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Abstract

Irinotecan (CPT-11) is one of the main agents used to treat colorectal cancer; unfortunately, it is associated with increased intestinal mucositis developing. Luteolin has been shown to prevent damage induced by this chemotherapeutic in mice; thus, in this research, we have investigated luteolin's action mechanism in human intestinal epithelial cells. The potential of luteolin in reducing inflammation and oxidative stress induced by irinotecan in Caco-2 cells was evaluated by PCR through mRNA expression of inflammatory and oxidative genes and by ELISA at the protein level. To assess whether luteolin's ability to control irinotecan-induced damage occurs in a PPARγ dependent manner, experiments were performed on PPARγ downregulated cells. Irinotecan downregulated PPARγ expression and upregulated inflammatory and oxidative genes, while luteolin upregulated PPARγ, HO-1, SOD and decreased expression of IL-1β and iNOS. Interestingly, when the cells were co-stimulated with luteolin and irinotecan, the flavonoid reversed the inflammation and oxidative imbalance evoked by the chemotherapeutic. However, when these experiments were performed in cells downregulated for PPARγ, luteolin lost the capacity to increase PPARγ and reverse the effect of irinotecan in all tested genes, except by IL-1β. The present study showed that the protective effect of luteolin against irinotecan is PPARγ dependent.

Keywords: Intestinal mucositis, Caco-2, inflammation, oxidative stress, chemotherapy, rosiglitazone.

Abbreviations

CPT-11, Irinotecan; COX, Cyclooxygenase; CTRL; Control; DAMP, damage-associated molecular patterns; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; DPPH, 2,2- diphenyl-1- picrylhydrazyl; GPX, glutathione peroxidase; HO-1, heme oxygenase-1; IL, Interleukin; iNOS, inducible nitric oxide synthase; NQO-1, NAD(P)H quinone oxidoreductase 1; NRF-2, factor erythroid 2-related factor; PGE2, prostaglandin E2; PPARγ, peroxisome proliferator-activated receptor-gamma; ROS, reactive oxygen species; ShPPARγ, Caco-2 cell line knockdown for PPARγ; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-alpha.

1 Introduction

Irinotecan (CPT-11), a chemotherapeutic drug analogous to camptothecin, is one of the leading agents used in treating colorectal cancer, acting inhibiting topoisomerase I [1]. Unfortunately, incorporating irinotecan into anticancer regimens is particularly associated with an increased risk of developing intestinal mucositis [2].

Intestinal mucositis is characterized by the mucosal barrier breakdown resulting in severe ulceration of the gastrointestinal tract and bacteria passing into the systemic circulation, increasing the risk of infections [3]. Sonis [4] has proposed that DNA damage, non-DNA damage, and ROS generation initiate an interesting and complex series of events that are still being defined, but that results in the activation of several transduction pathways resulting in the upregulation of up to 200 genes, many of which potentially influence mucosal toxicity.

Patients experiencing intestinal mucositis have nausea, vomiting, bleeding, abdominal pain, malnutrition, infections, sepsis, and diarrhea [5]. In fact, diarrhea is the main cause of patients' morbidity and mortality [6]. Currently, the treatments for mucositis are limited and largely target to oral rehydration and electrolyte replacement, as well as the use of pharmacologic agents to reduce fluid loss or decrease intestinal motility [3]; however, these approaches have low efficacy often leading to reduction of doses or interruption of the chemotherapeutic regime, consequently decreasing the chances of cancer remission [1].

In this way, searching for new therapeutic alternatives, we have previously evaluated the effect of the flavonoid luteolin in the prevention of irinotecan-induced intestinal mucositis in mice, evidencing that luteolin decreases oxidative stress, inflammatory process and maintains mucosal protective factors, such as mucus and expression of tight junctions, without interfering with the chemotherapeutic efficiency[7]. In the referenced study, it was observed that luteolin prevents the increase of cytokines such as TNF, IL-1β, and IL-6, without reducing PGE2 levels, in addition to ameliorating oxidative imbalance. This suggests that the modulation of transcription factors may be involved in the compound's mode of action, whereas modulation of COX does not appear to be part of the effect.

Actually, luteolin (3′,4′,5,7-tetrahydroxy flavone) is a flavone naturally found in several plant species, including broccoli, pepper, thyme, and celery [8,9], which exhibits a large number of biological activities reported in the literature and varied mechanisms of action described [10–16]. Among so many hypotheses, we call attention to the various reports suggesting that luteolin acts by activating the PPARγ pathway [17–20], which could justify our findings mentioned before. Thus, in this study, we have developed an *in vitro* model of irinotecan-induced damage in human intestinal epithelial cells and provide evidence that this flavonoid's effect on the attenuation of cellular damage induced by irinotecan is dependent on the PPARγ pathway.

2. Methods

3. Materials

Luteolin (≥98% purity, powder) was commercially obtained from Active-Pharmaceutica (Palhoça, SC, Brazil). All other drugs and reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

3.1 Cell culture

Human intestinal epithelial cell line Caco-2 (ATCC® CRL-2102TM) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Life Technologies, Cergy-Pontoise, France) supplemented with 20% fetal calf serum (FCS, Dutscher, Brumath, France), 1% penicillin-streptomycin (Invitrogen, Life technologies), and 1% non-essential amino acids (Invitrogen, Life technologies).

All cell lines were cultured as confluent monolayers at 37°C in a controlled, 5% CO2 atmosphere.

3.1.2 Generation of PPARγ knockdown cells

Generation of PPARγ knockdown Caco-2 cells and the analysis of silencing of PPARγ expression by quantitative reverse transcription PCR and western-blot have been previously described [21].

3.2 Experimental Design

Caco-2 cells were seeded in 12-well plates (0.5×10^6). To synchronize the cell cycle, a medium deprived of serum was used 16 h before stimulation. Firstly, cells were incubated with irinotecan (10, 30, and 100 μM; Trebyxan® Laboratório Químico Farmacêutico Bergamo Ltda, Brazil) to determine the appropriate concentration to induce an inflammatory and oxidative response. After, the effect of luteolin (98% purity, powder from Active-pharmaceutica Palhoça, SC, Brazil) was standardized in three different concentrations (3, 10, and 30 μM). Rosiglitazone (1 μM; Sigma-Aldrich) was also incubated to visualize the effect of a full PPARγ agonist [22] in these cells. When necessary, the DMSO vehicle (Sigma-Aldrich) was used as control.

In another set of experiments, cells were incubated with Luteolin (3, 10, and 30 μM), or Rosiglitazone (1 μM) with irinotecan (100 μM) at the same time for 24 hours. Thus, the supernatant was collected for the quantification of cytokines using ELISA kits. The cells were subsequently washed with sterile PBS and lysed for RNA extraction, or another dosage described below.

Cell stimulations were performed in 3 or 6 replicates.

3.4 Enzyme-Linked Immunosorbent Assay

The supernatant of cells was used to quantify the cytokines TNF-α, IL-33, and IL-1β and the results were expressed as pg/ml. Total ROS and SOD-1 were measured on the cell lysed. These results were expressed as pg/ml according to the protein level measured by the Bradford method.

ELISA kits from BD Biosciences (Franklin Lakes, New Jersey, USA) were used according to the manufacturer's instructions.

ROS

Total reactive oxygen species (ROS) were evaluated using a ROS assay Kit (Invitrogen, Thermo Fisher Scientific Logo) according to the manufacturer's instructions on the cultured cell supernatant and lysed cells.

3.5 RNA extraction

Firstly, cells were lysed by incubation in a solution containing large amounts of chaotropic ions. This lysis buffer immediately inactivated RNases, and total RNA was extracted with a Nucleospin RNA kit (Macherey-Nagel, Hoerdt, France). After RNAse inactivation, the total RNA was cleaned of traces of genomic DNA with a rDNase solution. The subsequent washing steps with different buffers removed salts, metabolites, and macromolecular cellular components, and then, pure RNA was finally eluted with RNase-free H_2O.

The RNA's purity was evaluated by UV spectroscopy on a Nanodrop system from 220 to 350 nm.

3.6 Quantitative RT-PCR

To performed quantitative reverse transcription-polymerase chain reaction (RT-PCR), 1 μg of total RNA was reverse-transcribed into cDNA using the High-Capacity cDNA Archive kit (Applied Biosystems).

Then, 2.5 μL of a 1:5 dilution of cDNA was employed for qPCR. ABI PRISM StepOnePlus detection system (Applied Biosystem) using Power SYBR® Green PCR master Mix (Applied Biosystem) was employed. Primer pairs were chosen with qPrimer depot software according to table 1. Quantification of qPCR signals was performed using ΔCt relative quantification method using GAPDH as a reference gene.

Table 1. Oligonucleotide Sequences for Quantitative RT-PCR

Genes	Forward sequences	Reverse sequences
GAPDH	5’-GACACCCACTCCTCCACCTTT-3’	5’-TTGCTGTAGCCAAATTCGTTGT-3’
PPARγ	5’-GCTGTCATTATTCTCAGTGGAGAC-3’	5’-GTCTTCTTGATCACATGCAGTAG-3’
IL-1β	5’- GATGCACCTGTACGATCACT - 3’	5’- GACATGGAGAACACCACTTG -3’
IL-33	5′-ACAGAATACTGAAAAATGAAGCC-3′	5′-CTTCTCCAGTGGTAGCATTTG-3′
iNOS	5’-CGGTGCTGTATTTCCTTACGAGGCGAAGAAGG -3’	5’-GGTGCTGCTTGTTAGGAGGTCAAGTAAAGGGC-3’
TNFα	5’-ATCAATCGGCCCGACTATCTC-3’	5’-ACAGGGCAATGATCCCAAAGT-3’
GPX	5′-GTG6TTG-GCT-TTT-CCC-TGC-AA-3’	5′-ACA-GCA-TAT-GCA-AGG6CAG-ATA-3’
NQO-1	5′-TGA-AGA-AGA-AAG-GAT-GGG-AGG-3’	5′-AGG-GGG-AAC-TGG-Aat-ATC-AC-3’
NRF-2	5′-TCA-GCC-AGG-CCA-GCA-CAT-CC-3’	5′-TCT-GCG-CCA-AAA-GCT-GCA-TGC-3’
HO-1	5′-TTG-CCA-GTG-CCA-CCA-AGT-TC-3’	5′-TCA-GCA-GCT-CCT-GCA-ACT-CC-3’

SOD	5′-ACA-AAG-ATG-GTG-TGG-CCG-AT-3’	5′-TCT-GGA-TCT6TTA-GAA-ACC-GCG-A-3’

3.7 Statistics

The data were analyzed by an investigator blinded to the experimental conditions. Each *in vitro* experiment was conducted at least three times independently. The Kolmogorov-Smirnov normality test was applied to verify the data normality. The data were expressed as mean ± SEM, and one- or two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was applied to verify the differences between means. Statistical analysis was performed using the software GraphPad Prism (RRID:SCR_002798) version 7.00 (GraphPad Software, La Jolla, CA, USA). A $p < 0.05$ was considered significant.

4. Results

4.1 Irinotecan concentrations to induce intestinal cell damage

Previously studies have demonstrated that irinotecan does not significantly reduce the cell viability of Caco-2 cells at 1 to 100 µM in 24 hours of incubation [23]. In this way, we have investigated the effect of irinotecan at 10, 30, and 100 µM during 24 hours of incubation, evaluating different target genes in the human epithelial intestinal cells to select the optimal concentration to maximize inflammation and oxidative stress without overly affecting viability.

4.2 Irinotecan-induced inflammation and oxidative imbalance in Caco-2 cells

As shown in figure 1, irinotecan at 100 μM decreased in 60% the PPARγ gene expression compared to control, and significantly increased the expression of the cytokines interleukin (IL)-1β (9.97-folds); interleukin (IL)-33 (17.01-folds); tumor necrosis factor-alpha (TNF-α) (16.44-folds); and inducible nitric oxide synthase (iNOS) (8.51-folds).

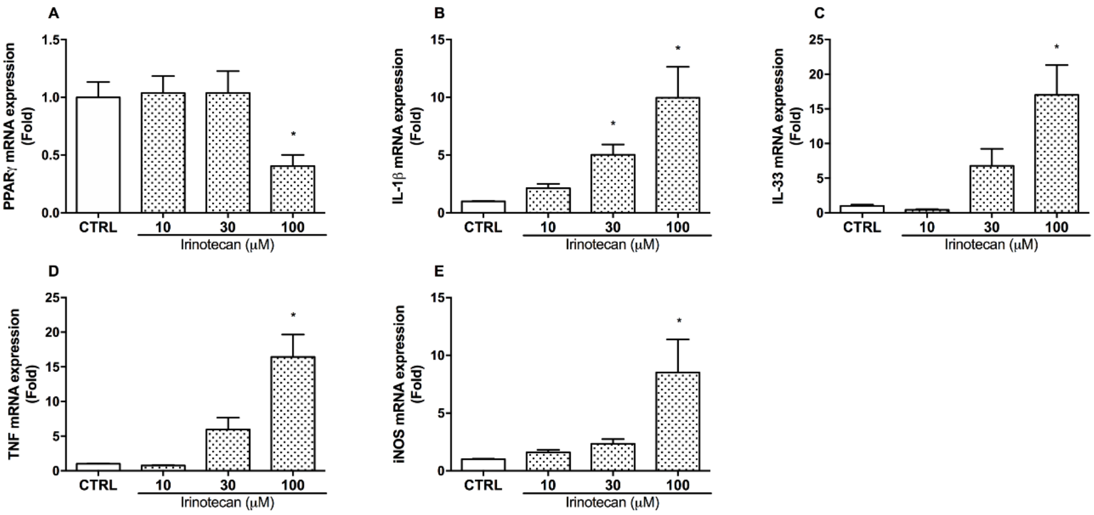
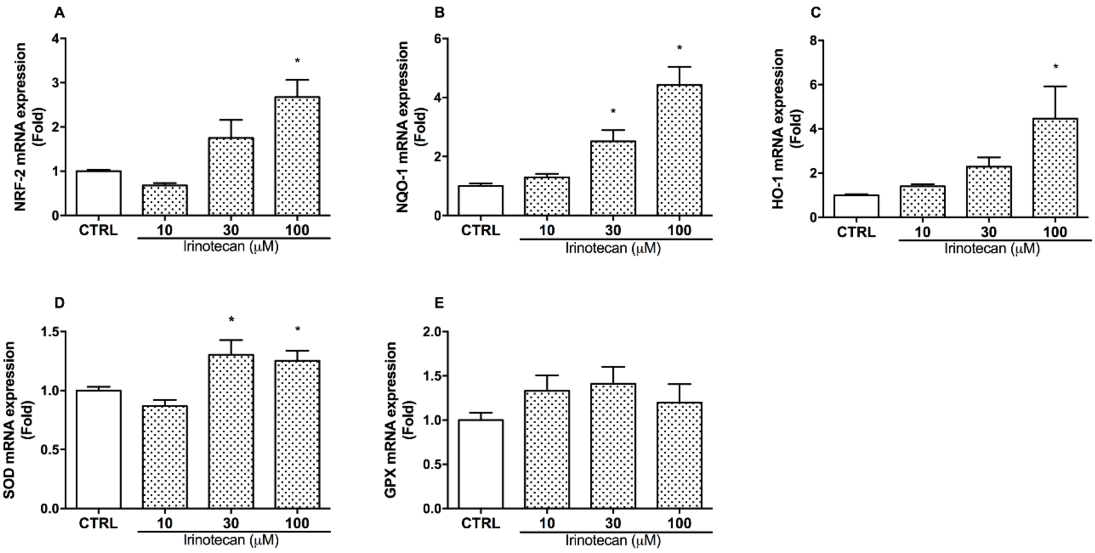


Fig 1. Effect of Irinotecan on peroxisome proliferator-activated receptor-gamma (PPARγ) (A) interleukin (IL)-1β (B); IL-33 (C); tumor necrosis factor-alpha (TNF-α) (D); and inducible nitric oxide synthase (iNOS) (E) expression. Cells were stimulated for 24 h with irinotecan. Results represent mean ± SEM (3) independent experiments in triplicate or sextuplicate, $9 < n < 12$) of the fold change of each gene expression normalized to GAPDH level. The expression level measured in control cells was used as a reference and defined as 1. * $p < 0.05$ compared to control (CTRL).

In figure 2, it is possible to observe that irinotecan upregulated the expression of factor erythroid 2-related factor (NRF-2 - 2.67-folds), NAD(P)H quinone oxidoreductase 1

(NQO-1 - 4.42-folds); heme oxygenase-1 (HO-1 - 4.46-folds), and superoxide dismutase (SOD - 1.25-folds), while it did not alter glutathione peroxidase mRNA expression



compared to control.

Fig 2. Effect of Irinotecan on nuclear factor erythroid 2-related factor (NRF-2) (A); NAD(P)H quinone oxidoreductase 1 (NQO-1) (B); heme oxygenase-1 (HO-1) (C); superoxide dismutase (SOD) (D); and glutathione peroxidase (GPX) (E) expression. Cells were stimulated for 24 h with irinotecan. Results represent mean ± SEM (3 independent experiments in triplicate or sextuplicate, $9 < n < 12$) of the fold change of each gene expression normalized to GAPDH level. The expression level measured in control cells was used as a reference and defined as 1. * $p < 0.05$ compared to control (CTRL).

4.3 Effect of luteolin on Caco-2 cells gene expression

As observed in the figure S2 (supplementary material), the cellular viability of Caco-2 cells incubated with luteolin at 1, 3, and 30 μM is more than 82%, therefore, the effect of luteolin on Caco-2 cells was evaluated at these three concentrations, as can be seen in figure 3. The luteolin at 30 μM caused a significant increase in the PPARγ mRNA expression (6.29-folds) compared to the control. Moreover, the flavonoid reduced by 88% the expression of IL-1β and by 53% the iNOS expression, which was also significative reduced by rosiglitazone (1μM) incubation (38%). Luteolin did not induce any change in the expression of TNF-α. The IL-33 expression from cells incubated only with luteolin is not shown because the flavonoid-induced gene expression did not reach the cycle threshold.

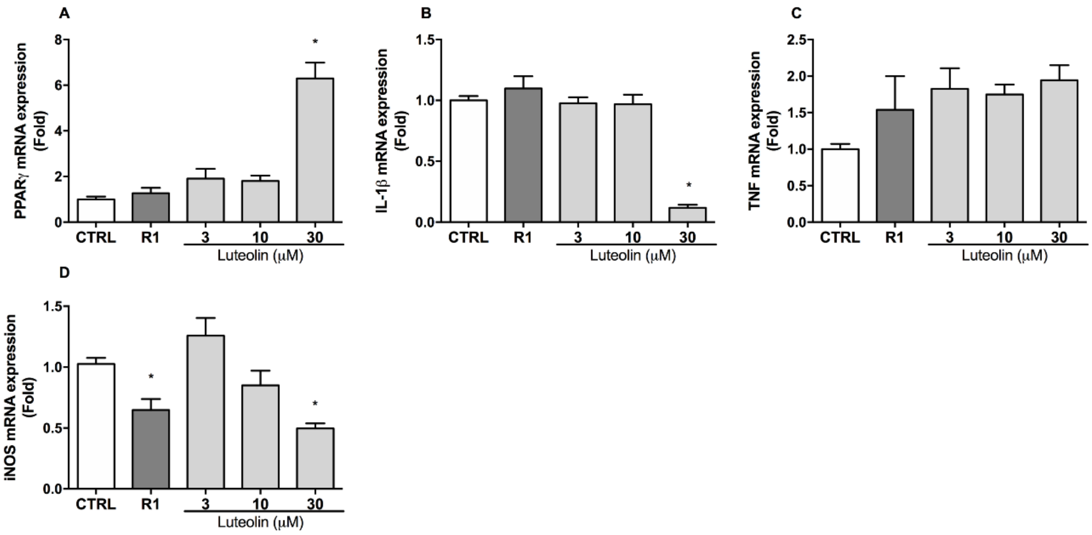


Fig 3 Effect of Luteolin on peroxisome proliferator-activated receptor-gamma (PPARγ) (A) interleukin (IL)-1β (B); tumor necrosis factor-alpha (TNF-α) (C); and inducible nitric oxide synthase (iNOS) (D) expression. Cells were stimulated for 24 h with luteolin or rosiglitazone 1μM (R1). Results represent mean ± SEM (3 independent experiments in triplicate or sextuplicate, $9 < n < 12$) of the fold change of each gene expression normalized to GAPDH level. The expression level measured in control cells was used as a reference and defined as 1. * $p < 0.05$ compared to control (CTRL).

Moreover, luteolin did not induce significative alteration on the NRF-2, NQO-1, and GPX mRNA expression, but led to a significative increase in the HO-1 (8.73-folds) and SOD (1.37-folds) expression, compared to control (Figure 4).

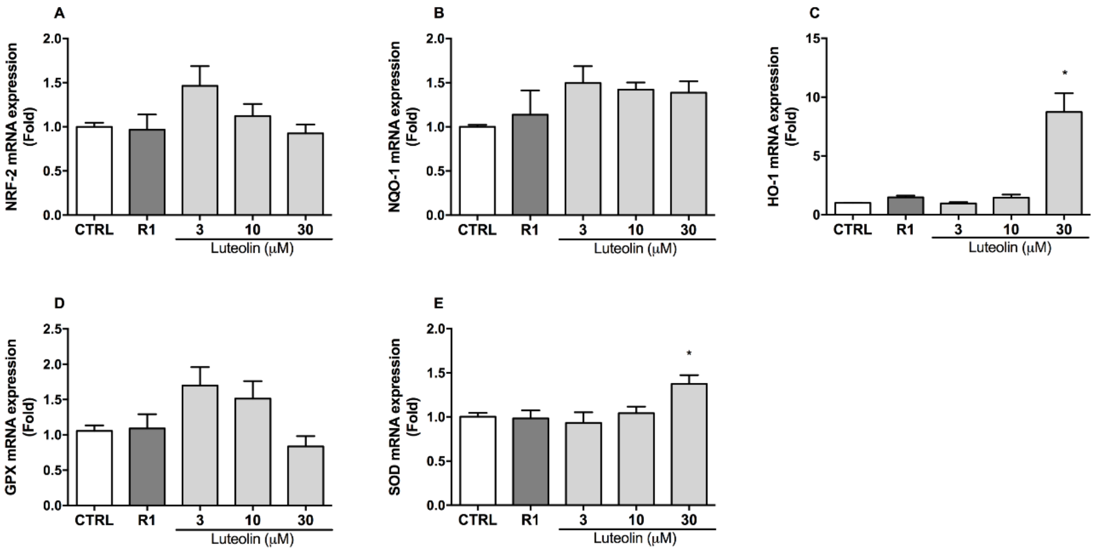


Fig 4. Effect of Luteolin on factor erythroid 2-related factor (NRF-2) (A); NAD(P)H quinone oxidoreductase 1 (NQO-1) (B); heme oxygenase-1 (HO-1) (C); superoxide dismutase (SOD) (D) and glutathione peroxidase (GPX) expression; (E). Cells were stimulated for 24 h with luteolin or rosiglitazone 1μM (R1). Results represent mean ± SEM (3 independent experiments in triplicate or sextuplicate, 9 < n < 12) of the fold change of each gene expression normalized to GAPDH level. The expression level measured in control cells was used as a reference and defined as 1. * $p < 0.05$ compared to control (CTRL).

4.4 Luteolin inhibits damage induced by irinotecan on Caco-2 cells

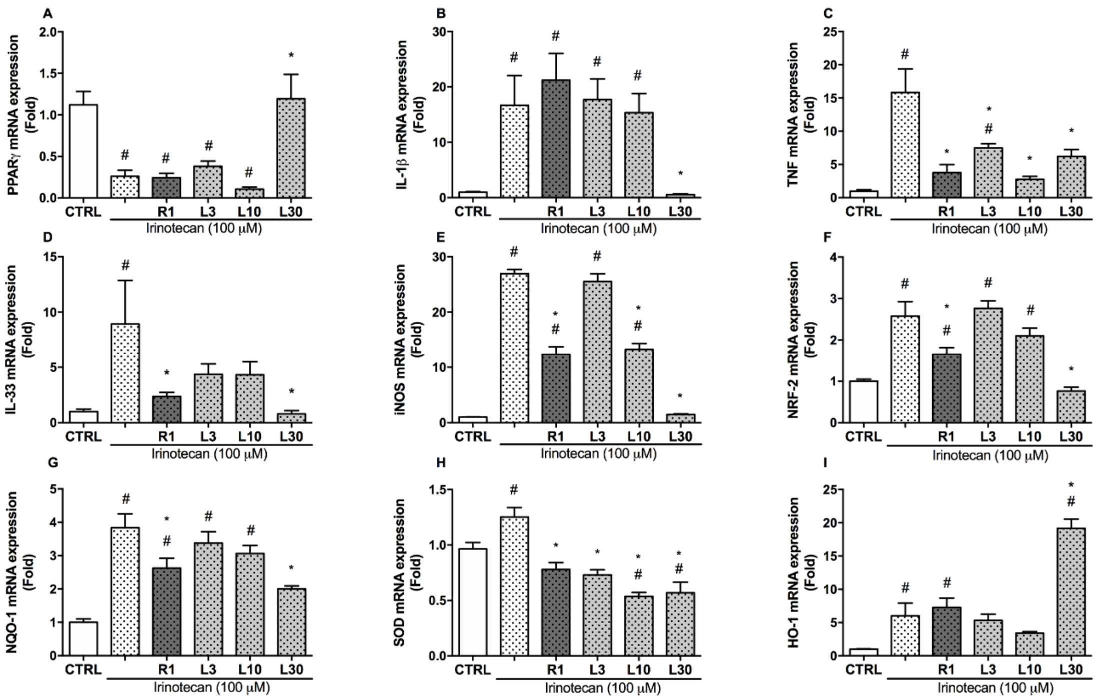
From the data obtained, we have selected the concentration of 100 μM of irinotecan to induce inflammatory and oxidative imbalance in the human intestinal epithelial cells.

Thus, we incubated the irinotecan with luteolin at the same time to evaluate the expression of genes that had been altered by the chemotherapeutic.

Interestingly, the PPARγ down-regulation induced by irinotecan was reversed by luteolin at 30 μM, reaching a mRNA expression similar to the control cells (Figure 5A). In accordance, the IL-1β up-regulation induced by irinotecan was significantly reduced by luteolin (96%), as well as the expression of TNF-α (60%), IL-33 (91%), iNOS (94%) (Figure 5 B, C, D, and E).

Besides, luteolin reversed the oxidative imbalance evoked by the chemotherapy, restoring expression of NRF-2, and decreasing the alteration produced in NQO-1 and SOD (Figure 5 F, G, and H). Otherwise, the HO-1 expression increased by luteolin itself (Figure 4C), was even more increased by the co-incubation of luteolin and irinotecan (Figure 5 I).

The PPARγ agonist rosiglitazone (1 μM) was able to reverse the damage induced by irinotecan in the TNF-α, IL-33, iNOS, NRF-2, NQO-1and SOD expression (Figure 5 C, D,



E, F, G, H).

Fig 5. Effect of Luteolin against gene expression disturbance induced by irinotecan. Peroxisome proliferator-activated receptor-gamma (PPARγ) (A) interleukin (IL)-1β (B); tumor necrosis factor-alpha (TNF-α) (C); IL-33 (D); inducible nitric oxide synthase (iNOS) (E); factor erythroid 2-related factor (NRF-2) (F); NAD(P)H quinone oxidoreductase 1 (NQO-1) (G); superoxide dismutase (SOD) (H); and heme oxygenase-1 (HO-1) (I) expression. Cells were stimulated for 24 h with luteolin 30μM (L30); rosiglitazone 1μM (R1) or L30 and R1 plus irinotecan 100 μM. Results represent mean ± SEM (3) independent experiments in triplicate or sextuplicate, $9 < n < 12$) of the fold change of each gene expression normalized to GAPDH level. The expression level measured in control cells was used as a reference and defined as 1. # $p < 0.05$ compared to control (CTRL); * $p < 0.05$ compared to irinotecan.

Besides the gene expression modulation, we measured the levels of secreted cytokines by Elisa. Irinotecan increased the levels of IL-β (1.56-folds), IL-33 (1.43-folds), and TNF-α (2.30-folds). Following the data observed at mRNA, cells incubated with luteolin 30 μM decreased by 48%, 44%, 43% the levels of the respective interleukins. Although luteolin incubation did not significantly decrease TNF-α levels compared to irinotecan, it was not significantly increased compared to basal (Figure 6 A, B, and C).

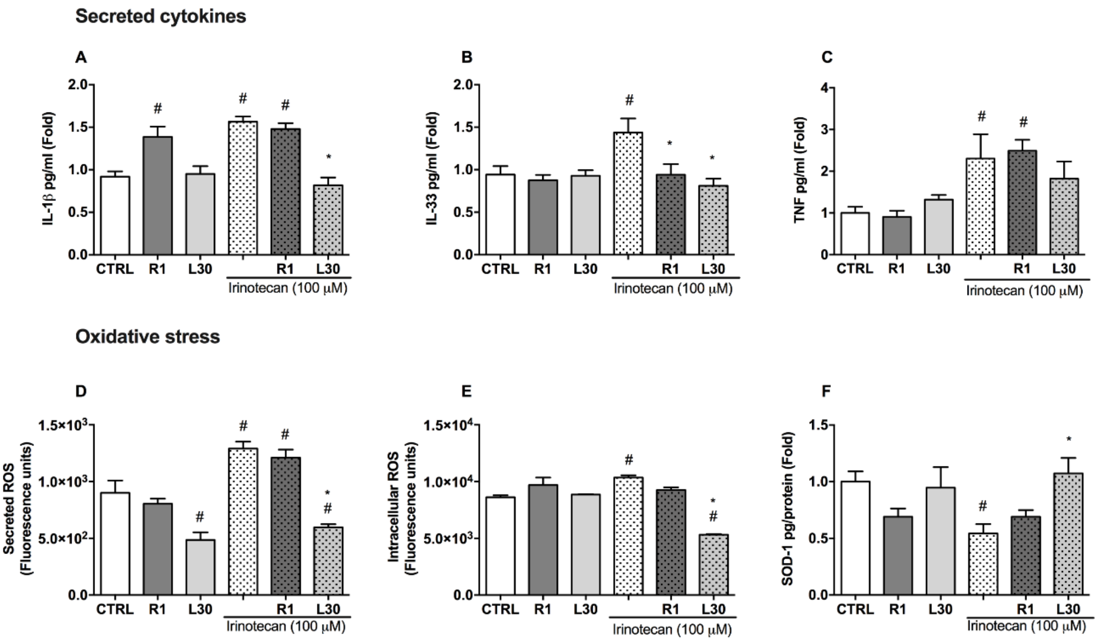


Fig 6. Effect of Luteolin against inflammation and oxidative stress induced by irinotecan. Interleukin (IL)-1β (A); IL-33 (B); TNF-α (C) and reactive oxygen species (ROS) amount (D) were measured by Elisa in the supernatant of cells stimulated for 24 h with luteolin 30μM (L30); rosiglitazone 1μM (R1) or L30 and R1 plus irinotecan 100 μM. ROS (E) and the levels of Superoxide Dismutase 1 (SOD-1) (F) were measured on the cell lysed. Results represent mean ± SEM (3 independent experiments in triplicate or sextuplicate, $9 < n < 12$). # $p < 0.05$ compared to control (CTRL); * $p < 0.05$ compared to irinotecan.

Moreover, total ROS present in the culture medium and the supernatant of the cell lysate were increased by irinotecan compared to control (Figure 6 D and E). In contrast, in the luteolin-incubated samples, it was significative reduced. Interestingly, the levels of SOD-1 were decreased by irinotecan at the protein level and reversed by luteolin (Figure 6 F).

4.5 Effect of luteolin is dependent on PPARγ

To identify if luteolin effects depend on PPARγ, we have investigated the mRNA expression of some genes altered by irinotecan in the previous data in PPARγ knockdown Caco-2 cells. To this end, we used a Caco-2 shPPARγ cell line that stably expresses a short hairpin anti-sense RNA against PPARγ, leading to specific downregulation of PPARγ [21] and Caco-2 shLUC as control cells (cells expressing a control shRNA directed against the luciferase gene). Compared to control cells, the expression of PPARγ in Caco-2 ShPPARγ cell line was significative reduced (Figure 7 A).

Interestingly, luteolin reversed the effect of irinotecan by decreasing PPARγ in control cells shLUC, but completely lost the capacity to increase the gene expression alone and reverse the gene downregulation induced by irinotecan in the cells shPPARγ (Figure 7 B). Moreover, luteolin and rosiglitazone showed decreased TNF-α, NQO-1, NRF-2, and SOD mRNA expression compared to irinotecan in the shLUC cells, but the same effects were not observed in the cells shPPARγ (Figure 7 D, E, F, and G). Conversely, the effect of luteolin of reversing IL-1β upregulation induced by irinotecan was maintained even in the PPARγ knockdown Caco-2 cells (Figure 7 C).

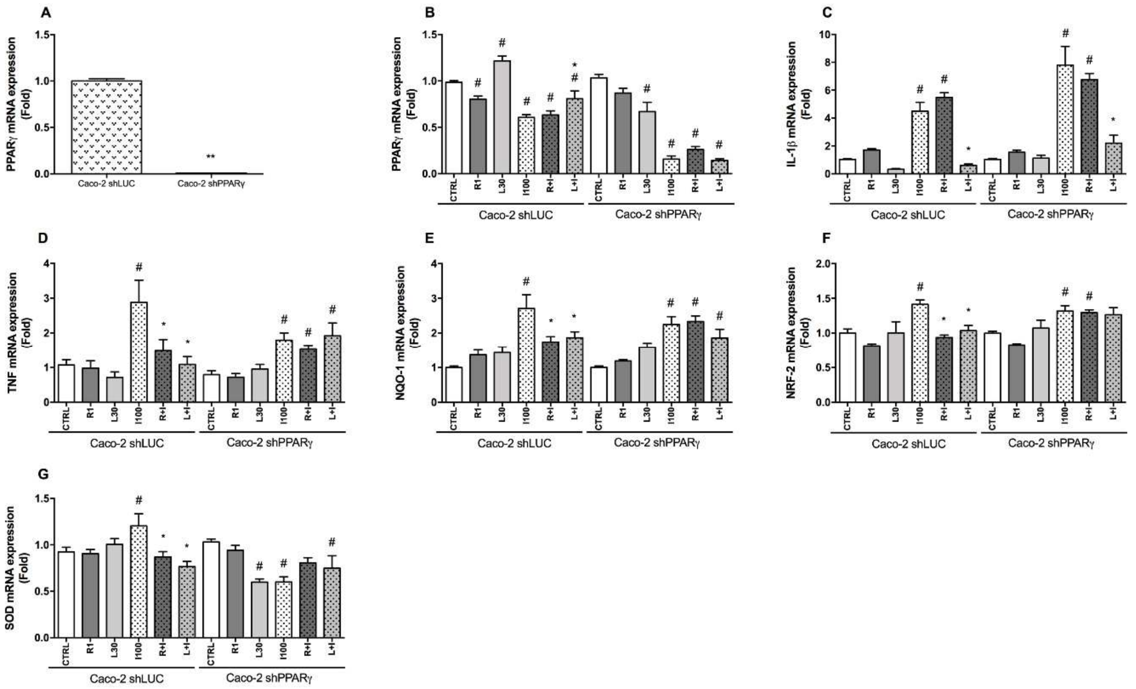


Fig 7. The effect of luteolin is strongly reduced in peroxisome proliferator-activated receptor-gamma (PPARγ) knockdown Caco-2 cells. Caco-2 cell line knockdown for PPARγ (ShPPARγ) expressed significantly fewer PPARγ expression compared to control cells (ShLuc) (A). The expression level measured in ShLuc cells (arbitrarily defined as one) was used as a reference. The results represent a triplicate of the same clone of ShLuc and ShPPARγ Caco-2 cells, respectively. The cells were stimulated for 24 h with luteolin 30 μM (L30); rosiglitazone 1μM (R1); irinotecan 100 μM (I100); or L30 and R1 plus irinotecan 100 μM (L+I and R+I, respectively). Peroxisome proliferator-activated receptor-gamma (PPARγ) (B) interleukin (IL)-1β (C); tumor necrosis factor-alpha (TNF-α) (D); NAD(P)H quinone oxidoreductase 1 (NQO-1) (E); factor erythroid 2-related factor (NRF-2) (F); and superoxide dismutase (SOD) (G) expression. Results represent mean ± SEM (3) independent experiments in triplicate or sextuplicate, $9 < n < 12$) of the fold change of each gene expression normalized to GAPDH level. The expression level measured in control

cells was used as a reference and defined as 1. # $p < 0.05$ compared to control (CTRL); * $p < 0.05$ compared to irinotecan.

5. Discussion

Irinotecan-induced intestinal mucositis produces mucosal changes associated with epithelial vacuolation, goblet cell hyperplasia, villous shortening, crypt cell apoptosis, and infiltration of leukocytes into the lamina propria [24]. Several lines of evidence have demonstrated that these changes appear to be related to specific inflammatory mediators that are crucial factors contributing to the pathogenesis of intestinal mucositis [1], as well as the reactive oxygen species generation [4]. Therefore, this research focused on evaluating gene expression of inflammatory and oxidative related genes in Caco-2-enterocytes exposed to irinotecan, subsequently evaluating the PPARγ dependent effect of luteolin on the attenuation of irinotecan-induced disorders.

Kontos et al. [23], have shown that the cell viability of Caco-2 cells incubated with irinotecan (1-100 μM) for 24 hours is more than 80%. In this way, to select the optimal concentration to maximize inflammation and oxidative stress, we have incubated cells with 3 to 100 μM of the chemotherapeutic, thus, selecting the higher concentration to continue the study since it induced changes in most of the evaluated genes. Caco-2 cells have been used to study methotrexate [25,26] and 5-fluoracil induced- mucositis [27], but as far as we know, this is the first study proposing an *in vitro* model for the study of cell damage induced by irinotecan, that resembles the *in vivo* intestinal mucositis. Therefore, we have first investigated the chemotherapeutic effect in different target genes involved in the intestinal mucositis process.

The data obtained showed that irinotecan upregulated the expression of IL-1β, TNF-α, IL-33, and iNOS. It is described that damage induced during intestinal mucositis results in the activation of transductions pathways, of which the NFκB-mediated inflammatory pathway plays an important role in mucosal injury [5,28], resulting in the production of pro-inflammatory cytokines, including TNF-α, IL-1β [4]. Moreover, Guabiraba et al. [29] reported that irinotecan induces direct epithelial cell damage by modulating the release of IL-33 and Lima et al. [30] have described that irinotecan increases immunoexpression of iNOS.

Besides, the results presented herein showed that irinotecan upregulated NRF-2 mRNA expression in the Caco-2 cells and the related genes NQO-1, HO-1, SOD and even caused a significant increase in reactive species of oxygen (ROS) (Figure E and F) into the cells. In fact, the increased ROS might lead to lipid peroxidation of cell-membrane-bound molecules, resulting in the upregulation of NRF2 [31]. This transcription factor is a key player in the cellular stress response, binding into cis-acting elements in the promoters of target genes; it encodes a series of cytoprotective proteins, including NAD(P)H:quinone oxidoreductase (NQO-1), heme oxygenase 1 (HO-1) and superoxide dismutase (SOD) [32,33].

Interestingly, irinotecan significantly decreased PPARγ mRNA expression. The peroxisome proliferator-activated receptor-gamma belongs to the nuclear receptor superfamily of ligand-activated transcriptional factors, which controls genes involved in cell differentiation, control of glucose homeostasis, and lipid metabolism [34]. Besides adipocytes, the other major tissue expressing PPARγ is the intestine [35,36], linked to the modulation of immune and inflammatory response. Numerous studies have suggested the therapeutic potential of targeting PPARγ to treat inflammatory bowel diseases, such as colitis [37], and the downregulation of PPARγ induced by irinotecan demonstrated in this work reinforces that this could be a new strategy in the management of intestinal mucositis which has been little explored so far.

Luteolin (Figure S1, supplementary material) is a naturally occurring flavonoid described as a PPARγ partial agonist [17] and proved to attenuate intestinal mucositis irinotecan induced in mice [7]. Interestingly, the intestinal cells stimulated with luteolin at 30 μM showed increased mRNA expression of PPARγ, as well as Ding et al. [38] have found that luteolin treatment (20 μmol/L) increases expression and transcriptional activation of PPARγ and its target genes adiponectin, leptin, and GLUT4 in 3T3-L1 adipocytes.

Moreover, luteolin stimulation enhanced HO-1 and SOD expression. Indeed, Polvani et al. [39] have described that PPARγ induces HO-1 expression in human vascular cells. These findings highlight the antioxidative potential of luteolin, since SOD is responsible for the catalysis of superoxide to hydrogen peroxide and has also been implicated in diverse roles in the cell, including that of a transcription factor [40], while heme oxygenases catalyze the degradation of heme to biliverdin and are related to the reduction of oxidative stress, diminished inflammatory response, and decreased rate of apoptosis [41].

Additionally, luteolin decreased L-1β and iNOS mRNA levels, similar to those found by other authors in different types of cells [16]. Although rosiglitazone is a known full PPARγ agonist, it did not induce the same response as luteolin in the target genes evaluated. Puhl et al. [17] have shown that luteolin acts as a potent anti-inflammatory agent through PPARγ in HCECs, but exhibits weak partial agonist behavior relative to the full agonist rosiglitazone in cell transactivation assays, probably, the different ways in which the ligands bind in the PPARγ receptor are responsible for the different responses of them. Further, it is worth noting that natural partial agonists, when compared to full synthetic agonists thiazolidinediones lead to slighter side effects [42].

Moreover, when the cells were co-stimulated with irinotecan and rosiglitazone, the PPARγ agonist was able to reverse the changes induced by the chemotherapeutic in the mRNA expression of TNF-α, IL-33, iNOS, NRF-2, NQO-1, and SOD, as well as luteolin 30 μM, that additionally reversed the changes in PPARγ, and IL-1β expression. The effect of the flavonoid in attenuating intestinal damage induced by irinotecan was confirmed at protein levels once the compound reversed the enhancement of IL-1β, and IL-33 generated by irinotecan. Regarding TNF-α expression, the data showed that luteolin effectively inhibited the irinotecan-induced upregulation, which is in agreement with the findings of the previous *in vivo* experiment, where luteolin reversed the increase in this cytokine induced by irinotecan in the duodenum of mice [7]. However, the results presented here also showed that the flavonoid itself did not reduce cytokine mRNA expression, indicating that TNF-α is regulated by the flavonoid when there is an aggressive stimulus, similarly to what was observed for rosiglitazone. Although luteolin reversed the up-regulation of TNF-α induced by irinotecan, only a slight decrease in the amount of cytokine secreted was observed. The fact that TNF-α exists in two forms, a membrane-bound and a soluble form [43] may explain this. Moreover, it is important to mention that the reduction in TNF-α levels *in vivo* is more evident, probably because it involves not only cytokine secretion by intestinal epithelial cells, but also by macrophages, lymphocytes, and neutrophils [43].

Conversely, the mRNA expression of SOD was increased by irinotecan and reversed by luteolin, but the cells incubated with irinotecan had decreased antigen level of SOD-1 detected by ELISA. Human SOD-1 is a polypeptide that forms a homodimer, with each monomer binding one copper and zinc ions within a disulfide-bonded conformer. The maturation of SOD-1 is dependent on a series of posttranslational modifications such as Zn(ii) and Cu(i) binding, disulfide bond formation, and dimerization. In contrast, the disruption of any of these steps results in an inactive protein [44]. Thus, irinotecan-induced NRF-2 pathway activation may have induced positive regulation of SOD mRNA, but protein maturation has not occurred, and antigen levels for this protein remained low. Then, with SOD diminished, oxidative stress becomes even more exacerbated, and intracellular ROS amount is increased in these cells. Besides, at present, three distinct isoforms of SOD have been identified in mammals, being SOD1 the most abundant enzyme found in the cytoplasm, nuclear compartments, and lysosomes of cells. In contrast, SOD2 has been localized to mitochondria and SOD3 has been detected in extracellular fluids [45], thereby, it is not possible to rule out the possibility that these two other protein isoforms are increased.

To this point, the results obtained in this study fomented our hypothesis that luteolin effects in attenuating damage irinotecan-induced are PPARγ depended. Thus, to confirm this theory, we investigated the mRNA expression of some genes altered by irinotecan in PPARγ knockdown Caco-2 cells. As expected, in the control cells (cells expressing a control shRNA directed against the luciferase gene - Caco-2 shLUC), the full PPARγ agonist rosiglitazone and the flavonoid luteolin were able to reverse the changes induced by irinotecan in the mRNA expression of TNF-α, NRF-2, NQO-1, and SOD, however in PPARγ knockdown cells (Caco-2 shPPARγ) both substances lost the activity.

In contrast, luteolin's effect on the mRNA expression of IL-1β was maintained in the PPARγ knockdown cells. During intestinal mucositis, TLR-2 and TLR-9 are activated by DAMPs and PAMPs in intestinal epithelial cells, activating the downstream cascade of the TIR domain, the differentiation adaptor protein (MyD88), which induces signaling pathways such as NFkB, IL-1, IL-18 [24], nevertheless, luteolin itself is a potent antioxidant molecule (IC50 of ~1.84 μg/ml was found in DPPH assay) [7], then, its directly scavenging properties on ROS formed during the pathophysiology of mucositis, at least in part, contribute to less DAMPS generation, decreased activation of TLR and consequently decrease induction of IL-1β expression, independently of PPARγ-pathway.

But still, the effects on mRNA expression of NRF-2, NQO-1, and SOD showed that outside the directly scavenging effect, luteolin activity is PPARγ dependent; in fact, natural ligands of PPARγ are produced during oxidative stress and PPARγ, if already expressed, may be one of the first responders directly inducing an arsenal of antioxidant molecules, inhibiting prooxidants and at the same time protecting the cells from apoptosis [39].

Furthermore, luteolin completely lost the capacity to increase PPARγ in knockdown cells, confirming the PPARγ-dependent effect of flavonoid luteolin against the damage induced by the chemotherapeutic. However, PPAR-γ ligands exert their anti-inflammatory effects often triggering cross talks with other signaling pathways [46]; thus, it is important to mention that PPARγ activation can also result in the NF-κB nuclear transcription factor repression signaling by various proposed mechanisms [47], contributing to decrease the transcription of inflammatory mediators. In addition, these cross talks with other signaling pathways also may explain why luteolin displays concentration-dependently effects for some genes but not others.

Finally, with the data presented here, it is acceptable to assume that the mechanism underlying the effects of luteolin in attenuating irinotecan-induced intestinal cell damage involves its direct scavenge property and increase in the PPARγ expression, regulating inflammation and oxidative stress by controlling gene expression of cytokines and oxidative genes. In addition, together with our previous study carried out in mice, this study supports the production chain in the search for new drugs for the treatment of intestinal mucositis, collaborating with the pre-clinical validation of the product, which demonstrates potential to be evaluated in clinical trials.

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Author Contributions

Thaise Boeing, Silvia Speca, and Anthony Martin Mena carried out the experimental work. Thaise Boeing, Silvia Speca, Priscila de Souza, Luisa Mota da Silva, and Sérgio Faloni de Andrade analyzed the data, wrote and corrected the manuscript. Laurent Dubuqoy, Benjamin Bertin, Pierre Desreumax, performed the study's design and contributed with reagents and analytical tools. All authors have read and approved the manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

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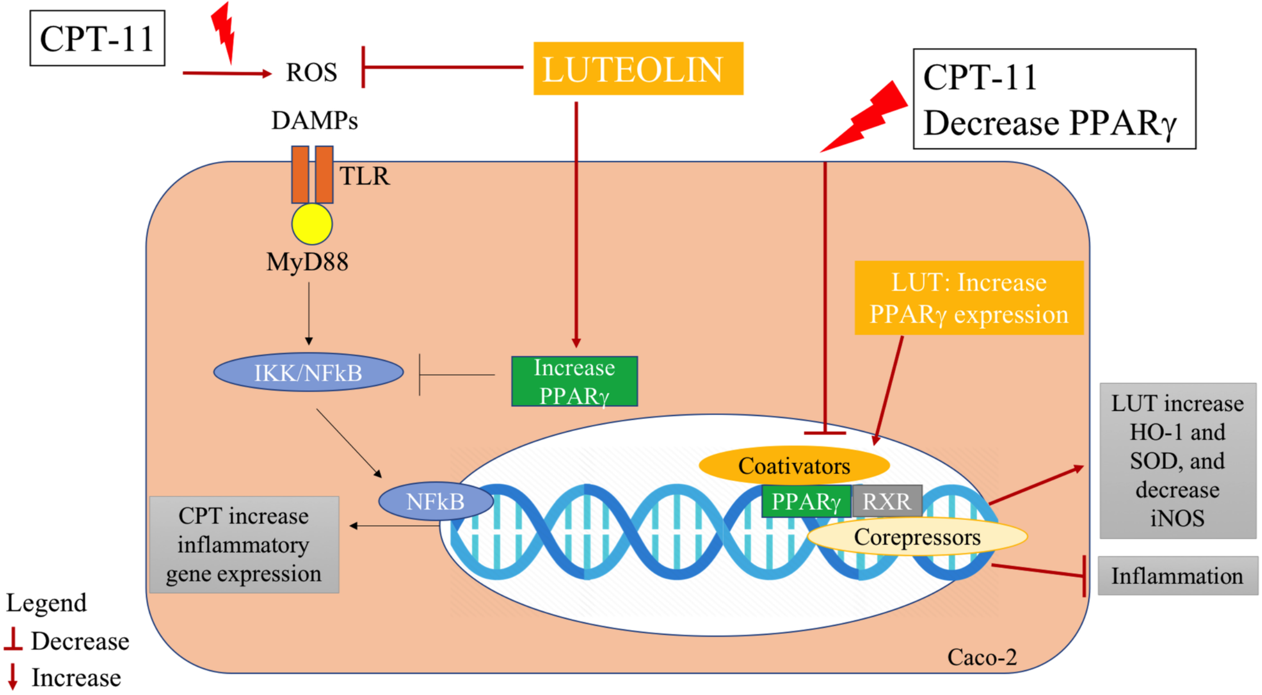
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PPARγ
LUT increase
HO-1 and
SOD, and
decrease
iNOS
Coativators
NFkB
PPARγ
RXR
CPT increase
inflammatory
gene expression
Corepressors
Inflammation
Legend
Decrease
Increase
Caco-2