with Ber-Ep4 showing that the undifferentiated cells of normal epidermis and of hair follicles are preserved despite being exposed to the same schedule and doses of cyclopamine as the BCC's. 4A : Ber-Ep4 labelling of the basal layer cells of the epidermis treated with cyclopamine. 4B and 4C : Higher power views from different areas of cyclopamine-treated epidermis showing Ber-Ep4 labelling of the basal cells. 4D : High power view of a hair follicle treated with cyclopamine yet showing normal labelling with Ber-Ep4. Original magnification is 400X for 4A and 1000X for 4B, 4C, 4D. Immunohistochemical detection procedure is the same as in Fig 3A, 3B; labelling is indicated by brown coloring.

Brief Description Of The Figures

Fig 5A shows an ulcerated BCC in the upper nasal region of a 68 years old man prior to treatment.

Fig 5B shows the same BCC as in Fig 5A at the 54th hour of cyclopamine application to its lower half.

Fig 5C shows a section from the cyclopamine-applied half of the BCC at the 54th hour. Hematoxylene-Eosine (H&E) staining, 400X original magnification.

Fig 5D shows a section from the untreated region of the same BCC. H&E, 400X original magnification.

Fig 5E shows a section from the cyclopamine applied half of the BCC at the 54th hour with immunohistochemical staining for the Ki-67 antigen. 200X original magnification.

Fig 6A shows a trichoepithelioma on the cheek of a 82 years old man prior to treatment.

Fig 6B shows the same skin region as in Fig 6A after 24 hours of treatment with cyclopamine.

Fig 6C shows a section from the excised skin region shown in Fig 6B with residual tumor cells. H&E, 400X original magnification.

Fig 6D shows another area from the same tissue as in Fig 6C. In addition to the numerous apoptotic cells and the formation of cystic structures by their removal, the tumor is seen to be infiltrated by mononuclear cells. H&E, 200X original magnification.

Fig 7A shows a pigmented BCC in the lower eyelid of a 59 years old man prior to treatment.

Fig 7B shows the same BCC as in Fig 7A on the third day of tretment with cyclopamine.

Fig 7C shows a section from the treated region of the BCC shown in Fig 7B. H&E, 200X original magnification.

Fig 7D shows close up view of an area of residual tumor cells in a section from the treated region of the BCC shown in Fig 7B. H&E, 400X original magnification.

Fig 7E shows a section from a punch biopsy material obtained from the BCC shown in Fig 7A prior to treatment. H&E, 400X original magnification.

Fig 7F shows a section containing part of the BCC nodule marked by the arrow in Fig 7A. The tissue was excised after 3 days of treatment and 6 days of non-treated follow up. H&E, 100X original magnification.

Table 1: Induction of the Differentiation and Apoptosis of Basal Cell Carcinoma Cells by Topical Cyclopamine

	Peripheral Palisading Cells of the BCC's Treated With		Non-Palisading Cells of the BCC's Treated With	
	Placebo	Cyclopamine	Placebo	Cyclopamine
% Of Cells Showing ≥ 2 Morphological Signs Of Apoptosis On H&E Stained Tissue Sections	0 ± 0	20 ± 8	0.2 ± 0.4	18 ± 11
% Of Cells Labelled With Ber-Ep4	100 ± 0	0 ± 0	91 ± 8	0 ± 0
% Of Cells Labelled With DO-7	58 ± 27	16 ± 11	67 ± 22	5 ± 3

Means ± standart deviations from at least 16 randomly selected high-power (1000 X) fields of the tissue sections of each tumor group are shown. $p < 0.001$ for the placebo vs cyclopamine-treated tumors for all the parameters, both for the palisading peripheral and the non-palisading (interior) tumor areas.

Color prints of the same figures as on page 12 (Fig 1A, 1B, 1C, 1D, Fig 2A, 2B, 2C, 2D, 2E, 2F, Fig 3A, 3B, 3C, 3D, 3E, 3F, 3G, Fig 4A, 4B, 4C, 4D), added as page 12a because the immunohistochemical data and findings, due to their nature, can be conveyed best in color rather than in gray-scale; we respectfully request consideration of this fact by the Patent Authority and the keeping of page 12a as part of this patent application. However the page 12a may be removed from the patent application if it is deemed necessary by the Patent Authority.

Color prints of the same figures as on page 12b (Fig 5A, Fig 5B, Fig 5C, Fig 5D, Fig 5E, Fig 6A, Fig 6B, Fig 6C, Fig 6D, Fig 7A, Fig 7B, Fig 7C, Fig 7D, Fig 7E, Fig 7F), added as page 12c because the immunohistochemical data and findings, due to their nature, can be conveyed best in color rather than in gray-scale; we respectfully request consideration of this fact by the Patent Authority and the keeping of page 12c as part of this patent application. However the page 12c may be removed from the patent application if it is deemed necessary by the Patent Authority.

The claims defining the invention are as follows:

1. Use of cyclopamine or a pharmaceutically acceptable salt or derivative thereof that is capable of inducing apoptosis and differentiation of the tumor cells that employ Hedgehog/Smoothened signaling for prevention of these processes, for the manufacture of a medicament, wherein said medicament is to be administered in an amount which causes decrease of size or disappearance of a tumor that employs Hedgehog/Smoothened signaling for prevention of said processes in tumor cells.

2. Use according to claim 1, for the treatment of basal cell carcinoma.

3. Use according to claim 1 or 2, wherein said medicament is formulated for topical or systemic administration.

4. Use according to claim 3, wherein for systemic administration, cyclopamine or a pharmaceutically acceptable salt or derivative thereof is in the form of an aqueous solution, or is entrapped in liposomes.

5. Use according to claim 3 or 4, wherein said medicament is a pharmaceutical form enabling controlled release.

6. Use according to any one of claims 1 to 3, wherein said medicament is adsorbed onto a dermal patch.

7, Use according to any one of claims 1 to 3, wherein said medicament is manufactured in the form of a cream, ointment, gel or hydrogel.

8. A method for inducing apoptosis and differentiation of tumor cells, comprising identification of the tumor cells that use Hedgehog/Smoothened signaling for prevention of these processes and contacting said tumor cells with a sufficient amount of cyclopamine or a pharmaceutically acceptable salt or derivative thereof that is capable of inducing apoptosis and differentiation of said tumor cells.

9. A method according to claim 8 for tumor treatment.

10. A method according to claim 8 or 9, for the treatment of basal cell carcinoma.

11. A method according to any one of claims 8 to 10, wherein cyclopamine or a pharmaceutically acceptable salt or derivative thereof is administered as a pharmaceutical composition selected from the group of compositions for topical, non-topical or systemic administration.

12. A method according to claim 11, wherein the composition for non-topical administration, is in the form of an aqueous solution, or is in the form of liposomes that have the cyclopamine or a pharmaceutically acceptable salt or derivative thereof entrapped therein.

13. A method according to claim 11 or 12, wherein the non-topical administration is direct intratumoral injection.

14. A method according to any one of claims 8 to 10, wherein cyclopamine or a pharmaceutically acceptable salt or derivative thereof is administered in a pharmaceutical form enabling controlled release.

15. A method according to any one of claims 8 to 10, wherein cyclopamine or a pharmaceutically acceptable salt or derivative thereof is adsorbed onto a dermal patch.

16. A method according to any one of claims 8 to 10, wherein cyclopamine or a pharmaceutically acceptable salt or derivative thereof is administered in the form of a cream, ointment, gel or hydrogel.

17. A use according to claim 1, substantially as hereinbefore described with reference to any one of Figures 1A to 1D, 2A to 2F, 3A to 3G or 4A to 4D.

18. A method according to claim 8, substantially as hereinbefore described with reference to any one of Figures 1A to 1D, 2A to 2F, 3A to 3G or 4A to 4D.

19. Cyclopamine or a pharmaceutically acceptable salt or derivative thereof that is capable of inducing apoptosis and differentiation of the tumor cells that employ Hedgehog/Smoothened signaling for prevention of these processes when used for inducing apoptosis and differentiation of tumor cells by causing decrease of size or disappearance of a tumor that employs Hedgehog/Smoothened signaling for prevention of said processes in tumor cells.

20. Cyclopamine or a pharmaceutically acceptable salt or derivative thereof when used according to claim 19, substantially as hereinbefore described with reference to any one of Figures 1A to 1D, 2A to 2F, 3A to 3G or 4A to 4D.

Dated 13 February, 2006
Sinan Tas

Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

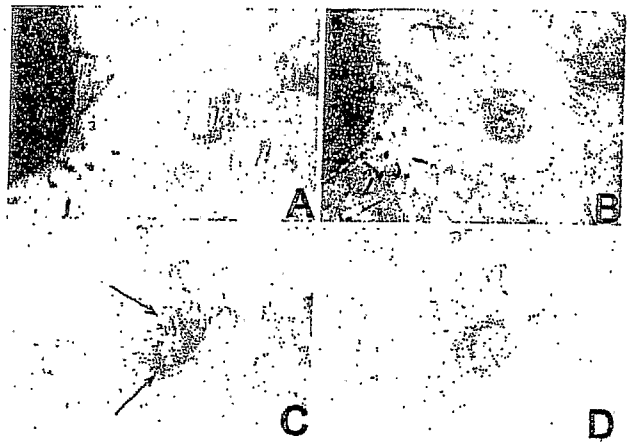


Fig 1A,1B,1C,1D

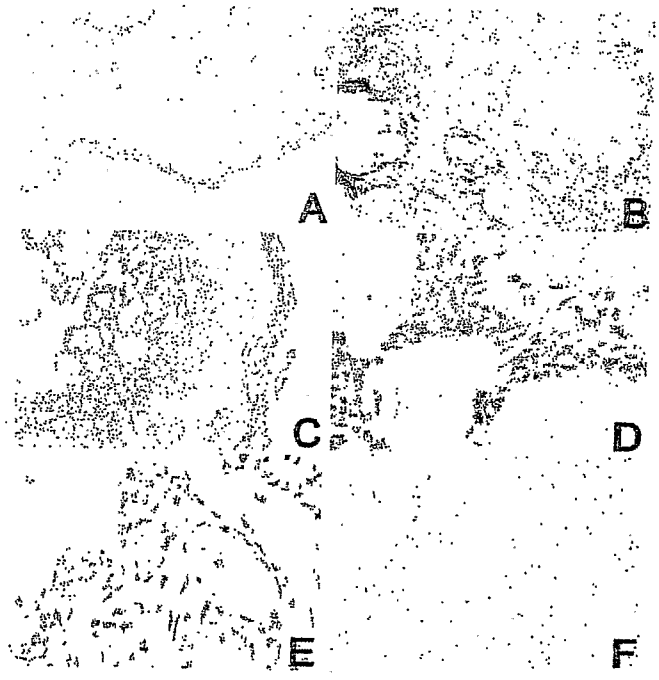


Fig 2A,2B,2C,2D,2E,2F

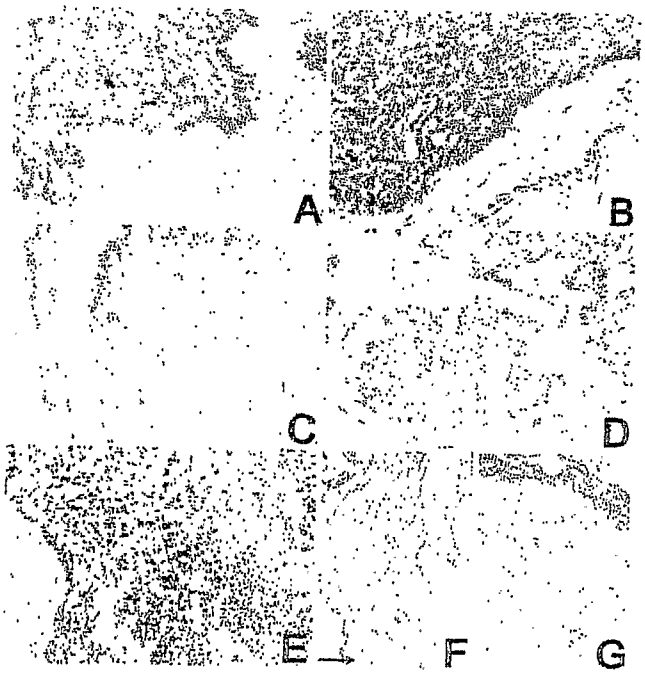


Fig 3A,3B,3C,3D,3E,3F,3G

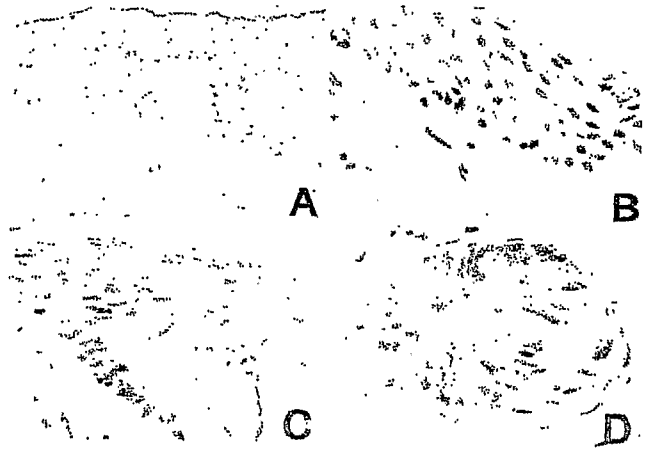


Fig 4A,4B,4C,4D

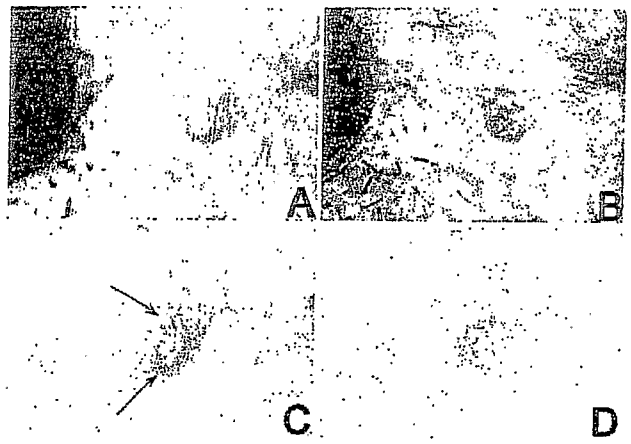


Fig 1A,1B,1C,1D

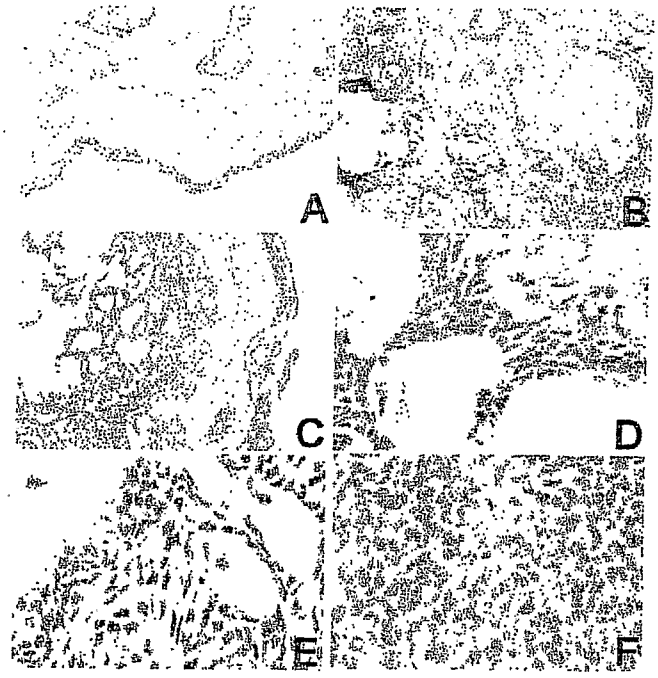


Fig 2A,2B,2C,2D,2E,2F

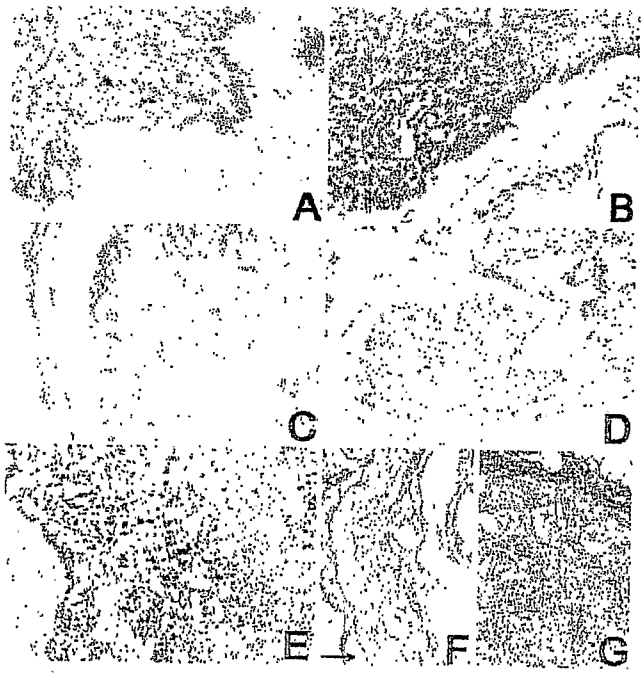


Fig 3A,3B,3C,3D,3E,3F,3G

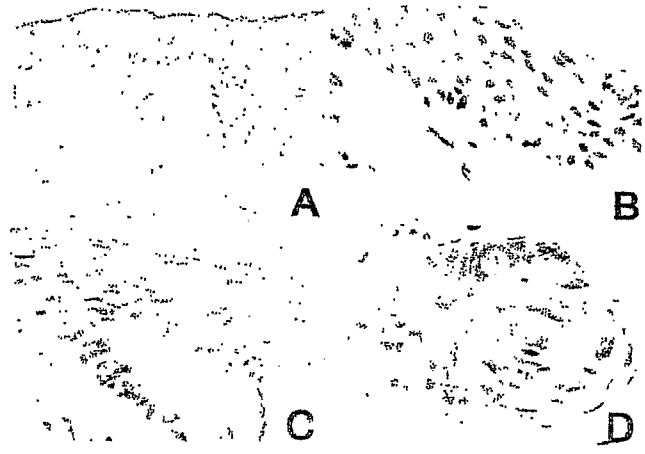


Fig 4A,4B,4C,4D

Fig 5A

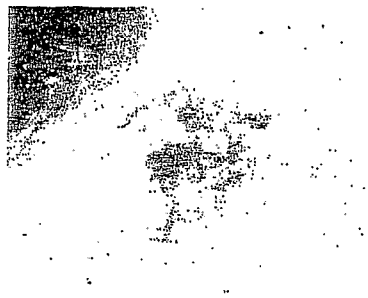


Fig 5B

Fig 5C

Fig 5D

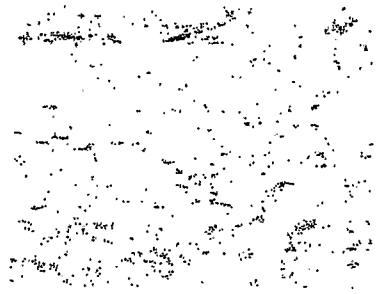


Fig 5E

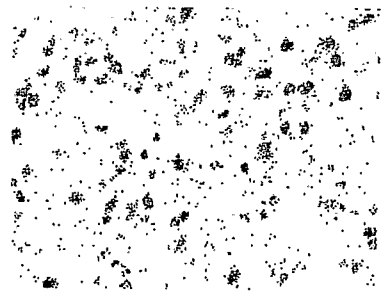


Fig 5F

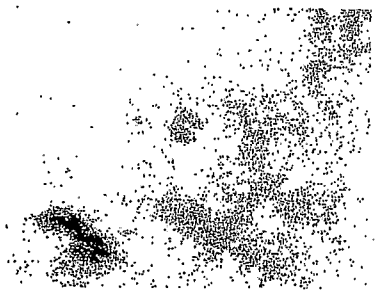


Fig 6A

Fig 6B

Fig 6C

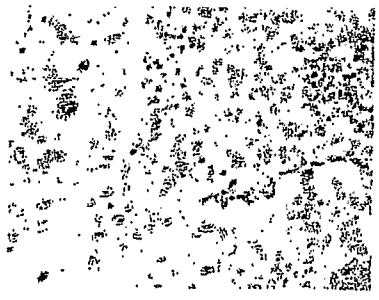


Fig 6D

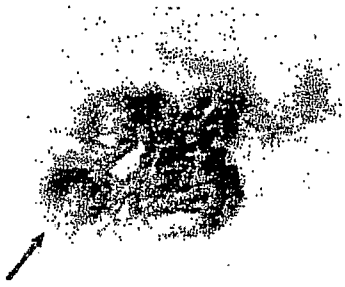


Fig 7A

Fig 7B

Fig 7C

Fig 7D

Fig 7E

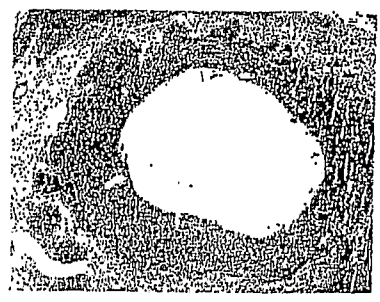


Fig 7F

Fig 5A

Fig 5B

Fig 5C

Fig 5D

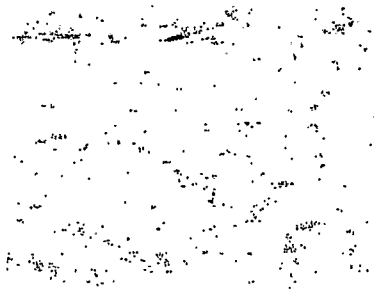


Fig 5E

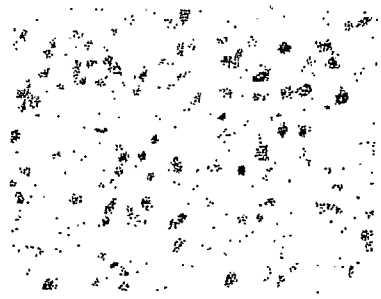


Fig 5F

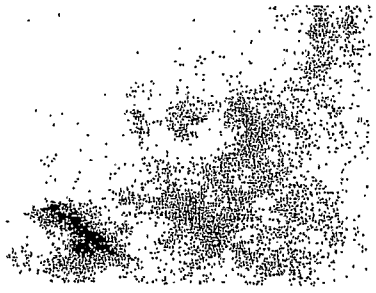


Fig 6A

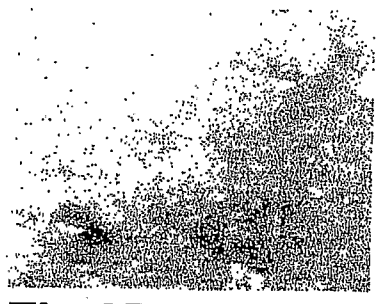


Fig 6B

Fig 6C

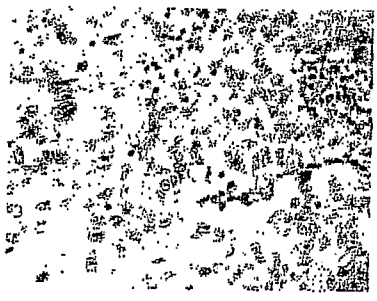


Fig 6D

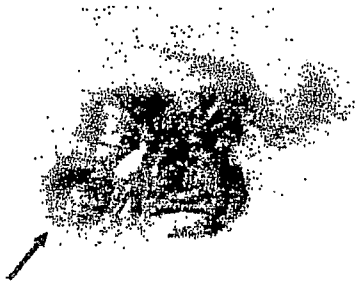


Fig 7A

Fig 7B

Fig 7C

Fig 7D

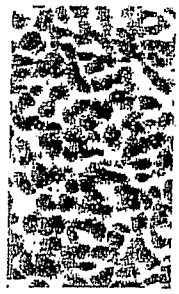


Fig 7E

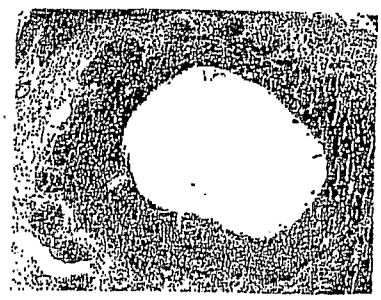


Fig 7F