Aspergillus fungi analysis | essay

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Micheli in 1729 named one of the oldest genera of fungi as *Aspergillus* . Micheli, while viewing the spore bearing structure of *Aspergillus* under the microscope, was reminded of the device used to sprinkle holy water by the Roman Catholic clergy as part of a liturgy called as *asperges* (Ainsworth, 1976). Many mycologists and industrial microbiologists were attracted to study about *Aspergillus* not because of their occurrence in nature, but because of their easy cultivation procedures in the laboratory. Several species of *Aspergillus* have economic importance such as the production of citric, gluconic, kojic and itaconic acid for industrial use (Joan Bennett, 2010). Also, the cholesterol lowering drug, Lovastatin, gained importance when it was approved for human use by Merck Inc. scientists. Lovastatin was isolated from *Aspergillus terrus* (Alberts, 1998). Other important secondary metabolites from various species of *Aspergillus* having a pharmacological activity include cholecystokinin and neurokinin antagonists, antifungal, antibacterial drugs amongst many compounds (Joan Bennett, 2010).

Apart from the economic importance presented by the species, *Aspergillus* can also cause adverse health effects by production of mycotoxins, induce allergic responses and by causing localized or systemic infections. The main reason being the inhalation of *Aspergillus* spores either by environmental or laboratory exposure.

Newman, Cragg and co-workers (2009, 2012, 2013) emphasize the pivotal role of natural products as anticancer agents. Products other than synthetic are natural or are a derivative of the natural product. Natural products from plants and bacteria play a very important role in the cancer drug discovery. Drugs include bleomycin, etoposide, paclitaxel, docetaxel, vincristine etc.

Fungi derived natural products also serve as an excellent source for compounds of pharmaceutical importance but the fungal diversity has not been explored to the maximum (Alexander Kornienko et al., 2015). There are a number of biotechnological, technological and physiological factors detrimental for the industrial development of secondary metabolites from fungi. Also, the amount of one metabolite produced by fungi, cost of its production could be a constraint.

A new drimane sesquiterpene lactone, SF002-96-1, was isolated from *Aspergillus* species by Silke Felix et al., 2013 which showed an inhibitory activity against Survivin, a member of the inhibitor of apoptosis (IAP) family . (no back references) Survivin plays a very important role in apoptosis and in cell cycle progression. High expression of survivin in tumors is one of the many reasons for increased drug resistance and hence poor patient survival. The structure of the compound was confirmed by HR-ESI mass spectrum, IR spectrum, COSY and NOESY spectrum and NMR techniques. Reporter gene assays were conducted to characterize the influence of the compound on the survivin expression by employing a human survivin promoter dependent transcriptional reporter by transient infection in the human colorectal carcinoma cell line Colo 320. SF002-96-1 inhibited the survivin promoter activity in a dose dependent manner. The IC_{50} value being 3. 42 μM (1. 3μg/ml). The effect of SF002-96-1 on Stat3, NF-κB and β-catenin activated T-cell factor (TCF) expression was also investigated as they are involved in regulating the surviving expression. The Stat3-dependent luciferase expression was strongly inhibited with an IC_{50} value of 1. 6μM (0. 6μg/ml) by SF002-96-1. NF-κB-dependent reporter gene expression was strongly

inhibited with an IC_{50} value of 2. 63μM (1μg/ml). But SF002-96-1 showed no significant inhibition against β-catenin/ TCF dependent luciferase expression even at a higher concentration of 8μM.

The effect of the compound on the transcription of the surviving gene was investigated by conducting quantitative real-time PCR experiments. Colo 320 cells were treated with SF002-96-1 at varying concentration for 8h. At a concentration of 18. 42μM (7μg/ml), the compound reduced the survivin mRNA levels to about 50%. The data was confirmed by performing Western blot experiments for the survivin protein expression. Starting at a concentration of 13. 16μM (5μg/ml) of the compound treated for 8h, a significant reduction in the endogenous levels of the survivin protein was observed which suggests that SF002-96-1 inhibits the transcription of surviving promoter gene. Chromatin immunoprecipitation(ChIP) assay was performed to investigate the binding of Stat3 and NF-κB to the survivin promoter. The results corroborated with the reporter gene assay indicating that the compound inhibited their binding to the surviving promoter.

Cell viability was assessed by measuring the reduction of the tetrazolium compound 2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium (XTT) into a colored formazan. SF002-96-1, at concentrations from 10. 5μM (4μg/ml), was observed to significantly decrease the cell viability in treated Colo 320 cells in a dose dependent manner. DNA fragmentation (approx. 200bp) was observed after a 5h treatment at a concentration of 13. 16 μM and 23. 31 μM concentrations of SF002-96-1. With an increase in the dosage level, the compound showed a concomitant increase in caspase-3 activity, indicating that an apoptotic

cascade is triggered by the compound. These results suggest that the compound has a potential to serve as a lead structure for developing a cancer drug. Drimane sesquiterpenes are also found in plants apart from fungi and marine organisms.

Two compounds, Norsolorinic acid (2008) and Asperfuranone (2010), were isolated, identified from *Aspergillus nidulans* and were investigated for their cytotoxic activities by Clay C. C. Wang et al.

Norsolorinic acid was isolated and purified by chromatographic techniques. The structure was determined by IR and NMR techniques. Cytotoxicity studies for the compound were investigated by conducting MTT assay and protein, ligand interaction studies. Norsolorininc acid exhibited proliferative inhibitory effect in a dose dependent manner. At a concentration of 20μM, MCF – 7 cells treated for 48 h, a 70. 2% proliferation inhibition was observed, the IC_{50} being 12. 7μM. The effect was also evaluated H184B5F5/M10 cell line (normal mammary epithelium) which confirmed that the compound exhibited cytotoxic effects only on the cancer cell line. Cell cycle analysis was investigated by using the flow cytometer which revealed no significant change in cell cycle distribution after treatment with the compound at 20μM for 24 h. Nucleosome ELISA kit was used to investigate the apoptotic cells. It was observed that there was an increase from 4. 4 fold at 10μM concentration of norsolorinic acid to 6. 8 fold at 20μM concentration for 48 h. p53 pan ELISA and WAF1 ELISA kits were used to understand the molecular mechanism of apoptosis induction by the compound. But there was no significant effect in the protein expression which determined that p53 and p21/WAF1 does not regulate apoptosis induced by norsolorinic acid.

The Fas/Fas ligand apoptotic system was investigated for a possible mechanism to confirm apoptosis induced by norsolorinic acid. Fas ligand ELISA and Fas/APO-1 ELISA kits were employed for conducting the study. An increased expression in the Fas/APO-1 receptor and membrane bound Fas ligand was observed in MCF-7 cells when treated for 6 h with the compound in a dose dependent manner. Maximum protein expression levels was observed at 20μM concentration for 48 h. To verify the results, Chang et al., pre-treated MCF – 7 cells with ZB4, an antagonistic anti-Fas antibody . The antiproliferative and pro-apoptotic effects were prevented. At a concentration of 20μM, cell proliferation was inhibited to 19. 2% from 70. 2% as earlier stated. Oligonucleosome DNA fragmentation of apoptotic cells decreased from 6. 8 fold to 2. 4 fold for a 48 h treatment. This experiment confirmed that norsolorinic acid induces apoptosis by Fas/Fas ligand system. Caspase-8 activity was investigated which resulted in maximum increase in caspase protein levels at 20μM concentration for 48 h treatment. A caspase-8 inhibitor , Z-IETD-FMK, was employed to assess the norsolorinic acid mediated anti proliferation and apoptosis. Results showed that there was a significant decrease in the antiproliferative and apoptotic activity which confirmed that caspase-8 activation is also required for apoptosis induction.

Fas/Fas ligand system plays a key role in signalling transduction pathway of apoptosis in cells and tissues (Nagata and Golstein, 1995) . Ligation of Fas by its ligand or by an agonistic antibody induces receptor oligomerization, hence formation of the death inducing complex, followed by the activation of caspase-8 which inturn activates the caspase cascade leading to apoptotic cell death.

Asperfuranone , is a novel polyketide, isolated from *Aspergillus nidulans* by Yi-Ming Chiang et al., 2009 which involved series of gene deletion experiments that revealed the action of two fungal polyketide synthases that encodes the biosynthetic pathway for the synthesis of the polyketide, Asperfuranone. Structural analysis for the polyketide involved ESI-MS, NMR techniques.

Antiproliferation activity of Asperfuranone was investigated by Clay C. C. Wang et al., 2010 in human non-small cell lung cancer A549 cells. Maximum effect on proliferation inhibition was observed when A549 cells were treated with asperfuranone at a 20μM concentration for a 48 h treatment period. 64. 5% inhibition was observed, the IC_{50} value being 15. 3μM. Inhibitory effect of the compound was also assessed on two other cancer cell lines – liver cancer PLC/PRF/5 cells and breast cancer MDA-MB-231 cells. The IC_{50} values being 18. 6μM and 16. 5μM for the liver and breast cancer cell lines respectively with the data not shown in the research article.

Cell cycle analysis revealed that at a concentration of 15μM of the compound, the population of cells from G0/G1 phase increased from 30. 7% to 52. 6% when compared to the untreated control. 72. 3% of cells entered G0/G1 phase when treated with 20μM of asperfuranone. By conducting the TUNEL assay, 36. 33% of cells were induced to undergo apoptosis at a concentration of 20μM for a 48 h treatment period. Insights into the anti-cancer mechanisms involved assessing levels of certain proteins that play a crucial role in apoptosis. p53 protein levels were assessed by ELISA. The protein level was found to increase in a dose dependent manner. The protein

level reached maximum expression for a treatment period of 12 h at a 20μM concentration.

The p21/Waf1/Cip1 expression was also assessed for the p53 – expressing A549 cells as the protein is thought to be responsible for G0/G1 cell cycle arrest. There was an increase in p21/Waf1/Cip1 protein level that was apparent during the 6h treatment, which reached maximum induction of the protein level when the compound was treated for 24h. This p21/Waf1/Cip1 protein level was found to increase in a dose-dependent manner. Therefore it was suggested that the cell cycle arrest caused by asperfuranone might operate through induction of p21/Waf1/Cip1 protein in a p53 dependent event in A549 cells . Fas/APO-1 and Fas Ligand ELISA kits were employed to understand a possible role of Fas/FasL apoptotic system in the asperfuranone mediated apoptotic mechanism. The protein expression was found to increase in a dose dependent manner with a maximum effect being observed when A549 cells were treated with asperfuranone for 24 h. ZB4, earlier mentioned in while assessing the effect of Norsolorinic acid on MCF-7 cells (Clay C. C. Wang et al., 2008), treated cells showed a decrease in cell proliferation inhibition from 64. 5% to 19. 8%. Induction of apoptosis was also decreased from about 36. 33% to 12. 74% suggesting that asperfuranone plays a key role in inducing apoptosis via increasing p53, p21 and Fas protein levels. Asperfuranone also increased caspase-8 levels which was confirmed by employing the caspase-8 inhibitor (Z-IETD-FMK). Results showed that induction of apoptosis was completely abolished with the inhibition of caspase-8.

Defects in the Fas/Fas ligand system can be one of the reason for tumor progression. Fas/FasL system was assessed for both Norsolorinic acid and Asperfuranone which makes the Fas/Fas ligand system a target for therapeutic strategies.

Polyketides are a diverse group of natural products which includes polyphenols, polyenes and macrolides having biological activities with antimicrobial and anticancer properties (Preetida J. Bhetariya et al., 2011) . There are a number of secondary metabolites produced by *Aspergillus* species of pharmaceutical importance.

Protease inhibitors (PIs) encompass a large group of proteins that regulate the hydrolytic activity of proteolytic enzymes. The protease/PI balance is necessary for cellular homeostasis. Any disturbance in the homeostatic pathway can cause pathological conditions like cancer development. The main feature includes their ability to form strong protease-PI complex, inhibiting the protelytic activity (Laskowsky and Kato, 1980). Valentina et al., 2014 evaluated the cytotoxic and protease inhibitory effect of biomolecules secreted by *Aspergillus fumigatus* . Proteins from the culture filtrate were analysed quantitatively by Bradford method, molecular weight was determined by SDS-PAGE supported by an electropherogram and densitometric analysis. There was no significant difference observed between the proteinaceous products during the short fermentation of the fungi as per the experiments conducted by Valentina et al. but there were several new products detected in a time dependent manner i. e. with an increase in the incubation time under aerated (20% O_2) conditions. Cytotoxic results for the *A. fumigatus* culture filtrates are expressed as

percentage inhibition rate on cell line proliferation. The cell lines under study were human epithelial colorectal adenocarcinoma Caco-2 cells and a contaminant of HeLa cervical cancer WISH cells. The inhibitory effect of *A. fumigatus* culture filtrates were examined on papain and trypsin like proteinases. Inhibitory effect was found to be significant on papain like proteinases when *A. fumigatus* was cultured under aerated conditions. Culture filtrate from Day 3 exhibited a 41. 95% inhibition and from Day 6 exhibited 43. 21% inhibition. But there was no significant inhibitory effect observed for trypsin like proteinases. The data suggests that the protease inhibitory activity can be significant during infection and may interplay with the host cells but a lot research is yet to be conducted to prove the outcome.

Halophilic microorganisms are also a very promising source of bioactive compounds (Ventosa et al., 1998) having antitumor and antimicrobial activities. Lin Xiao et al., 2013 investigated the cytotoxic effects of a moderately halophilic *Aspergillus* sp. Nov. F1 that was isolated from a marine solar saltern in China. On extraction with ethyl acetate and purification using the Sephadex LH-20 column chromatography, three secondary metabolites were identified – Ergosterol, Rosellichalasin and Cytochalasin E. Structural analysis was determined by ESI-MS, NMR techniques. The anticancer activity was determined by MTT assay on A549, HeLa, BEL-7402 and RKO cell lines. Amongst the three metabolites tested for cytotoxicity, ergosterol showed the highest potent cytotoxic activity to the human colon cancer RKO cell line with the IC50 value being 3. 3±0. 5 μM. (Back reference papers requested. Awaiting the response)

Phenylahistin is a fungal diketopiperazine metabolite isolated from *Aspergillus ustus* by Kanoh et al., 1997 as a racemic mixture. Enantiomers were separated by HPLC (Kanoh et al., 1999) and the structure determined by spectroscopic techniques. (-)- phenylahistin exhibited antitumor activity *in vitro* against tumor cell lines- A549 , A431 dermal, K562 leukemia, HeLa ovary, WiDr colon, MCF-7 breast, TE-671 CNS with the IC_{50} values ranging from 0. 18 to 3. 7μM. (+)- phenylahistin exhibited less potent activity.

Kanoh et al., 1999 performed *in vivo* studies that showed a potent antitumor activity of (-)- phenylahistin against Lewis lung carcinoma and P-388 cells. The product has shown to have a potent vascular disrupting when bound to the colchicine site on β-tubulin (Milward et al., 2012). The synthetic analogue of the compound named NPI-2358 (plinabulin) has entered clinical trials with NSCLC (Non-small cell lung cancer) patients. In combination with docetaxel, results were quite promising indicating antitumor activity.

Cytochalasins are a group of fungal metabolites that bind to actin filaments, blocking polymerization and hence the elongation of actin (Andrew, 2004). Cytochalasin B (originally named phomin) was the first cytochalasin to be isolated by Rothweiler, W. & Tamm, C. (1966) from ???. Cytochalasin E was first isolated from *Rosellina nectarix* and *Helminthosporium dematioideum* by Alridge et al. in 1972. Cytochalasin E was later isolated from many other fungi belonging to different genera. Cytochalasin E was produced by *Aspergillus clavatus* (Doubravka V, 1983) and *Aspergillus* sp. Nov. F1 (Ventosa et al., 1998) among other species. Cytochalasin E exhibited antiangiogenic activity as it was found to be particularly potent and selective inhibitor of endothelial cells *in vitro* and inhibited angiogenesis *in vivo*

(Udagawa et al., 2000). A dosage level of 2mg/kg administered on alternate days in the lewis lung tumor model exhibited a 72% inhibition. Elucidation of non-actin target of Cytochalasin E hopefully will result in the development of more number of specific analogues which may reveal new cell signalling mechanisms involved in tumor angiogenesis.

Compound name	Origin	*in vitro* testing	Reference
SF002-96-1, drimane sesquiterpene lactone	*Aspergillus* sp. Strain IBWF002-96	Colo 320	Silke Felix, 2013
Fumigaclavine C	*Aspergillus fumigatus* (marine)	MCF – 7	Yong-Xin Li, 2013
Nigerapyrones	*Aspergillus niger* (marine)	MCF – 7, HepG2, Du145, NCI H460 , MDA-MB-	Liu et al., 2011a- hard copy (4m endophytic fungi

		231	and antitumor activities)
Naphtho-g-pyrones, TMC 256	*Aspergillus tubingensis* GX1-5E	MCF – 7, MDA-MB-435, Hep3B, Huh7, SNB19, U87 MG	Huang et al., 2011b request sent – (4m endophytic fungi and antitumor activities)
Norsolorinic acid	*Aspergillus nidulans*	MCF -7	Clay C. C. Wang et al., 2008
Asperfuranone	*Aspergillus*	A549	Clay C. C. Wang

	nidulans		et al., 2010
Phenylahistin	*Aspergillus ustus*	A549 , A431 dermal, K562 leukemia, HeLa ovary, WiDr colon, MCF-7 breast, TE-671 CNS	Kanoh et al., 1997