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Deguelin Suppresses Pancreatic Tumor Growth and Metastasis by Inhibiting Epithelial to Mesenchymal Transition in an Orthotopic Model[1]

Srinivas Reddy Boreddy, Ph.D. and **Sanjay K. Srivastava, Ph.D.**[*]

Department of Biomedical Sciences and Cancer Biology Center, Texas Tech University Health Sciences Center, Amarillo, Texas 79106, USA

Abstract

Deguelin is known to suppress the growth of cancer cells; however, its anti-metastatic effects have not been studied so far in any cancer model. In the present study, we aimed to evaluate the anti-metastatic potential of deguelin *in vivo* and in TGFβ1-stimulated cells. Our results demonstrate that tumor growth, peritoneal-dissemination and liver/lung metastasis of orthotopically implanted PanC-1-luc cells were significantly reduced in deguelin-treated mice along with the induction of apoptosis. Furthermore, deguelin-treated tumors showed increased epithelial signature such as increased expression of E-Cadherin and cytokeratin-18 and decreased expression of Snail. Similar observations were made when PanC-1, COLO-357 and L3.6pl cells were treated *in vitro* with deguelin. Moreover, E-cadherin was transcriptionally up-regulated and accumulated in the membrane fraction of deguelin-treated cells as indicated by increased interaction of E-Cadherin with β-catenin. TGFβ1-induced down-regulation of E-Cadherin and up-regulation of Snail were abrogated by deguelin treatment. In addition, deguelin inhibited TGFβ1-induced Smad3 phosphorylation and Smad4 nuclear translocation in PanC-1 cells. Furthermore, when TGFβ1-induced NFkB activation was inhibited, TGFβ1-induced Snail up-regulation or E-Cadherin down-regulation was blocked. Deguelin also significantly down regulated the constitutive phosphorylation and DNA binding of NFkB in a dose dependent manner. Interestingly, overexpression of either NFkB or Snail completely abrogated deguelin-mediated EMT inhibition, whereas overexpression of NFkB but not Snail rescued cells from deguelin-induced apoptosis. Hence, deguelin targets NFkB to induce reversal of EMT and apoptosis but downstream effectors might be different for both processes. Taken together, our results suggest that deguelin suppresses both pancreatic tumor growth and metastasis by inducing apoptosis and inhibiting epithelial to mesenchymal transition.

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[*]**To whom request for reprints should be addressed:** Sanjay K. Srivastava, Ph.D., Department of Biomedical Sciences, Texas Tech University Health Sciences Center, Suite 1103, 1406 Coulter, Amarillo, Texas-79106. Phone: 806-356-4750, Ext 224; Fax: 806-356-4770; Sanjay.Srivastava@ttuhsc.edu.

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Keywords

NFkB; TGF-β; RKIP; Snail; Metastasis; Apoptosis

Introduction

Pancreatic cancer is the fourth leading cause of cancer related deaths in the United States of America (1). Most of the patients with pancreatic cancer develop metastasis and die because of the debilitating metabolic effects of their unrestrained growth (2). Several well-known factors such as late detection and resistance to chemo- and radiotherapy contribute to poor survival rate. Pancreatic cancer commonly metastasize to the abdominal cavity, lymph nodes and liver (3).

Emerging evidences suggest that EMT is a key process in tumor cell invasiveness and metastasis. Previous reports have shown a close association between pancreatic cancer progression and EMT (4). In fact several pancreatic cancer cell lines and surgically resected pancreatic tumors have shown strong EMT characteristics (4-6). Interestingly, increase in fibronectin or vimentin and decrease in E-Cadherin expression in tumors correlates with poor survival (5). A recent report has shown that 43% (13/30) of primary pancreatic tumors and 53% (8/15) of metastatic tumors have increased levels of N-Cadherin, indicating the potential role of EMT in pancreatic cancer metastasis (6).

Tumor growth factor β1 (TGFβ1) can induce and maintain EMT during embryogenesis and cancer progression (7). It induces EMT by canonical Smad signaling and/or non-canonical TGFβ/TAK1/IKK/NFkB signaling, but both the pathways merges on Snail to repress E-Cadherin expression (8). The most important suppressors of E-Cadherin are Snail-related zinc-finger transcription factors such as Snail, Slug and SIP-1/ZEB (9). Interestingly, Snail knockout animals die at gastrulation stages and show defects in EMT indicating the role of Snail in EMT induction (10). Recently, NFkB was identified as a central mediator of EMT by regulating Snail expression (11, 12). Huber *et al*. have shown that the activation of NFkB promoted the transition to a mesenchymal phenotype even in the absence of TGFβ (11). Moreover, inhibition of NFkB in metastatic cells resulted in reversal of EMT suggesting that the IKK-2/IkBα/NFkB pathway is required for induction and maintenance of EMT in epithelial cancer cells (11).

Recognition of EMT as a potential mechanism for metastasis may offer new targets for therapeutic intervention (13). Such therapeutic intervention might prevent tumor invasion and block metastasis, if applied at an early phase of tumor growth. If a primary tumor has already metastasized to distant sites, anti-EMT approach alone may not be sufficient.

Deguelin, a rotenoid isolated from *Mundulea sericea* Willd. (Leguminosae) has shown to be a potential anti-cancer agent in various cancer models such as breast, colon and lung cancer (14-16). Intravenous injection of deguelin showed a mean residence time (MRT) of 6.98 h and terminal half-life (t1/2) of 9.26 h. About 58 and 14% of the deguelin was eliminated via feces and urine, respectively, within 5 days of intragastric (i.g) administration of deguelin (17). Previous reports have shown that deguelin inhibits the survival of various cancer cells

by targeting the key survival pathways such as AKT, Wnt, NFkB and cell cycle proteins (14-16, 18-20). However, the anti-metastatic potentials of deguelin have not been investigated in any cancer model. The present study was designed to elucidate the role of deguelin on EMT and metastasis in pancreatic cancer.

Results

Deguelin inhibits primary tumor growth and spontaneous in vivo metastasis of pancreatic tumors

Previous reports have shown that deguelin inhibits the growth of cancer cells (18, 21, 22). In the present study, we evaluated whether deguelin could inhibit the metastasis of pancreatic cancer along with primary tumor growth. To determine the anti-metastasis potential of deguelin *in vivo*, mice that were orthopically implanted with PanC-1-luc cells were treated with 5mg/kg deguelin (1:1 corn oil:DMSO, daily, i.p) and tumor growth and metastasis were monitored using IVIS Bio Imaging Station. Our results show that deguelin substantially reduced the primary tumor growth by 73% (7.6×10^7 *vs* 1.8×10^7 photons/sec; difference = 5.7×10^7 photons/sec, 95% Cl = 6.6×10^7 to 12×10^8 photons/sec, $p>0.087$), as compared to control tumors (Fig. 1A&B). Primary tumor weight was about 63% lesser in deguelin-treated mice, as compared to control mice (Fig. 1C). Deguelin was well tolerated by the mice as depicted by no weight loss or signs of acute or delayed toxicity (Fig. 1D). Interestingly, deguelin-treated mice developed much lesser metastatic lesions in peritoneum (Fig. 2A). As shown in Fig. 2B, control tumors migrated to an average of about 20mm away from the pancreas, whereas deguelin-treated tumors migrated around 5-6mm, indicating significant inhibition of tumor cell migration by deguelin (20.3 *vs* 6.6mm; difference = 13.7mm, 95% Cl= 7 to 20.8, $p<0.0006$). In fact, 8 out of 10 mice in treated group showed no metastatic lesions whereas, in the control group, 8 out of 10 mice demonstrated metastatic lesions (Fig. 2C). Compared to control, deguelin-treated mice exhibited reduced migration of PanC-1 cells into various organs such as liver, lung, spleen, intestinal mesentery nodules and abdominal cavity (Fig. 2C&D, indicating that deguelin inhibits the metastasis of primary implanted tumors to liver and lungs. As liver is the preferential site of metastasis for most of the pancreatic tumors, we imaged the luminescence in liver. Luciferin was injected 10 min before sacrificing the mice, livers were excised and luminescence was measured by the imaging system. As shown in Fig. 2E, liver from deguelin-treated mice showed no or modest luminescence, whereas control mice livers demonstrated strong luminescence indicating that deguelin significantly inhibits the metastasis of pancreatic tumors to liver.

Deguelin inhibits Epithelial to Mesenchymal Transition (EMT) in vivo

Since the primary objective of the present study was to evaluate the anti-metastatic potential of deguelin, we focused further studies on metastasis. Inhibition of pancreatic tumor metastasis and migration by deguelin *in vivo* prompted us to examine whether the anti-metastatic effect of deguelin was due to the inhibition of EMT. Interestingly, our immunofluorescence studies show that E-Cadherin expression was increased around 3 fold and vimentin expression decreased by 80% in the tumors of deguelin treated mice, as compared to control tumors (Fig. 3A&B), providing a critical clue that deguelin inhibits EMT in tumors. Similarly, western blot analysis of tumors shows that E-Cadherin

expression was significantly increased, whereas N-Cadherin, vimentin and Snail expressions were decreased by deguelin treatment. In addition, an increase in cleavage of caspase 3 and PARP was observed in deguelin-treated tumors, as compared to control tumors (Fig. 3C). Western blots were quantitated and presented as bar diagram (Fig. 3D).

Deguelin inhibits the migration and invasion of pancreatic cancer cells

Migration and invasion are the critical steps in metastasis of primary tumors as these processes help in escaping the primary tumor cells from either blood stream or lymph nodes. Since deguelin inhibited *in vivo* migration of PanC-1 xenografts, we wanted to see whether deguelin could inhibit the migration and invasion of pancreatic cancer cells *in vitro*. Initially, we evaluated the cytotoxicity of deguelin in pancreatic cancer cells and found that IC_{50} (24h) values for PanC-1, COlO-357 and L3.6pl were 62, 27 and 12μM, respectively (Fig. 4A). Cells were treated with sub-lethal dose of deguelin and migration and invasion were evaluated. Our results show that control PanC-1 cells migrated into 92% of the wound area by 36h; whereas deguelin treated PanC-1 cells migrated into 49% (Fig. 4B&C) of the wound area showing 44% of inhibition by deguelin (859 *vs* 100μm^2, difference = 759μm^2, 95% Cl = 625 to 893, $p<0.07$). Similar observations were made in COLO-357 cells (Fig. 4B&C). Similarly, deguelin (10μM) also inhibited the invasion of PanC-1, COLO-357 and L3.6pl cells (58, 77 and 59%, respectively) as evaluated by Boyden's chamber (Fig. 4D).

Deguelin inhibits EMT in pancreatic cancer cells in vitro

Since deguelin inhibited the migration and invasion of pancreatic cancer cells from their parental colonies as compared control cells, we next wanted to see whether deguelin suppress mesenchymal (metastatic) properties of pancreatic cancer cells and induce epithelial cell signature along with apoptosis induction. Similar to our *in vivo* observations, our results show that deguelin significantly down regulated vimentin, up regulated cytokeratin 18 and E-Cadherin expression and induced cleavage of caspase-3 and PARP in all the three cell lines in a dose and time-dependent manner (Fig. 5A & B).

Since Snail is considered to be the main transcriptional repressor of E-Cadherin, we monitored the expression of Snail in response to deguelin treatment. Interestingly, our results show that Snail expression was significantly reduced in all the three cells lines in a dose and time-dependent manner (Fig. 5A&B). However, Snail and E-Cadherin were not altered by deguelin treatment in normal human pancreatic ductal epithelial cells (HPDE-6) (Fig. 5A, right panel).

To differentiate between the cytotoxic effects of deguelin with that of reversal of EMT, we used gemcitabine, which is a known chemotherapeutic drug used in clinics against pancreatic cancer. Our results show that gemcitabine treatment decreased the survival of PanC-1 and COLO-357 cells similar to that of deguelin (Fig. 5C & Fig. 4A). However in contrast to deguelin, gemcitabine treatment failed to induce E-Cadherin expression or reduce vimentin expression in pancreatic cancer cells (Fig. 5C). In fact, previous reports have shown that gemcitabine and tamoxifen treated cells acquire EMT phenotype (23-25). Hence, deguelin is unique as it not only suppresses pancreatic tumor growth but also suppress metastasis by inhibiting EMT.

Deguelin transcriptionally increases membrane-bound E-Cadherin and down regulates cytosolic E-Cadherin

Next, we wanted to see whether deguelin induces E-Cadherin expression transcriptionally or by altering the post-translational degradation. Our RT-PCR analysis shows that E-Cadherin was transcriptionally up regulated in deguelin treated PanC-1 (Fig. 5D). Surprisingly, the increase in E-Cadherin protein expression by deguelin treatment in the whole cell lysates did not correlated exactly as mRNA levels (Fig. 5B & D). E-Cadherin is known to mis-localize in various portions of the cell such as cytosol and nucleus hence, we wanted to see whether deguelin alters the localization of E-Cadherin. Interestingly, our results show that membrane bound E-Cadherin was significantly increased whereas, cytosolic E-Cadherin was down regulated. However, nuclear E-Cadherin was unaltered in deguelin-treated PanC-1 cells (Fig. 5D, right panel). Furthermore, β-catenin interaction was increased in deguelin-treated cell lysates that was immunoprecipitated with E-Cadherin (Fig. 4E), indicating that E-Cadherin is accumulated in membrane fraction. Immunofluorescence studies further confirmed the localization of E-Cadherin and its repressor Snail (Fig. 5E, right panel).

Deguelin suppresses TGFβ1-induced EMT

Family of TGFβ growth factors can initiate and maintain EMT in variety of biological systems (26). Hence, we wanted to see whether deguelin would inhibit TGFβ1-induced EMT in pancreatic cancer cells. As shown in Fig 6A, TGFβ1 (10ng/mL) treated PanC-1 cells showed elongated morphology including protruding lamellipodia (Fig. 6A, white arrows) and scattered from parental colonies (Fig. 6A, black arrows), as compared to control cells. However, deguelin treatment substantially reduced TGFβ-induced cell morphology in PanC-1 cells (Fig. 6A). Interestingly, TGFβ1 enhanced the invasion of PanC-1 and COLO-357 cells in Boyden's chamber by 2.12 and 1.7 fold respectively, as compared to control cells. Nonetheless, deguelin treatment completely blocked TGFβ1-induced cell invasion in PanC-1 cells, as compared to TGFβ1 alone treated cells (Fig. 6B).

Deguelin targets TGFβ1 canonical signaling pathway to inhibit EMT

Single dose of TGFβ (10ng/mL) for 72h significantly induced the mesenchymal signature in PanC-1 cells (Fig. 6C). Nonetheless, deguelin treatment completely blocked TGFβ1-induced Snail expression and restored TGFβ1-reduced E-Cadherin expression in both PanC-1 and COLO-357 cells (Fig. 6C, right panel). In addition, deguelin reduced the phosphorylation of Smad-3 (Ser-423/425) in PanC-1cells (Fig 6D). Interestingly, Smad-4 expression was not altered by deguelin treatment however; deguelin treatment completely blocked TGFβ1-induced shuttling of Smad4 from cytosol to nucleus (Fig. 6D, right panel).

Deguelin down regulates Snail expression by inhibiting TGFβ1 non-canonical NFkB pathway

Interestingly, deguelin treatment increased E-Cadherin expression in L3.6pl, BxPC-3 and MIA PaCa-2 cells (data not shown), which do not have intact functional TGFβ1signaling due to mutation in Smad4 (L3.6pl and BxPC-3) or lack of TGFβ receptors (MIA PaCa-2) (27). TGFβ1 is also known to induce EMT in various cancer types by Smad-independent pathways such as, NFkB (28). Based on these facts, we hypothesized that deguelin could

also target non-canonical TGFβ pathway to inhibit EMT by modulating NFkB. To evaluate the role of NFkB in TGFβ1-induced EMT, NFkB was silenced using SiRNA in PanC-1 and COLO-357 cells. As expected, our results show that NFkB-silenced PanC-1 or COLO-357 cells were completely insensitive to TGFβ1-induced EMT (Fig. 7A). Furthermore, we observed maximum NFkB phosphorylation (Ser-536) and nuclear accumulation after 2h of TGFβ1 treatment in PanC-1 cells (Fig. 7A, *right panel*). Since NFkB was a prerequisite for TGFβ1-induced EMT, we next wanted to evaluate the effect of deguelin on NFkB signaling pathway. Our results show that deguelin significantly inhibit the constitutive IkB (Ser-32/34) and NFkB (Ser-536) phosphorylation in pancreatic cells without affecting the protein levels (Fig 7B). Similarly, TGFβ1-induced phosphorylation of IkB (Ser-32/34) and NFkB (Ser-536) were also drastically reduced by deguelin treatment (Fig. 7B, right panel). Furthermore, deguelin significantly reduced the transcriptional activity, DNA binding activity (Fig. 7C&D) and nuclear localization (Fig. 7D, right panel) of NFkB in PanC-1 and COLO-357 cells.

Ectopic expression of Snail or NFkB abrogates EMT inhibitory effects of deguelin

To confirm the role of NFkB and Snail in deguelin-mediated inhibition of EMT, PanC-1 and COLO-357 cells were transiently transfected with plasmid-DNA encoding either NFkB or Snail gene. As expected, over expression of Snail alone significantly diminished the expression of E-Cadherin, cytokeratin and RKIP, whereas up-regulated the expression of vimentin in PanC-1 and COLO-357 cells (Fig. 8A&B). However, when Snail overexpressing cells were treated with deguelin, EMT inhibition mediated by deguelin was almost completely blocked in PanC-1 and COLO-357 cells (Fig. 8A&B), indicating the critical role of Snail in deguelin mediated EMT inhibition. Interestingly, NFkB was not altered significantly by Snail over expression, indicating that NFkB may not be regulated by Snail in our model (Fig. 8A&B).

Similarly, when NFkB was overexpressed in PanC-1 or COLO-357 cells, E-Cadherin expression was reduced. On the other hand, expression of N-Cadherin and vimentin was increased in both the cells (Fig. 8C&D), indicating the role of NFkB in EMT. Interestingly, NFkB overexpression also resulted in the increased expression of Snail, providing direct evidence on the role of NFkB in inducing EMT through Snail (Fig. 8C&D). Nonetheless, when NFkB overexpressing PanC-1 or COLO-357 cells were treated with deguelin, E-Cadherin induction or vimentin and Snail down regulation was abolished (Fig. 8C&D). Interestingly, Snail overexpression did not protected the cells from deguelin-induced apoptosis, whereas NFkB overexpression significantly recued the cells from deguelin-induced apoptosis (Fig. 8A-D).

Discussion

In the present study, we investigated the role of deguelin on pancreatic cancer tumor growth and metastasis *in vivo* and *in vitro*. Our results show that deguelin significantly inhibits the peritoneal dissemination and liver metastasis of orthotopically implanted PanC-1-luc cells in nude mice along with induction of apoptosis. Furthermore, deguelin significantly down regulated the mesenchymal markers and increased the expression of epithelial markers *in*

vivo and *in vitro*, indicating that deguelin targets EMT to inhibit metastasis of pancreatic tumors along with primary tumor growth. In the present study, we established that deguelin targets TGFβ1 canonical Smad signaling and non-canonical NFkB signaling pathway to down regulate Snail expression leading to the induction of E-Cadherin. Deguelin also suppresses primary pancreatic tumor growth by targeting NFkB.

Since pancreatic tumors have high tendency to metastasize to peritoneal organs such as liver, spleen and intestinal mesentery nodules, *peritoneal dissemination* and liver metastasis were used as parameters to measure pancreatic tumor metastasis. As compared to control tumors, deguelin treated tumors reduced peritoneal dissemination and blocked metastasis to liver. Interestingly, control tumors were more diffused and less compact as compared to deguelin treated tumors. Based on these observations, we hypothesized that deguelin target tight junction proteins, which are necessary to bind epithelial cells to each other, to prevent tumor diffusion (metastasis). E-Cadherin is a tight junction protein that is differentially expressed in compact and diffused tumors (29, 30), and plays a critical role in tumor metastasis (31, 32). E-Cadherin expression was significantly increased by deguelin treatment; whereas mesenchymal markers were decreased *in vitro* and *in vivo* confirming that deguelin inhibits EMT in pancreatic cancer. It appears that up-regulation of E-Cadherin by deguelin in our model was due to the inhibition of Snail, an endogenous repressor of E-Cadherin.

Surprisingly, the increase in E-Cadherin mRNA by deguelin did not exactly correlated with the protein expression of E-Cadherin in PanC-1 cells. Furthermore, the extent of Snail down regulation also did not correlated with E-Cadherin up regulation as expected. One plausible reason could be that concomitant with Snail down regulation, deguelin might be activating other E-Cadherin repressors such as Slug and Twist. However, both the repressors Slug and Twist were down regulated by deguelin treatment (data not shown). Another possibility which exists is the localization of E-Cadherin. Primarily, E-Cadherin is expressed as transmembrane protein that interacts with β-catenin to maintain cell-cell adhesion (33). Salahshor *et al*. have reported that 24% pancreatic tumors show abnormal localization of E-Cadherin in the cytosol and nucleus (34). Thus we hypothesized that overall increase in E-Cadherin expression by deguelin treatment was due to the increase in membrane bound E-Cadherin. In agreement with our hypothesis, deguelin significantly down regulated E-Cadherin expression in cytosolic fraction whereas increased its expression in the membrane fraction of the cells. Our hypothesis was also strengthened by the observation that E-Cadherin and β-catenin interactions were increased in deguelin-treated PanC-1 cells, confirming that more E-Cadherin was accumulated in membrane fraction.

EMT can be induced or regulated by various growth factors such as TGFβ1, FGF, HGF, PDGF, Wnt and Notch (7). TGFβ is known to induce EMT by Smad-dependent (canonical) and Smad-independent (non-canonical) pathways (28). It is possible that deguelin specifically targets R-Smads to inhibit TGFβ1 signaling as Smad3 activation (Ser-423/425) was significantly suppressed by deguelin in TGFβ1primed cells, without affecting the protein levels of Smad4. TGFβ1 modulate NFkB activity in certain epithelial cells (8, 35) and NFkB is essential for TGFβ1-induced EMT in certain cancers such as breast cancer (11). Interestingly, when NFkB was silenced in PanC-1 and COLO-357 cells and treated

with TGFβ1, TGFβ1-induced Snail induction and E-Cadherin down-regulation was completely prevented. NFkB overexpression induced Snail expression and down regulated E-Cadherin in PanC-1 and COLO-357 cells. On the other hand, TGFβ1 treatment resulted in the phosphorylation of IkB (Ser-32) and NFkB (Ser-536). Taken together, these observations indicate that NFkB-Snail pathway is indispensable for TGFβ1-induced EMT in pancreatic cancer cells. Interestingly, deguelin significantly inhibited both constitutive and TGFβ1-induced activation of IkB (Ser-32/34) and NFkB (Ser-536) in PanC-1 cells and orthotopically implanted tumors. Furthermore, overexpression of either NFkB or Snail completely abrogated EMT inhibition by deguelin, indicating that deguelin targets NFkB-Snail pathway to inhibit EMT in pancreatic cancer. Interestingly, ectopic expression of NFkB induced Snail expression but Snail overexpression did not induced NFkB expression, indicating that NFkB acts upstream and regulates Snail in our model.

There could be two possible mechanisms by which deguelin inhibit NFkB activation in pancreatic cancer cells: (a) TGFβ1 induce NFkB nuclear localization by TGFβ Activated Kinase (TAK1) dependent IkB phosphorylation (8), and (b) RKIP mediated inhibition of NFkB activation by interacting with various up stream kinases such as NIK and TAK1 (36-38). Deguelin significantly inhibited TAK1-induced phosphorylation of IkB (Ser-32) in TGFβ-treated cells. Inhibition of IkB phosphorylation by deguelin lead to stabilization of NFkB-IkB interaction thus prevent nuclear translocation of NFkB and obligatory TGFβ1-induced Snail induction. Since, deguelin treatment significantly induced RKIP expression, it could lead to the inhibition of NFkB activation.

To evaluate whether deguelin-induced reversal of EMT and apoptosis induction were through a common pathway, induction of apoptosis (Cl. Caspase-3 and Cl. PARP) was evaluated in deguelin-treated NFkB or Snail overexpressing PanC-1 and COLO-357 cells. Interestingly, deguelin-induced apoptosis was completely blocked in NFkB overexpressing cells but not in Snail overexpressing cells. Nevertheless, EMT reversal by deguelin was significantly blocked in both NFkB and Snail overexpressing cells. These results suggest that upstream regulator, such as NFkB play significant role in both deguelin-induced reversal of EMT and apoptosis process, however the downstream effectors are different. For example, Snail overexpression failed to protect the cells from deguelin-induced apoptosis but significantly prevented EMT reversal suggesting that Snail or EMT plays no role in the apoptosis induction through deguelin in our model. A schematic diagram of deguelin targets presented in Fig. 9.

In conclusion, deguelin suppresses both pancreatic tumor growth and metastasis by inducing apoptosis and inhibiting epithelial to mesenchymal transition.

Materials and Methods

Chemicals and antibodies

Deguelin and TGF-β1 were purchased from Sigma Chemicals (Sigma, St. Louis, MO). All the antibodies were procured from Cell Signaling Technology Inc., (Danvers, MA).

Cell culture

Human pancreatic cancer cell lines Panc-1 and L3.6pl cells were kind gift from Dr. Thomas L. Brown (Wright State University, Dayton, OH) and Dr. Ming H. Wang (Texas Tech Health Sciences Center, Amarillo, TX), respectively. COLO-357 cells were kind gift from Dr. Fazlul H. Sarkar (Wayne State University, Detroit, MI) and Dr. Paul Chiao (MD Anderson Cancer Center, Houston, TX). PanC-1 cells stably expressing luciferase (PanC-1-luc) was a kind gift from Dr. Frank C Marini (MD Anderson Cancer Center, Houston, TX). Human pancreatic ductal epithelial cells (HPDE-6) were generously provided by Dr. Ming-Sound Tsao (University of Toronto, Toronto, Ontario, Canada). All the cell lines were maintained in DMEM medium except HPDE-6, which were cultured in keratinocyte-SFM medium as described by us earlier (39).

Orthotopic implantation of PanC-1 luciferase cells

PanC-1-luc cells were orthotopically implanted in the pancreas of nude mice, as described by us earlier (40). All the experiments involving animals were approved by the Institutional Animal Care and Use Committee (IACUC). Twenty 4-6 week old female athymic nude mice (Charles River, Wilmington, MA) were kept on an antioxidant-free AIN-76A diet (TestDiet, Richmond, IN) during the experiment. The mice were used in each group to get better statistical difference. Sample size was calculated using the software developed by DuPont and Plummer. The following parameters were used to calculate the sample size: α (0.1), δ (0.5), σ (0.5) and power (0.9) (41). Animals were anesthetized by Ketamine-Xylazine-Acepromazine mixture and a small left abdominal flank incision was made. Around 1×10^6 exponentially growing PanC-1-luc cells suspended in 20μL PBS were injected into the subcapsular region of the pancreas. The peritoneum and skin incisions were closed sequentially with absorbable suture. Animals were imaged on the next day of surgery for basal luminescence using IVIS Bio Luminescent System equipped with Living Image software (Caliper LifeSciences, MA). After seven days, mice were randomly separated into two groups with 10 mice in each group. Mice in the experimental group received 5mg/Kg deguelin (daily, i.p, 37 days), whereas the control mice received vehicle alone. Dose of the deguelin was selected based on previously published literature (42-44). Tumor luminescence and animal weight was measured twice a week for six weeks. At the end of the experiment (Day 45), mice were sacrificed; tumors and pancreas were excised from each mouse weighed and snap frozen for western blot, and immunohistochemical analysis. Tumor migration from the site of injection was measured by Living Image software (Caliper LifeSciences, MA).

Cytotoxicity, wound healing, transwell and RT-PCR analysis

PanC-1, COLO-357 and L3.6pl cells were treated with various concentrations deguelin and cytotoxicity, wound healing and transwell assays were performed as described by us earlier (45, 46). Total RNA was extracted from control and deguelin-treated cells and RT-PCR analysis was performed as described us earlier (46) using E-Cadherin sense and antisense primers 5’-CGC CCT ATG ATT CTC TGC TCG-3’ and 5’-TCG TCC TCG CCG CCT CCG TA-3’, respectively.

TGF-β1 treatment

Cells were plated in a 6-well plate at a density of 0.3×10^6 cells/well and left overnight. Next day, cells were serum starved overnight and media was replaced with fresh media containing 10ng/mL TGF-β1. After 48h of incubation, various concentrations of deguelin were added to TGF-β1-treated cells and co-incubated for additional 24h. Cells were collected and subjected to either western blot analysis or immunostained and observed under microscope.

NFkB transcriptional activity and DNA binding (EMSA)

PanC-1 or COLO-357 cells were treated with deguelin (10μM) for 24h and nuclear and cytosolic fractions were collected. NFkB DNA binding activity and transcriptional activity was measured as described by us earlier (47).

Transient transfections

PanC-1 or COLO-357 cells were transiently reverse-transfected with either NFkB or Snail over expressing plasmids using FuGene 6 (Roche Diagnostics Corporation, IN) transfection reagent, according to manufacturer's instructions. Briefly, either 2μg of NFkB-plasmid-DNA or 1.5μg of Snail-plasmid-DNA for PanC-1 and 3μg of NFkB-plasmid-DNA or 2μg of Snail-plasmid-DNA for COLO-357 was diluted separately in Opti-MEM media to which FuGene transfection reagent was added in a ratio of 1:3 (w:v) and incubated at RT for 1h in a 6-well plate. After incubation, Panc-1 or COLO-357 cells were trypsinized and around 0.3×10^6cells were added separately to DNA-FuGene complexes formed in the 6-well plates and incubated for another 5h. Medium was replaced with fresh media and incubated for additional 48h. Transfected cells were either treated with 0.1% DMSO or deguelin (10μM) for 24h. Cells were collected and subjected to western blot analysis.

Western blot analysis

Treated and control cells were collected and lysed in lysis buffer containing protease and phosphatase inhibitors by incubating on ice for 20 min. The protein concentration was estimated by Bradford reagent (Bio-Rad Hercules, CA). Forty microgram of protein was resolved on 10-12% SDS-PAGE and transferred on to PVDF membrane for western blot analysis as described by us previously (45, 48).

Statistical analysis

All the statistical analysis was performed using Prism 5.0 software (Graph Pad software Inc., San Diego, CA). Results were expressed as mean ± SD of the at least three independent experiments. Data was analyzed by Student's *t*-test or one way ANOVA followed by Bonferroni's post hoc analysis for multiple comparisons. Differences were considered statistically significant at $p<0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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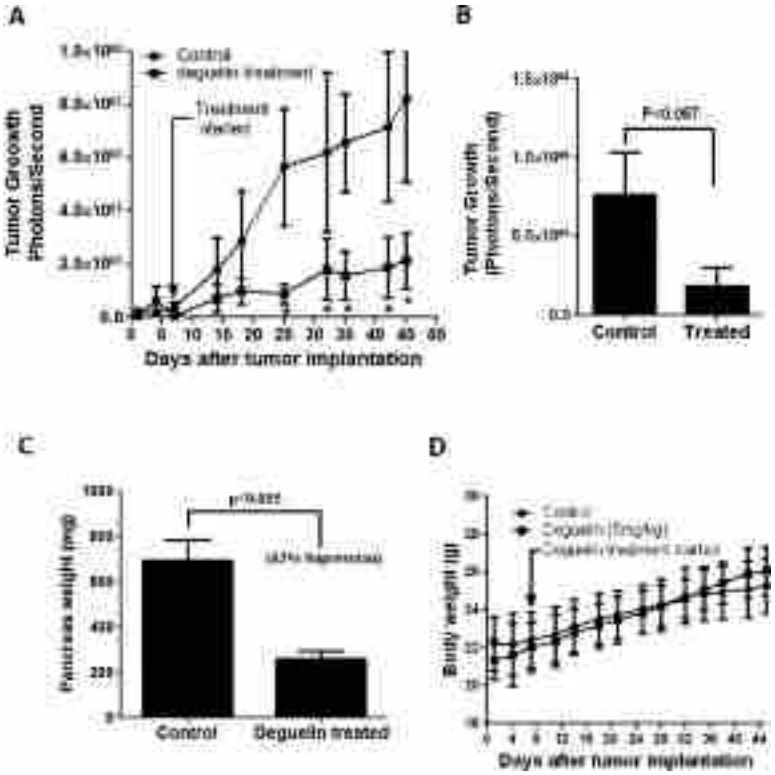

Figure 1. Deguelin suppresses primary tumor growth of pancreatic tumors

Mice were orthotopically implanted with PanC-1-luc cells and treated with deguelin (5mg/Kg, daily, i.p) for 37 days. **A)**. Tumor growth was monitored by measuring luminescence of control and deguelin-treated mice twice a week by IVIS imaging station. **B)**. Tumor growth curve (photons/second; at the termination of the experiment) of control and treated mice. **C).** Weight of the pancreas with tumor. At the end of the experiment, pancreas along with the primary tumor from the control and deguelin-treated mice were carefully excised, weighted and presented as a bar graph. **D).** Weight of the control and deguelin-treated mice presented as bar graph. Values are Mean±SD, *=p<0.05.

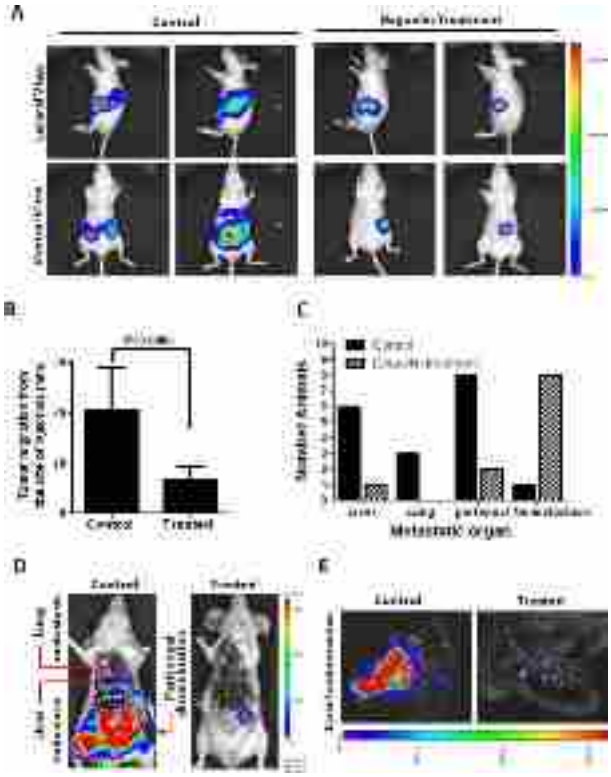

Figure 2. Deguelin inhibits in vivo metastasis of pancreatic tumors

Mice were orthotopically implanted with PanC-1-luc cells and treated with deguelin (5mg/Kg, daily, i.p). Tumor luminescence was measured twice a week by IVIS *in vivo* imaging station. **A)** A representative images showing peritoneal dissemination of control and deguelin-treated animals were presented **B)** Distance of the secondary tumors that migrated from pancreas was measured by Living Image Software. **C)** At the end of the experiment animals were euthanized and pancreatic tumor metastasis to various organs were counted. **D)** At the end of the experiment, animals from control and deguelin-treated were imaged at maximum time scale (5 min) to visualize micro metastasis and representative images were presented. **E)** At the end of the experiment, animals were given with luciferin and animals were euthanized. Intact livers were carefully removed from control and deguelin-treated mice and imaged for metastatic lesions. Values are Mean±SD.

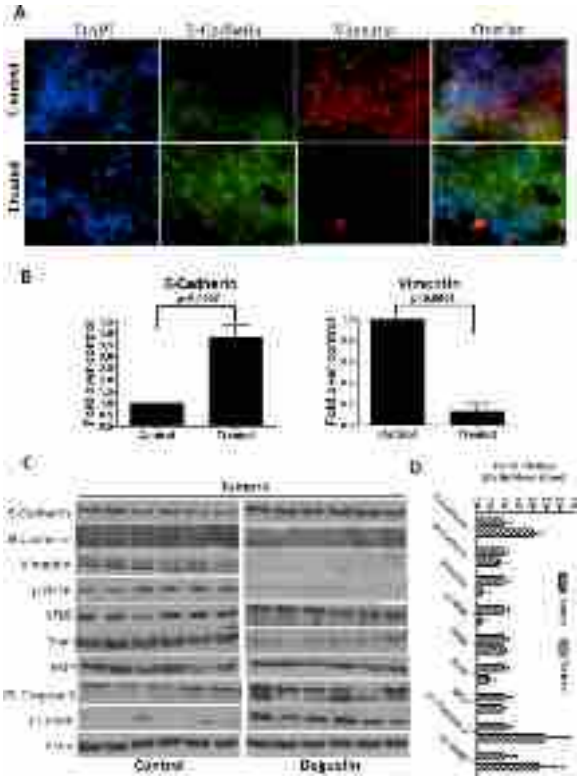

Figure 3. Deguelin inhibits EMT in vivo

A) At the end of the experiment, control and deguelin-treated tumors were excised and immunostained for E-Cadherin and vimentin. DAPI was used as an internal reference. **B**) Immunofluorescence of tumor section was quantified by Image J software and presented as bar diagram. Values are Mean±SD of at least three slides. **C)** Tumors from control and treated mice were homogenized and equal amount of protein was subjected to western blot. **D)** Tumor western blots were quantified using UN-SCAN-IT software and presented as bar diagram. Values are Mean±SD of six separate tumors. *=p <0.05.

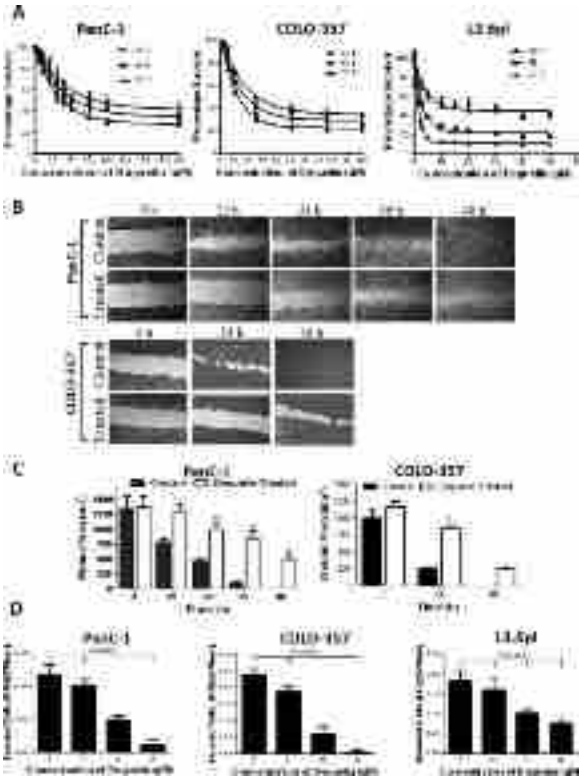

Figure 4. Deguelin inhibits proliferation, migration and invasion of pancreatic cancer cells

A) Cells were plated in a 96-well plate, treated with various concentrations of deguelin and viability was measured by SRB assay. **B)** Confluent monolayers of PanC-1 and COLO-357 cells were scratched with 1mL pipette tip, treated with 5μM deguelin and photographed using Nikon microscope. **C)** Wound area of control and treated cells was quantified by Image J software. Values are Mean±SD. *=p<0.05. **D)** Invasion of pancreatic cells was measured by Boyden's Transwell assay according to manufacturer's instructions. Values are Mean±SD. *=p<0.05.

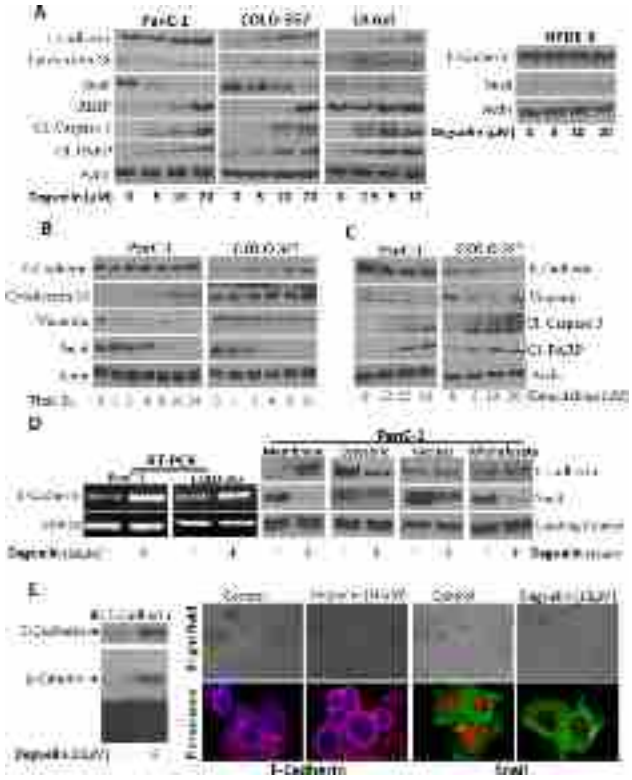

Figure 5. Deguelin inhibits EMT in vitro

Cells were treated with deguelin either in **A)** dose or **B**) time-dependent manner and EMT markers were evaluated western blot. **C)** PanC-1 and COLO-357 cells were treated with gemcitabine for 72h and EMT markers were evaluated by western blot. **D)** PanC-1 cells were treated with 10μM deguelin for 24h and membrane, cytosolic, nuclear, and whole cell lysates were subjected to western blot whereas, mRNA was analyzed by RT-PCR for E-Cadherin expression. **E**) Equal protein from control and deguelin-treated cells were immunoprecipitated with E-Cadherin antibody and immunoblotted for β-catenin. **D)** *(right panel)*. PanC-1 cells were treated with deguelin and immunostained for E-Cadherin, Snail (red) and actin (green).

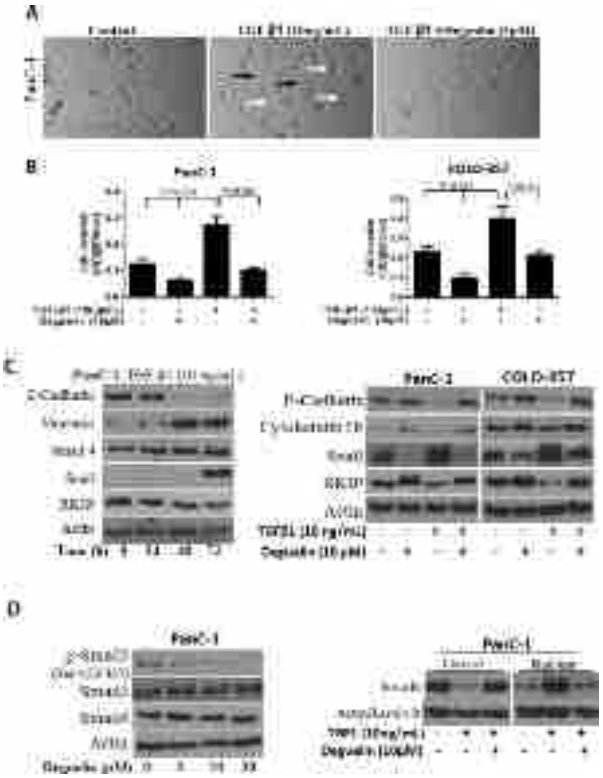

Figure 6. Deguelin inhibits TGFβ1-induced EMT in pancreatic cancer cells

PanC-1 cells were serum starved and treated with TGFβ1 or deguelin or both. **A)** After 72h of treatment, cells were either evaluated for morphological change (white arrows show lamellipodia whereas, black arrows show space between cells) or **B)** invasion by Boyden's Transwell assay or **C)** EMT markers by western blot. **D)** PanC-1 cells were treated with deguelin for 24h and whole cell lysates were evaluated for phosphorylation and expression of Smad3/4 or **D)** (*right panel)* nuclear localization of Smad4.

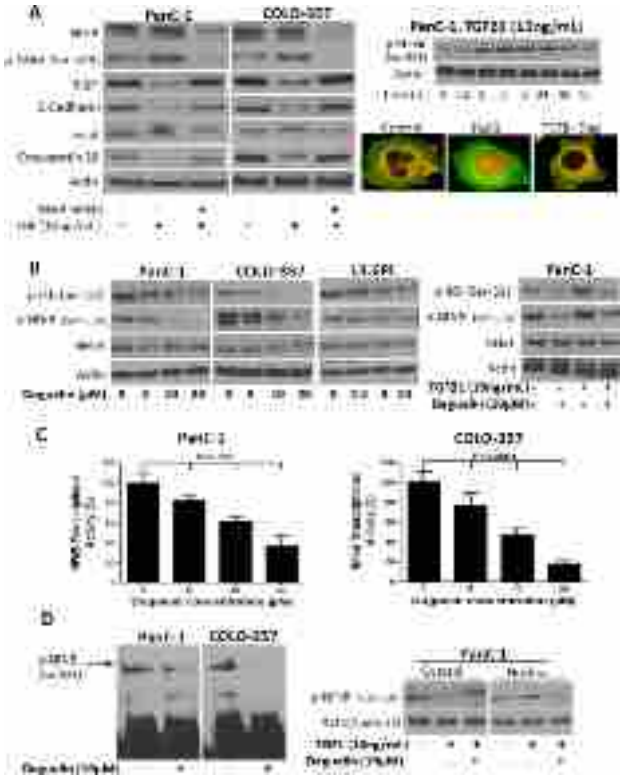

Figure 7. Role of NFkB in TGFβ1-induced EMT

A) PanC-1 and COLO-357 cells were transfected with 100pM NFkB siRNA, treated with TGFβ1 for 72h and whole cell lysates were analyzed for EMT markers by western blot. **A)** *(right upper panel)*. PanC-1 cells were treated with TGFβ1 for various time points and phosphorylation of NFkB (Ser-536) was monitored by western blotting. **A)** *(right bottom panel)*. PanC-1 cells were treated with TGFβ1 for 2h and immunostained for NFkB (red). **B)** Pancreatic cancer cells were treated with deguelin or **B)** *(right panel)* pretreated with TGFβ1 followed by deguelin and phosphorylation of IkB (Ser-32) and NFkB (Ser-536) was evaluated by western blot. **C)** PanC-1 and COLO-357 cells were treated with deguelin and NFkB transcriptional activity was measured using commercially available kit from Panomics (Fremont, CA) and **D)** DNA binding activity was measured by EMSA kit (Panomics, CA), according to manufacturer's instructions. **D)** *(right panel)*. PanC-1 cells were pretreated with deguelin, primed with TGFβ1 and nuclear and cytosolic fractions were subjected to western blot analysis to evaluate NFkB localization.

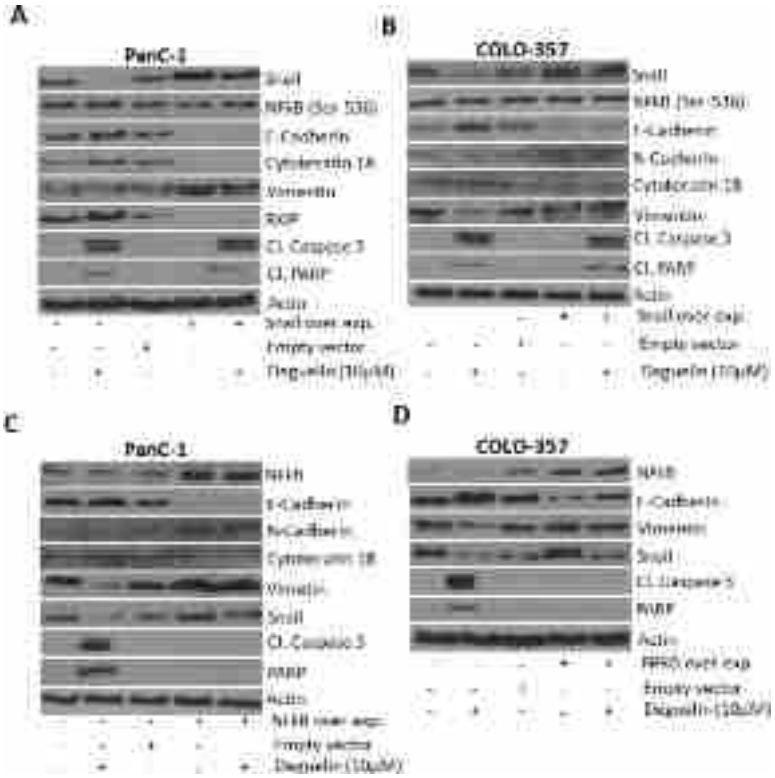

Figure 8. Overexpression of Snail or NFkB abrogates deguelin-induced epithelial markers in pancreatic cancer cells

PanC-1 and COLO-357 cells were reverse transfected with **A)** &**B)** NFkB plasmid or **C) & D)** Snail plasmid in PanC-1 and COLO-357 cells, respectively, using FuGene transfection reagent according to manufacturer's instructions and treated with deguelin. Equal amount of protein from control and treated cells were analyzed for EMT markers.

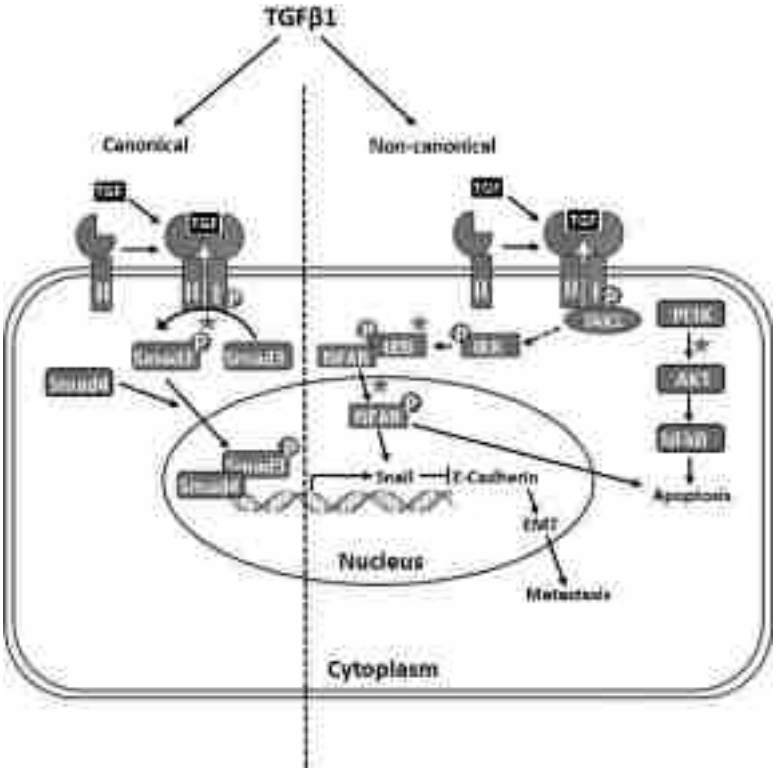

Figure 9. Deguelin targets canonical and non-canonical TGFβ1 pathway to inhibit EMT and metastasis

TGFβ1 is known to induce EMT by both Smad-dependent and Smad-dependent pathways. In canonical pathway, upon binding to its receptor TGFβ1 leads to the activation of R-Smads such as Smad2 and Smad3. The activated R-Smads binds to co-Smads such as Smad4 and translocate into the nucleus leading to Snail up regulation and eventually EMT. In TGFβ1-non canonical pathway, TGFβ1 receptors activate various downstream molecules such as NFkB and MAPK. TGFβ1 is known to activate TAK1, which in turn phosphorylates NFkB inhibitor IkB leading to the proteosomal degradation of IkB. IkB-free NFkB translocate to nucleus and induce Snail expression, which leads to E-Cadherin down regulation.★ indicates deguelin potential targets.