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Abstract

Effects of dietary flavonoid luteolin alone or in combination with established chemotherapy drugs Tasigna (nilotinib) or Adrucil (5-fluorouracil, 5-FU) were explored on Bcl-2-positive (PL5, MiaPaCa-2) and Bcl-2-negative (Panc1, Capan-2) pancreatic adenocarcinoma (PDAC) cell growth, viability and responses to growth factors that are implicated in pancreatic tumor development, progression and resistance to anti-cancer agents. Growth inhibition dose-response curves following 48 h exposure to luteolin, Tasigna, Adrucil or DMSO control in 1% FBS-containing medium were generated by fluorometric AlamarBlue assay. Luteolin down-regulated basal activation levels of pro-survival serine/threonine protein kinase Akt, non-receptor protein tyrosine kinases c-Src and FAK, and latent cytoplasmic transcription factor STAT3, also reduced the expression of Bcl-2, cyclin D1 and vimentin, but increased the activation of ERK1/2, p38 MAPK and Rb protein, and promoted the caspase-3 and PARP-1 cleavage in a dose-dependent manner as determined by immunoblotting and immunofluorescence analyses. Restored Akt phosphorylation by PP2A inhibition did not rescue cells from luteolin-induced apoptosis, which occurred in c-Myc-dependent manner and was further augmented by ERK1/2 inhibition in Bcl-2(-) PDAC cells. By contrast, c-Myc inhibiton increased luteolin-induced apoptotic signals in Bcl-2(+) PDAC cells. An ability of luteolin to synergize with drugs for acceleration of PARP-1 cleavage was cell type-dependent and consistently more pronounced in IGF-1-treated cells, suggesting that EGF engaged the compensatory survival mechanism(s) possibly mediated by ERK1/2. Luteolin may yield additional benefit in combined chemotherapy applications in preclinical *in vivo* studies of pancreatic cancer.

Synergistic anti-tumor effect by a combination treatment of dietary flavonoid luteolin and chemotherapy drugs Tasigna or Adrucil in human pancreatic cancer cells

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Introduction

Pancreatic cancer (PC) is the fourth leading cause of cancer-related death for both gendres in the United States and European Union countries. It is one of the most fatal types of tumors due to its scarce symptoms until cancer is well developed, with the strikingly short length of time between diagnosis and patient death (usually < 6 months; 6% 5-year survival rate) [1].

Chemotherapy and radiation therapy are the most common treatment options that are offered to relieve symptoms, improve the quality of life and/or increase survival rates in patients with advanced inoperable PC. However, currently apporved drugs for PC produce responses only in a minority of patients, and have been reported to cause severe complications as well as unwanted hematologic or cardiac toxicity side effects that are associated with the dosage and length of the treatment. Therefore, the development of more effective, less toxic and more individualized combination therapy strategies against invasive tumors is in high demand [2, 3].

PC risk has been reported to decrease with increased consumption of fresh vegetables and fruits that are rich in flavonoids – the large class of polyphenolic compounds [4-6]. The administration of dietary flavonoids has been shown to inhibit pancreatic tumor growth *in vitro* [7-9] and *in vivo* [10-12]. There is also an increasing body of evidence that simultaneous use of anti-cancer drugs with dietary phytochemicals act synergistically in suppressing malignant cell growth and/or promoting their death [13, 14].

The anti-cancer activity of the dietary flavonoid luteolin (3',4',5,7-tetrahydroxyflavone) (**Fig. 1**) have been demonstrated in many types of human solid tumor [15-17], however, its impact on human pancreatic adenocarcinoma (PDAC) cell physiology has not been extensively studied, whereas its effects on short-, medium- and long-term signaling of chemoresistance-related proteins and potential chemosensitizing effects in combination with the conventional anti-cancer drugs have not been addressed.

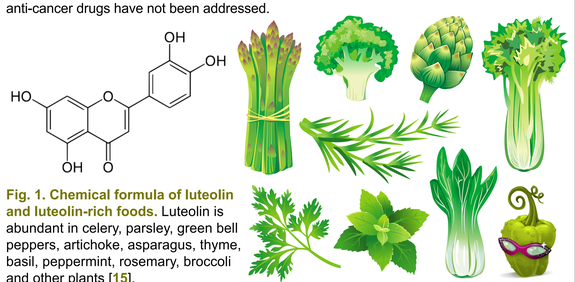

Fig. 1. Chemical formula of luteolin and luteolin-rich foods. Luteolin is abundant in celery, parsley, green bell peppers, artichoke, asparagus, thyme, basil, peppermint, rosemary, broccoli and other plants [15].

Expression of chemoresistance-related proteins in PDAC cell lines

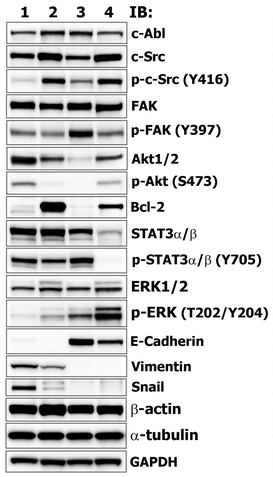

Fig. 2. The total and/or basal activation levels of proteins known to be associated with PC chemoresistance were detected by Western blotting and compared in slightly differentiated non-metastatic Panc-1 (*lane 1*), poorly differentiated highly metastatic MiaPaCa-2 (*lane 2*), moderately-to-well differentiated low-metastatic Capan-2 (*lane 3*) and PL5 (Panc 04.03) (*lane 4*) PDAC cell lines. Cells were grown in 100×20 mm tissue culture dishes in a complete (10% FBS) DMEM media until 80-90% confluent and lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM HEPES (pH 7.4), 1 mM EGTA, 1% Triton X-100, 10% glycerol diluted in dH2O plus the phosphatase and protease inhibitor cocktails). Proteins from whole-cell lysates were resolved by LDS-PAGE at 140V using 4-12% gradient Bis-Tris gels (Invitrogen) followed by electrotransfer onto nitrocellulose membranes at 30V for 90 min. Blots were kept in 3% BSA blocking buffer for 1h, then incubated with 1°Ab against the phosphorylated (p-) or non-phosphorylated protein forms overnight at RT, washed (5x, 7 min/each) and treated with respective 2°Ab for 1 h followed by a final wash. The blocking, Ab dilution and washing solutions contained Tris-buffered saline with 0.5% Triton X-100. The chemiluminescent signals of protein bands were detected by ECL using SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology) in KODAK Image Station 440CF. IB - immunoblotting; Ab - antibody.

Results I

In vitro PDAC cell chemosensitivity to luteolin, 5-fluorouracil and nilotinib

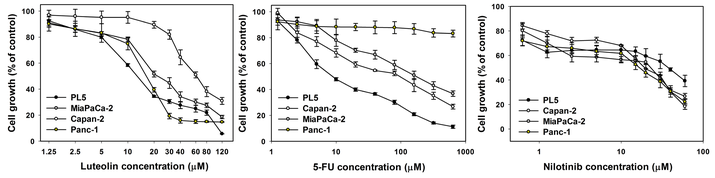

Fig. 3. PL5 (*black circles*), Capan-2 (*grey circles*), MiaPaCa-2 (*white circles*), and Panc-1 (*yellow-green circles*) cells were plated in quadruplicate into 24-well plates at a density of 20,000 cells/ml. After 24 hours, complete culture medium was changed into fresh low-serum-containing media (1% FBS) with DMSO solvent-only (control) or indicated increasing doses of luteolin (left panel), 5-FU (middle panel) or nilotinib (right panel). Cell growth/viability 48 hours after treatment was determined by AlamarBlue oxidation-reduction indicator assay (Invitrogen), which is based on conversion of blue non-fluorescent dye resazurin to pink highly fluorescent resorufin by mitochondrial enzymes. Fluorescence was measured with a microplate reader (BioTek) at Ex/Em wavelength set of 530/590. Results are expressed as percentages of control, which was arbitrarily assigned 100% growth, and represented as the mean ± standard deviation (SD, error bars) of the quadruplicate wells.

Luteolin suppresses EGF and IGF-1-induced PDAC cell growth

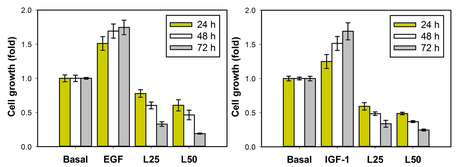

Fig. 4. MiaPaCa-2 cells were treated with luteolin for 24 h (*yellow-green bars*), 48 h (*white bars*) or 72 h (*grey bars*) in serum-free media either with 5 nM EGF (left panel) or 5 nM IGF-1 (right panel). Cell growth was determined by AlamarBlue assay as described under Fig. 3. Results are expressed as fold above basal growth level, and represented as mean ± SD obtained from three independent experiments measured in quadruplicates.

Long-term effects of luteolin on basal MAPK signaling and cell cycle-related proteins

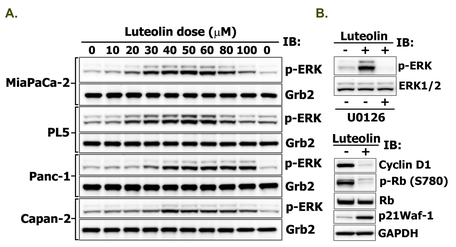

Fig. 5. A. Luteolin increases basal ERK1/2 activation. PDAC cells were treated with the indicated doses of luteolin or DMSO-only in low-serum-containing media (0.1% FBS) for 24 h and lysed. Floating cells were collected, spun down, lysed and mixed with respective first lysates. Proteins from whole-cell lysates (WCL) were transferred to NC membranes that were probed by IB with antibodies against the indicated proteins. **B. Upper panel. MEK1/2 inhibition abrogates luteolin-induced ERK activation.** Panc1 cells were pretreated with 5 μM MEK inhibitor U0126 for 30 min before adding 40 μM luteolin (+) or DMSO (-) for 24 h. **Bottom panel.** Blots of WCL from luteolin-treated (40 μM, 24 h) PL5 cells were probed with antibodies against the indicated proteins.

Luteolin decreases mitochondrial membrane potential (ΔΨm, MMP) and induces apoptosis via caspase-3 and PARP cleavage in PDAC cells

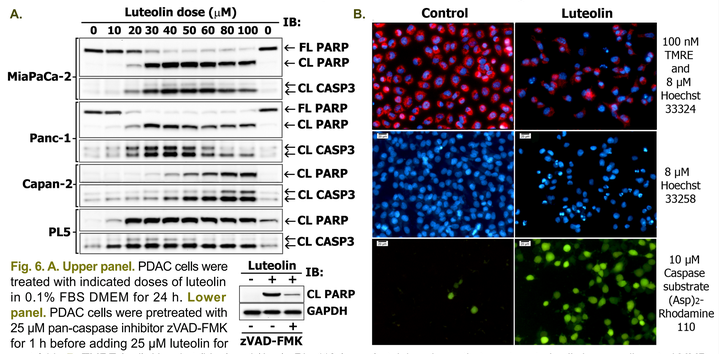

Fig. 6. A. Upper panel. PDAC cells were treated with indicated doses of luteolin in 0.1% FBS DMEM for 24 h. **Lower panel.** PDAC cells were pretreated with 25 μM pan-caspase inhibitor zVAD-FMK for 1 h before adding 25 μM luteolin for next 24 h. **B.** TMRE (red), Hoechst (blue) and (Asp)2-Rho110 (green) staining shows that most control cells have undisrupted MMP, intact nuclei and no caspase-3 activation, and luteolin-treated cells have reduced MMP, fragmented nuclei and active caspase-3.

Results II

Short-, medium- and long-term effects of luteolin on PI3-kinase/Akt signaling

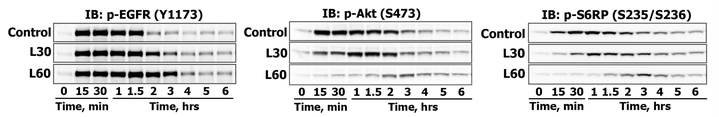

Fig. 7. Luteolin does not affect EGFR autophosphorylation, but inhibits EGF-induced Akt activation, which decreases activating phosphorylation of S6 ribosomal protein (S6RP) that functions downstream of p70 S6 kinase. Serum-starved Capan-2 cells were pretreated with DMSO-only (control), 30 μM or 60 μM luteolin (L) for 30 min prior to stimulation with 2 nM EGF for the indicated time intervals (in min or hours).

The roles of Akt, PP2A, p38 MAPK and ERK1/2 in luteolin-induced apoptosis

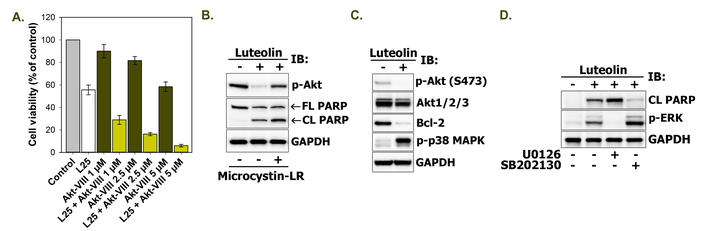

Fig. 8. A. Akt inhibition augments luteolin-induced apoptosis. PL5 cells grown in 24-well plates were treated with DMSO-only (control), 25 μM luteolin, indicated doses of Akt inhibitor VIII or their combinations. Cell viability 24 h after treatment was determined by AlamarBlue assay. Results are expressed as percentages of control, which was arbitrarily assigned 100% viability, and represented as the mean ± SD of the triplicate wells. **B. Partially restored Akt phosphorylation by PP2A inhibition does not rescue PDAC cells from luteolin-induced apoptosis.** Panc-1 cells were pretreated with 0.5 nM PP2A inhibitor microcystin-LR (IC_{50} = 0.04 nM) for 1 h prior to treatment with 20 μM luteolin for next 24 h in 0.1% FBS media. **C. Inhibition of basally active Akt by chronic luteolin exposure correlates with reduced levels of anti-apoptotic protein Bcl-2 expression and activation of stress-induced p38 MAPK.** PL5 cells were treated with DMSO-only (-) or luteolin (40 μM, 24 h in 0.1% FBS-containing media). **D. Inhibition of ERK further increases PARP cleavage, whereas p38 MAPK inhibition prevents it.** MiaPaCa-2 cells were pretreated with 10 μM MEK inhibitor U0126 or 10 μM p38 MAPK inhibitor SB202130 for 1 h prior to addition of 30 μM luteolin for next 24 h in 0.1% FBS media. Blots of WCL were probed with antibodies against cleaved (CL) form of PARP, p-ERK1/2 or GAPDH.

Effects of luteolin on Src/FAK/STAT3 activation and expression of vimentin and c-Myc

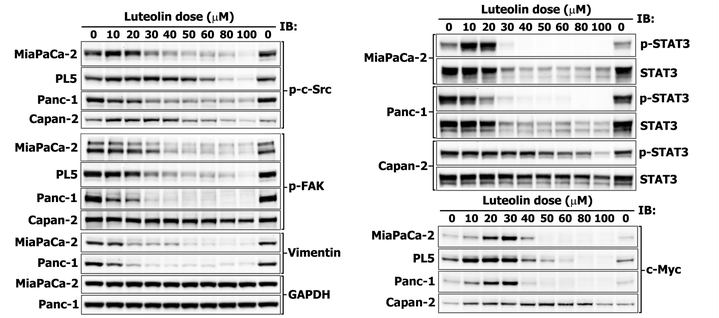

Fig. 9. Chronic luteolin exposure inhibits basal activating phosphorylation of c-Src on Y416, c-Src-independent autophosphorylation of FAK on Y397, activating phosphorylation of STAT3 on Y705, and down-regulates STAT3 and EMT marker vimentin total protein levels. Dual effects of luteolin on immediate-early gene c-Myc expression depends on its dose. PDAC cells were treated with DMSO-only (-) or increasing doses of luteolin for 24 h in 0.1% FBS media.

Oposing roles of c-Myc in luteolin-induced apoptosis

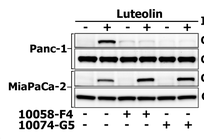

Fig. 10. Inhibition of c-Myc-Max binding by 10058-F4 or 10074-G5 promotes PARP cleavage in Panc-1, but not in MiaPaCa-2 cells. Panc-1 or MiaPaCa-2 cells were either left untreated or were pretreated with 15 μM 10058-F4 or 10 μM 10074-G5 (Sigma-Aldrich) inhibitors for 1 h before adding 30 μM luteolin or DMSO-only (-) for next 24 h in 0.1% FBS media. Blots of whole-cell lysates were probed with antibodies against cleaved (CL) form of PARP or GAPDH (loading control).

Results III

Synergistic effects of low-dose combinations of luteolin and Tasigna or Adrucil on PDAC cells

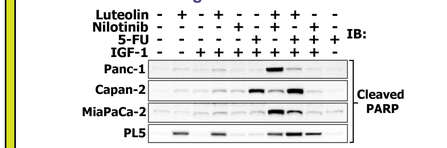

Fig. 11. Serum-starved PDAC cells were treated with luteolin (30 μM for Capan-2, 15 μM for MiaPaca-2, Panc-1 and PL5), nilotinib (2.5 μM), 5-FU (10 μM for PL5, 20 μM for Capan-2, MiaPaca-2 and Panc1) or their combinations in the presence of 5 nM IGF-1 in serum-free medium for next 24 h.

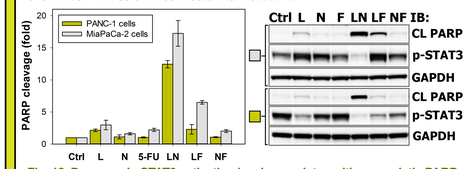

Fig. 12. Decrease in STAT3 activation levels correlates with synergistic PARP cleavage in PDAC cells upon combined treatment with luteolin and nilotinib. Blots from WCL of 24h luteolin (L, 15 μM), nilotinib (N, 2.5 μM) or 5-FU (F, 20 μM) -treated MiaPaca-2 (grey bars) or Panc-1 (yellow-green bars) were probed with antibodies against cleaved PARP, p-STAT3 (Y705) or GAPDH (loading control).

Conclusions

1. Cytostatic effects of luteolin on PDAC cell growth may be mediated via inhibition of cyclin-dependent kinases by upregulated p21Waf-1, leadingto downregulation of cyclin D1, a key player for G1/S transition, and subsequent activation of retinoblastoma tumor suppressor protein Rb, which induces cell cycle arrest.
2. Luteolin reduces PDAC cell viability and promotes intrinsic mitochondrial apoptotic pathway via caspase-3 activation and PARP cleavage in c-Myc dependent and independent manners. ERK protects PDAC cells from luteolin-induced apoptosis, but is a subject to multiple negative feedback regulations (e.g. by p38 MAPK).
3. Luteolin-induced suppression of activated Akt can be caused by direct inhibition of p110 catalytic subunit of the PI3-kinase (IC_{50} = 8.65 μM *in vitro*), the attenuation of pathways that act upstream of PI3K/Akt such as c-Src/FAK and JAK/STAT3, and/or enhancement of PP2A phosphatase activity. The decrease in total Akt, STAT3 and vimentin levels may be a consequence of elevated caspase-3 activity.
4. With luteolin, lower doses of individual chemotherapeutic agents can be used to achieve the same or better beneficial effects on survival and improvement of symptoms with lower toxicity. Such combinations may also help in overcoming an intrinsic or acquired tumor resistance to individual chemotherapeutic drugs.

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