Anti-inflammatory therapies in TRAMP mice: delay in PCa progression

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Abstract

The aim of this study was to characterize the structural and molecular biology as well as evaluate the immediate and late responses of prostatic cancer in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model after treatment with goniothalamin (GTN) and celecoxib. The treated mice received GTN (150 mg/kg, gavage) or celecoxib (10 mg/kg, gavage) from 8 to 12 weeks of age. They were killed at different ages: the immediate-response groups at 12 weeks and the late-response groups at 22 weeks. The ventral prostate was collected for light microscopy, immunohistochemistry, western blotting, TUNEL, and ELISA. Morphological analyses indicated that GTN treatment delayed the progression of prostatic adenocarcinoma, leading to a significant decrease of prostatic lesion frequency in both experimental period responses to this treatment, mainly high-grade prostatic intraepithelial neoplasia and well-differentiated adenocarcinoma. Also, the celecoxib treatment showed a particular decrease in the proliferative processes (PCNA) in both the experimental periods. Despite celecoxib diminishing the COX2 and IGFR1 levels, GTN presented higher action spectrum considering the decrease of a greater molecular number involved in the proliferative and inflammatory processes in prostatic cancer. Goniothalamin attenuated the pro-inflammatory response in TRAMP prostatic microenvironment, delaying prostate cancer (PCa) progression. Celecoxib treatment was efficient in the regulation of COX2 in the TRAMP mice, mainly in the advanced disease grade. Finally, we concluded that inflammatory process control in early grades of PCa was crucial for the downregulation of the signaling pathways involved in the proliferative processes in advanced cancer grades.

Key Words
- inflammation
- prostate cancer
- TRAMP
- anti-inflammatory therapies
- goniothalamin

Introduction

Cancer is one of the greatest public health concerns not only in the United States but also in other countries. Prostate cancer (PCa) is the second main cause of death among men, estimating around 220,800 new cases and 27,540 deaths in 2015 in United States (Siegel et al., 2015).

The relationship between chronic inflammation and increased PCa development has been investigated over
the past few years. And, studies have demonstrated the association between inflammatory cell occurrence and its mediators in the prostatic microenvironment, not only in the PCa precursor lesions but also in the early PCa grades (De Marzo et al. 1999, Nelson et al. 2003, Hamid et al. 2011, Thapa & Ghosh 2015).

The imbalance between the cellular proliferation mechanism and apoptosis in the PCa is directly influenced by glandular tissue response to pro-inflammatory cytokines, growth factors, and steroid hormones, creating a permissive microenvironment for neoplastic process development (Hamid et al. 2011). In addition, it is well known that the tissue microenvironment is a fundamental compound for tumor development and maintenance; thus, knowledge about this is relevant to evaluate the carcinogenesis process in clinical trials (Bissell & Hines 2011).

Therefore, taking into consideration the prostatic lesions and chronic inflammation, studies have evaluated therapeutic alternatives for both prostatic lesion prevention and contention, particularly PCa, by means of inflammation controller agents. Among them, nonsteroidal anti-inflammatory drugs (NSAIDs) have been tested for cancer chemoprevention in both human beings and other animal species (Rodriguez & Gonzalez-Perez 2004). In addition to this, similar to other COX2 blockers, celecoxib acts on inflammation control, decreasing angiogenesis and cellular proliferation, also inducing apoptosis mechanisms (Sobolewski et al. 2010, Gravitz 2011). However, the extended periods of COX2 blocker use have been questioned due to the increase of cardiotoxic risk, suggesting the necessity for constant collateral effect evaluation, not only in therapies using one drug but also in case of drug association (Gravitz 2011, Jendrossek 2013).

Regarding goniothalamin (GTN), astyryl lactone compound isolated from the plants of the Goniothalamus genus and found in an enantiomeric form (R), it has demonstrated selective toxicity against different cancer cell lineages (Sam et al. 1987, Al-Qubaisi et al. 2011). According to Vendramini-Costa et al. (2010) both enantiomerically pure (R) and racemic (racGTN) forms showed anti-proliferative features in an Ehrlich solid tumor model mice.

Transgenic adenocarcinoma of the mouse prostate model (TRAMP), which was used in the study, expresses viral SV40 oncoprotein in the prostatic luminal cells under the control of a rat androgen-responsive probasin promoter gene, blocking the activity of important tumor suppressor genes (Greenberg et al. 1995, Gingrich et al. 1999, Huss et al. 2003). This animal model develops prostatic hyperplasia that was graded as prostatic intraepithelial neoplasia (PIN) from 6 to 12 weeks old mice and well-differentiated adenocarcinoma from 10 to 16 weeks old mice, and also primary and metastatic tumors from 18 to 24 weeks old mice (Greenberg et al. 1995, Gingrich et al. 1999).

Thus, taking into consideration the involvement of the inflammatory process in PCa development and progression, we suggest that the use of drugs that show preventive action in the inflammation and proliferation control could be a promising target in chemoprevention cancer therapies. So, GTN therapy in the TRAMP mice model arose from the necessity not only of evaluating the inflammatory role in prostate tumor growth in different drug evaluation periods, but also of comparing the anti-inflammatory and anti-carcinogenic effects in relation to a widely known NSAID, celecoxib.

The aim of this study was to characterize the structural and molecular biology of the ventral lobe of the prostate considering the inflammatory process, as well as the immediate and late glandular responses to GTN and celecoxib treatments in preventing cancer development and progression in the TRAMP mice model.

Materials and methods

Animals

All male transgenic TRAMP mice (C57BL/6-Tg (TRAMP) 8247Ng/JX FVB/Unib F1/J) used in this study were divided into seven experimental groups. The control animals (T8, T12, T22) were treated (gavage) with carboxymethyl cellulose (CMC) 0.05% or phosphate-buffered saline (PBS) +1% Tween 80 (10mL/kg) vehicles (n=40). The T1GTN and T2GTN groups both received 150mg/kg GTN (gavage) (modified from Vendramini-Costa et al. 2014), three times a week for 30 days only (in 8 to 12-week-old mice) and all the animals in both groups were killed at different ages (T1GTN at 12 weeks of age and T2GTN at 22 weeks of age) (Supplementary Fig. 1, see section on supplementary data given at the end of this article). The T1CEL and T2CEL groups were treated (gavage) with 10mg/kg celecoxib (modified from Sozer et al. 2011), five times a week for 30 days only (in from 8 to 12-week-old mice) and all the animals in both these latter groups were killed at different ages (T1CEL at 12 weeks of age and T2CEL at 22 weeks of age) (Supplementary Fig. 1). All mice were provided by the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB) at the University of Campinas (UNICAMP) and received water and solid diet ad libitum (Nuvilab,
Colombo, PR, Brazil). Ethics Committee on Animal Use (CEUA) – UNICAMP/Protocol: 3119-1.

Drugs

Goniothalamin was prepared according to a published procedure (de Fatima et al. 2005) and it was emulsified with 1% Tween 80 (Synth, Diadema, SP, Brazil) and dissolved in PBS, pH 7.0 (Vendramini-Costa et al. 2014). Celecoxib was obtained from CELEBRA (Pfizer Pharmaceuticals LLC, Caguas, Puerto Rico), and diluted in CMC 0.05% (Sozer et al. 2011). The acute toxicity of GTN has been evaluated in a previous study (Vendramini-Costa et al. 2010).

Morphological analysis

Ventral prostate samples were collected from five animals and fixed in Bouin’s solution for 24h. Then, the tissues were rinsed in 70% ethanol, dehydrated, and embedded in paraffin and plastic polymers (Paraplast, Sigma Aldrich). The samples were cut using the Hyrax M60 microtome (Zeiss) and then stained with hematoxylin–eosin and Masson’s trichrome (Junqueira et al. 1979). The slides were photographed using a Nikon Eclipse E-400 photomicroscope (Nikon).

For each animal, ten random fields were captured at 40x magnification, which were divided into four quadrants. Then, in each quadrant the predominant morphological feature was classified according to the following specifications: (1) normal tissue (NT); (2) low-grade prostatic intraepithelial neoplasia (LGPIN); (3) high-grade prostatic intraepithelial neoplasia (HGPIN); (4) well-differentiated adenocarcinoma; and (5) undifferentiated adenocarcinoma (Fig. 1A, B, C, and D). Thus, the percentage of each lesion for each experimental group was established. The morphological classification of different degrees of prostatic lesions in TRAMP mice was partially based on descriptions made by Roy-Burman et al. (2004). The presence of undifferentiated adenocarcinoma was calculated considering the total number of 22-week-old mice (Fig. 1D).

Morphometric analyses (nuclear and cytoplasmatic areas)

Morphometric analysis was performed using the same images from the counting of different lesion degrees in TRAMP mice. Imaging Software NIS-Elements was used to measure nucleus and cytoplasm areas in healthy and hyperplasic tissues. For each experimental group, 800 cells in healthy regions and 800 cells in hyperplasic regions were measured.

Immunohistochemistry

Prostate ventral lobe samples were collected from five animals in each experimental group; the same were used for light microscopy analyses. The COX2, STAT3, IGFR1 and PCNA antigens were detected, respectively, using the following antibodies: mouse monoclonal anti-COX2 (sc-376861; Santa Cruz Biotechnology), rabbit polyclonal anti-STAT3 (sc-7179; Santa Cruz Biotechnology), rabbit polyclonal anti-IGFR1 (sc-712; Santa Cruz Biotechnology), and mouse monoclonal anti-PCNA (ab-29; Abcam). The pattern of protocols was the same as those described by Kido et al. (2014) and Montico et al. (2015) and all the primary antibodies were diluted in a 1:50 ratio, except for PCNA 1:250. Then, the sections were incubated for 2h with HRP-conjugated secondary antibodies, goat anti-mouse IgG (W4021; Promega), and goat anti-rabbit IgG (W4018;
Promega). Subsequently, peroxidase activity was detected using a 3,3'-diaminobenzidine (DAB) (Sigma Aldrich). Harris' hematoxylin was used for counterstaining. The DAB precipitate indicated positive antibody reaction and the frequency of antigen immunoreactivity was graded according to the frequency and positivity of antigens.

Figure 2
Photomicrographs of the ventral prostate of TRAMP mice from T8 (A and B): Glandular acini with folded mucosa. Secretory epithelium with columnar cells and basal cells (Bc), and also LGPIN (*) presence. Prostatic stroma with smooth muscle cells (Smc) interspersed with collagen fibers. T12 (C, D and E): Presence of LGPIN (*), HGPIN †, and invasion of epithelial cells in the stroma (well-differentiated adenocarcinoma) (arrow). Ventral lobe showing cells with nuclear atypia and pale and abundant cytoplasm (dotted arrow). T1GNT (F and G): Glandular acini with folded mucosa and scarce prostatic stroma. Decrease of epithelial proliferation foci. T1CEL (H, I and J): Proliferative lesions such as LGPIN (*), HGPIN †, and occasional points of well-differentiated adenocarcinoma (arrow). Atrophied secretory epithelium regions (a) and reduction of folded acinar mucosa. The stromal features were similar to the T1GNT group. Ep: epithelium; St: stroma; L: lumen. Sections stained with hematoxylin–eosin (A, C, F, H and J); Masson's trichrome (B, D, E, G and I) (scale bar = 25 µm). A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-15-0540.

Figure 3
Photomicrographs of TRAMP mice ventral prostate from T22 cancer group (A, B, C and D): Hyperplastic glandular acini with different proliferative lesion grades such as HGPIN †, and undifferentiated adenocarcinoma (triangle). Hyperplastic prostatic stroma with smooth muscle cells interspersed with collagen fibers and inflammatory cells. T2GNT (E, F and G): Simple secretory epithelium with cubic and columnar cells. Decrease of proliferative lesions and stromal fibrillar elements in relation to the cancer group and T2CEL. T2CEL (H, I and J): Folded acinar mucosa and proliferative lesions such as LGPIN (*), HGPIN †. Fibrocellular feature of prostatic stroma, highlighting abundant amount of collagen fibers (green). Ep: epithelium; St: stroma; L: lumen. Sections stained with hematoxylin–eosin (A, B, E, G, H and J); Masson's trichrome (C, D, F and J) (scale bar = A–D, F, G, I and J: 25 µm; E and H: 50 µm). A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-15-0540.
in sectioned tissues: 0 for negative staining (0%), 1 for weak staining (33%), 2 for moderate staining (33–66%), and 3 for intense staining (greater than 66%) (modified from Tuxhorn et al. 2002a, b, Tomas & Kruslin 2004). The immunohistochemical analyses were followed by a negative control parameter in which the primary antibody was not used.

**PCNA**

PCNA immunolabeling was used in this study as a cellular proliferation marker. The prostatic samples were the same as used in the immunohistochemistry analysis for the other proteins. The experiment was carried out using the multipoint system (Weibel 1963) with 710 intersection points. Ten random fields were captured at 40 magnification for each animal. PCNA-positive-cell quantification was determined by brown-stained nucleus count, coinciding with the grid intersection, divided by the total number of points. The result was expressed as the relative frequency of PCNA-positive cells in all experimental groups.

**Apoptosis**

The DNA fragmentation from apoptotic cells was detected by Dead End Fluorometric TUNEL System (Promega) according to the manufacturer’s instructions. Apoptotic nuclei were identified and photographed using an Olympus IX71 inverted-II microscope, equipped with a fluorescence system (Olympus). Ten random fields were captured at 40 magnification for each animal. The quantification for apoptotic cells was similar to that for PCNA, following the same counting method. The result was expressed as relative frequency of apoptotic cells in all experimental groups.

**Western blotting**

Prostate ventral lobe samples from five animals were frozen and then homogenized by the Polytron homogenizer (Kinematica Inc., Lucerne, Switzerland) in a diluted RIPA extraction buffer (Millipore) and protease inhibitor cocktail (Sigma Aldrich). The ventral prostate extract were centrifuged at 18,659 g for 15 min at 4 °C, and then protein quantification using Bradford reagent (Bio-Rad Laboratories) was carried out. A total of 50 μg protein was applied and separated by electrophoresis to the SDS-polyacrylamide gel under reducing conditions. Subsequently, the proteins were electrically transferred to nitrocellulose membranes (Amersham Life Science, Arlington Heights, IL, USA). The membranes were blocked with 3% bovine serum albumin (BSA) diluted in tris-buffered saline and Tween 20 (TBS-T) for 1 h and incubated overnight with the primary antibodies in a dilution range of 1:350–1:500: mouse monoclonal.
Figure 5
Goniothalamin and celecoxib effects on the relative frequency of PCNA positive cells in different evaluation periods in TRAMP mice (scale bar = 25 µm) (one-way ANOVA: ****P < 0.0001). A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-15-0540.

anti-COX2 (sc-376861; Santa Cruz Biotechnology), mouse monoclonal anti-NFκB (ab 13594; Abcam), mouse monoclonal phosphoSTAT3 (3E2; Cell Signaling Technology), and rabbit polyclonal anti-IGFR1 (sc-712; Santa Cruz Biotechnology). After that, the membranes were incubated for 2 h with secondary HRP-conjugated anti-rabbit and anti-mouse antibodies in a dilution range of 1:4000–1:6000 in 1% BSA. The bands were detected by chemiluminescence solution (Pierce Biotechnology) for 5 min and captured using Gene Gnome equipment and GeneSys image acquisition software (Syngene Bio Imaging, Cambridge, UK). The antibody for mouse monoclonal
anti-beta-actin (sc-81178; Santa Cruz Biotechnology) was used as an endogenous control. The intensity of antigen bands was quantified by densitometry using the Image J (https://imagej.nih.gov/ij/; NIH) software for image analyses and was expressed as the mean percentage in relation to beta-actin band intensity.

**ELISA**

Blood samples were centrifuged (Sigma 3-18 K refrigerated centrifuge) at 857 g for 10 min. The plasma obtained was used to determine TNF-alpha (KMC3012), IL1beta (KMC0012), IL17 (KMC3021), and IL6 (KMC0062)

Figure 6

Goniothalamin and celecoxib effects on relative frequency of apoptotic cells in different evaluation periods in TRAMP mice (scale bar = 25 µm) (one-way ANOVA). A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-15-0540.
concentrations using commercial reagents for the enzyme immunoassay (Novex, Life Technologies). Sample absorbance was read using Multi-Mode Microplate Reader Model Synergy H1M equipment (Bio-Tek Instruments) at a 450-nm wavelength.

Statistical analyses

The statistical analysis for prostatic lesion incidence (morphology, PCNA, and TUNEL) and protein levels among the experimental groups was carried out by variance analysis (ANOVA) followed by Tukey’s multiple range test or t-student test, with the level of significance set at 5% (Zar 1999). The results were expressed as the mean ± s.d.

Results

Goniothalamin and Celocoxib treatments delay the prostate adenocarcinoma development

The prostatic tissue from control TRAMP mice (8 and 12 weeks old) showed predominant lesions characterized as LGPIN and HGPIN (Fig. 2A, B, C, D, and E; Supplementary Fig. 2A and B). These lesions presented epithelial cell stratification, occupying different extension in the acinus lumen. The hyperplastic epithelial cells presented a significant increase of nuclear and cytoplasmatic areas (Supplementary Fig. 3A and C), evident nucleoli, and chromatin condensation. Also, rare hyperplastic epithelial cell herniation regions toward the underlying smooth muscle cell layers were identified. The HGPIN frequency was greater ($P < 0.05$) in the T12 group than in the T8 group, 25% and 7%, respectively (Fig. 4E). The prostatic stroma in the T8 control group showed fibrocellular features with smooth muscle cells, collagen fibers placed concentrically around of the prostatic acini, in addition to blood vessels (Fig. 2A and B). Also, thick fibromuscular layer areas around epithelial proliferation were verified. The prostatic stroma changes were similar between T8 and T12 control groups; however, the frequency of these lesions was directly proportional to the age of the mice (Fig. 2A, B, C, D, and E).

The immediate response to GTN treatment showed maintenance of prostatic tissue morphology, highlighting the incidence of 7.5% for HGPIN and 2.5% well-differentiated adenocarcinoma ($P < 0.05$), in contrast to the T12 control group with 25% and 9.5%, respectively.

**Figure 7**

Western blotting analysis of cyclooxygenase-2 (COX2), phosphorylated signal transducer and activator of transcription 3 (pSTAT3), factor nuclear kappa B (NFκB), insulin-like growth factor receptor 1 (IGFR1) protein levels in the ventral prostate of the experimental groups. Different lowercase letters indicate statistically significant differences among the groups (one-way ANOVA: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).
These results were confirmed by means of a significant decrease in PCNA immunolabeling ($P < 0.0001$) and an increase in apoptotic cells ($P < 0.001$) (Figs 5C and 6C). Moreover, scarce prostatic stroma was verified in comparison with T8 and T12 control groups, showing decreased fibrillar element frequency, as well as hypertrophy and hyperplastic areas (Fig. 2F and G; Supplementary Fig. 2E).

The immediate response to celecoxib anti-inflammatory treatment (T1CEL) did not indicate statistically significant decrease of the premalignant and malignant lesions (Fig. 4A, C, E, and G). However, in general, there was a decrease in the incidence of lesions when compared with the T12 control group (Fig. 2H, I, and J; Supplementary Fig. 2F). In addition, PCNA immunohistochemistry showed a significant reduction of the proliferative process ($P < 0.0001$), and TUNEL showed a significant increase in apoptotic cells ($P < 0.001$) (Figs 5D and 6D). The morphological analyses also confirmed delay in prostatic lesion progression as well as the...

Figure 8
COX2, STAT3, and IGFR1 immunoreactivities in the ventral prostate of TRAMP mice from T8 (A, E and I), T12 (B, F and J), T1GTN (C, G and K), and T1CEL (D, H and L). Epithelial and stromal reactivities were graded according to Table 1 (scale bar = 25 µm; insets: 10 µm). A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-15-0540.
occurrence of occasional epithelial atrophied regions and reduction of folded acinar mucosa. The prostatic stroma showed structural features similar to those seen in the immediate response of the GTN treatment (Fig. 4H, I, and J; Supplementary Fig. 2F).

Different ventral prostate lesion grades were identified in the TRAMP 22 cancer control group (T22), particularly HGPIN, well-differentiated adenocarcinoma, and undifferentiated adenocarcinoma (Fig. 3A, B, C, and D). The HGPIN lesion showed the highest frequency in this group, 48.5%, characterized by the occurrence of epithelial cell stratification within the acinar lumen (Figs 3A and C; 4F). Furthermore, the well-differentiated adenocarcinoma was identified in 6% of the total area evaluated, showing atypical epithelial cells in the prostatic stroma (Fig. 4H).

Partial morphological prostatic tissue maintenance was verified in late response to GTN treatment (T2GTN), in relation to the T22 cancer group, thus delaying PCa progression (Supplementary Fig. 2G). This is a fact, considering the significant increase of normal prostatic tissue (21.5%) and LGPIN frequency (45%) ($P<0.05$), as well as the significant decrease in PCNA immunolabeling ($P<0.0001$) (Figs 3E, F, and G; 4B and D; 5G). There was no significant lesion-incidence decrease in late response to celecoxib treatment in relation to the T22 cancer group ($P>0.05$). Nevertheless, significant PCNA immunolabeling reduction was seen ($P<0.0001$) (Fig. 5E, F, and H). Despite identifying stromal recovery in the celecoxib treatment, the tissue response was less intense than that was found in GTN treatment (Fig. 3E, F, G, H, I, and J; Supplementary Fig. 2H). Regarding the TUNEL evaluation, there was no significant difference in late-response groups (Fig. 6G and H).

The quantification of undifferentiated adenocarcinoma was performed only in the 22-week-old mice groups, taking into consideration the fact that older the
mice, the greater the occurrence of aggressive cancer (Fig. 3B and D; Supplementary Fig. 2D). Undifferentiated adenocarcinoma was verified in 60% of mice in the T22 cancer group, in contrast to that observed in the late-response to GTN and celecoxib treatments, which represented 20% and 40%, respectively.

**Goniothalamin led to a decrease of the great majority of pro-inflammatory mediators evaluated in the ventral prostate lobe**

COX2 expression increased according to lesion progression in the ventral prostate lobe in T8, T12, and T22 control groups (Figs 7A and B; 8A and B; 9A). The celecoxib treatment significantly reduced the COX2 protein levels in both immediate- and late-response groups ($P<0.01–0.001$), highlighting the celecoxib-specific COX2 inhibition and the extremely significant results (Fig. 7A and B). GTN treatment also reduced the levels of this enzyme in both the evaluation periods ($P<0.01$). However, the late response to celecoxib treatment exhibited better results than treatment with GTN in the same period, if considered the significance level. The immunohistochemistry evaluation in the different treatments also indicated decreased COX2 reactivity in relation to T8, T12, and T22 control groups (Figs 8A, B, C and D; 9A, B and C; Supplementary Tables 1 and 2, see section on supplementary data given at the end of this article).

The immediate response to GTN treatment significantly reduced the NFκB protein level ($P<0.01$), whereas both celecoxib-treated groups (T1CEL and T2CEL) and the late response to GTN treatment (T2GTN) did not significantly reduce the NFκB protein level (Fig. 7C and D).

The pSTAT3 protein level dropped significantly only in the late response to GTN treatment ($P<0.05$) (Fig. 7E and F). The immunohistochemical evaluation indicated lower STAT3 in the epithelial compartment (cytoplasmatic immunolabeling) in immediate response to GTN (Fig. 8G; Supplementary Table 1). Regarding the stromal compartment, STAT3 immunoreactivity decreased only in the immediate response to celecoxib treatment. Whereas pSTAT3 immunoreactivity (nuclear immunolabeling) decreased in the immediate and late responses to both treatments in the epithelial and stromal compartments (Figs 8E, F, G and H; 9D, E and F; Supplementary Tables 1 and 2).

**Goniothalamin effect on the IGFR1 in the different prostatic lesion development grades**

The immunohistochemical and protein-level evaluation showed gradual increase in IGFR1 in the ventral prostate lobe in the T8 and T12 control groups (Figs 7G, 8I, J, K and L; Supplementary Table 1). On the other hand, IGFR1 was reduced in the immediate responses in the GTN and celecoxib treatments (Fig. 7G; Fig. 8L, J, K and L; Supplementary Table 1). IGFR1 protein levels did not change significantly ($P>0.05$) in the late response GTN and celecoxib treatments (Fig. 7H). However, the immunohistochemical analyses verified that the T22 control group had a reduction in the IGFR1 reactivity in relation to the other control groups, T8 and T12 (Supplementary Tables 1 and 2). This result was highlighted in the mice presenting undifferentiated adenocarcinoma.

**Goniothalamin led to decreased IL1beta and TNF-alpha plasmatic levels in TRAMP mice**

TNF-alpha and IL1beta concentrations significantly reduced ($P<0.001$, $P<0.001$) immediately after GTN treatment (Fig. 10A and C). IL6 and IL17 plasmatic levels did not show a statistically significant difference among
the groups (T8: 8.7±0.4 pg/mL; T12: 8.87±3.2 pg/mL; T1GTN: 8.33±3.0 pg/mL; T22: >35 pg/mL; and T2GTN: 33.47±1.8 pg/mL) and (T8: 19.94±5.8 pg/mL; T12: 16.88±0.8 pg/mL; T1GTN: 13.37±3.0 pg/mL; T22: 19.2±2.5 pg/mL; and T2GTN: 17.72±4.7 pg/mL), respectively.

Discussion

The results herein showed that GTN treatment delayed the prostatic adenocarcinoma progression, decreasing the incidence of glandular lesion by means of reducing pro-tumorigenic effect related to the inflammatory process. Despite celecoxib diminishing the COX2 and IGFR1 levels, GTN presented higher action spectrum considering the decrease of a greater molecular number involved in proliferative and inflammatory processes.

Inflammation is a tissue reaction that includes a series of responses from the immunological system, including the release of cytokines and recruitment of defense cells. Under normal conditions, inflammatory processes are solved by endogenous anti-inflammatory mediators; however, the persistent accumulation and activation of leukocytes can create a constant inflammatory condition (Hanada & Yoshimura 2002). According to the same authors, current clinical approaches are focusing on inhibition or suppression of pro-inflammatory mediators as a way to discover new hallmarks and targets for treating inflammatory disease.

Nowadays, different studies have shown the involvement of the inflammatory process in the development and progression of several cancer types, including PCa (De Marzo et al. 1999, Hamid et al. 2011, Vendramini-Costa & Carvalho 2012, Thapa & Ghosh 2015). It is known that the etiology of prostatic inflammation is multifactorial, involving infectious agents, factors related to aging, eating habits among others (De Marzo et al. 2007). Boehm et al. (1997) demonstrated that the acute inflammation in induced prostatitis increased the proliferative process of epithelial and stromal cells. Other studies verified inflammatory cell occurrence in the prostate of elderly animals due to changes in aging process, highlighting the hormonal imbalance as a determining factor in triggering prostatic inflammation, particularly, when the estrogen level is higher than the testosterone level (Yatkin et al. 2009, Montico et al. 2011, Kido et al. 2014). In addition, the interaction between an inflammatory process and sexual hormones led to favorable conditions for androgen- and estrogen-activating lymphocytes, which are essential for the immune response in the prostatic tissue (Djavan et al. 2009).

Therapies with different natural compounds, which show anti-inflammatory, anti-proliferative, and antioxidant properties, have been evaluated in various types of cancer (Cragg et al. 2009). Studies in vitro indicated cytotoxic activity of the GTN natural enantiomer, especially in tumor cell lineage, such as multiresistant breast cells, and also lung, melanoma, kidney, colon, ovary, and PCas (de Fatima et al. 2005). Also, other studies demonstrated pro-apoptotic activity of the R-GTN by means of caspases pathway; increase of BAX pro-apoptotic protein expression; and inhibition of nitric oxide synthase (NOS) (Inayat-Hussain et al. 2003, de Fatima et al. 2005). Vendramini-Costa et al. (2010) verified that racGTN, the same used in the study herein, showed antiedematogenic activity with simultaneous tumoral development inhibition in the solid tumor of Ehrlich. The same authors observed similar action between racGTN and the Piroxicam, nonsteroidal and anti-inflammatory, by means of significant inhibition of the inflammatory edema in an Ehrlich tumor. These results indicated the racGTN anti-inflammatory action as being favorable to anti-proliferative activity (Vendramini-Costa et al. 2010).

The results herein show that celecoxib and GTN treatments led to reduction in proliferative process in both the experimental periods. Also, significant COX2-level reduction was detected in the ventral prostate of TRAMP mice in the different treatments. However, the celecoxib action was more pronounced than GTN action in the late-response group, if considered the significance level.

Regarding the therapies, which have cancer chemopreventive action, epidemiological studies demonstrated a PCa development risk decrease in men treated regularly with nonsteroidal anti-inflammatory drugs (NSAIDs) (Mahmud et al. 2006, Daniels et al. 2009). Among the various types of NSAIDs, the selective inhibitors of COX2 can be highlighted as this enzyme is overexpressed in inflammatory processes and also in different cancers (Liu & Rose 1996, Tsuji et al. 1997, Kulkarni et al. 2001). It is well known that the COX2 overexpression, resulting from inflammatory process, increases the angiogenic factor production and the carcinogenic potential of cells by means of pro-carcinogenesis oxidation to carcinogens, thus promoting cellular growth and decreasing the apoptosis (Kirschenbaum et al. 2001, Hamid et al. 2011, Vendramini-Costa & Carvalho 2012). High levels of COX2 related to proliferative lesions associated to inflammation suggest a risk factor for CaP development (Yatkin et al. 2009). According to Narayanan et al. (2006) celecoxib administration, added to the diet for 16 weeks, resulted in a reduced PIN and adenocarcinoma
incidence in the TRAMP mice model, due to an increase of apoptotic index and simultaneous pro-inflammatory mediator inhibition, such as NFκB p65 and COX2. The celecoxib dose-dependent effects (400, 600, and 1000 ppm) in the early grade of the neoplastic lesions indicated an important role in the prostatic tumoral growth prevention, showing the action mechanism of this anti-inflammatory drug (Narayanan et al. 2006). In this study, the celecoxib dose was lower than that used by Narayanan et al. (2006). In contrast to the results shown here, Vendramini-Costa et al. (2015) did not identify a relationship between the anti-inflammatory role of GTN and decrease in Cox2 gene expression. However, GTN inhibited release of inflammatory prostaglandins, which is a by-product of COX2 action. Thus, we concluded that celecoxib treatment was more efficient for COX2 enzyme control, particularly, during late response in relation to GTN treatment.

In addition, not only celecoxib but also GTN treatment reduced IGFR1 immunoreactivity and protein level, evaluated immediately after both treatments, pointing to the relationship between the inflammatory process control and the decreased mitogenic process. However, the evaluation in the late response to both treatments did not show any significant reduction in IGFR1 protein-level. Montico et al. (2014) observed high-level IGFR1 in the prostatic dorsolateral lobe in 12- and 22-week-old TRAMP mice, similar to the results found herein. The same authors verified the occurrence of tissue remodeling and mitogenic factors involved in cellular invasion, proliferation, and angiogenic processes, which are the positive markers of neoplastic lesion onset. In the great majority of cancers such as that of colon, endometrium, and breast IGFR1 overexpression was associated with the aggressive phenotype of the disease, such as therapy resistance (Wu & Yu 2014). The interaction between pro-inflammatory mediators and growth factors could stimulate the mitogenic action of these factors, such as the inflammatory prostaglandins in the increase of the insulin-like growth factor 1-binding sites (Hakeda et al. 1991). Also, according to Kaplan et al. (1999) the loss of IGFR1 expression in advanced lesions could indicate the androgen-independent character of PCa, in addition to metastatic potential.

In the results herewith, the IGFR1-level maintenance in the late-response groups, in both the treatments, indicated mitogenic process persistence in the prostate of these animals, not suggesting, however, an independent-androgen profile in the prostatic tumor. We concluded that both treatments interfered in IGFR1 regulation, being more efficient in immediate-response groups and contributing to the inhibition of the proliferative process.

The results indicated dual and additional effects on the STAT3 and pSTAT3 pathways in the different periods of evaluation. The immunohistochemistry analysis demonstrated a STAT3 decrease, particularly in the prostatic cellular cytoplasm in the GTN immediate-response group. On the other hand, there was a significant pSTAT3-level decrease in the GTN late-response group. Regarding celecoxib treatment, a decrease of pSTAT3 and STAT3 immunoreactivities was verified in both immediate and late responses to this drug. However, the pSTAT3 protein levels did not significantly decrease.

Maintenance of STAT3 activation has been associated to different cancers, such as that of neck, breast, and lung, due to the chronic stimulation that increases the production of inflammatory cytokine, particularly from those of the IL6 family, and to the decrease of the suppressor of the cytokine signaling (SOCS) expression, which are negative cytokine signaling regulators (Sansone & Bromberg 2012). Thereby, STAT3 is considered to be a cellular survival function regulator and a potent inducer of anti-apoptotic genes, such as Bcl2 and BclX- (Levy & Lee 2002). In CaP, STAT3 also acts as a key molecule in androgen receptor (AR) activation, which is a potent regulator of cellular proliferation and survival, besides acting by means of alternative pathways, which do not need testosterone (Bishop et al. 2014). According to Yang et al. (2005) STAT3 plays two distinct functions in the dependent transcription of cytokines: first, being part of the primary response by means of STAT3 dimer action (product of tyrosine phosphorylation) and second, being part of a large amount of STAT3 unphosphorylated action. The same authors showed unphosphorylated STAT3 relevance in a transcription, as it is required for cellular survival, growth, and differentiation in CDC2, CYCLIN B1, MRAS, and EF21 gene overexpression, which increase in cancer.

Thus, we concluded that GTN acted in the STAT3 activity in both cytoplasmatic and nuclear levels in different periods of the treatment responses, interfering in the prostatic neoplastic lesions. Despite the celecoxib treatment influencing STAT3 immunoreactivity, the GTN action led to stronger tissue responses in lesion progression delay, probably due to GTN interaction in various pathways, which are involved in the neoplastic process.

In addition, the results herein showed a significantly decreased NFκB protein level and also reduced TNF-alpha and IL1beta plasmatic levels in the groups evaluated immediately after GTN treatment, suggesting
possible GTN pathways. Celecoxib treatment showed no statistically significant difference in the NFκB levels during the different experimental periods.

NFκB is known to be one of the main inflammatory transcriptional regulators, which is strongly activated in cancer. Studies have demonstrated not only the critical role of NFκB in the regulation of prostatic cancer progression, from androgen-dependent to androgen-independent cancer but also the upregulation of the AR activity (Chen & Sawyers 2002, Jin et al. 2008). The NFκB is kept activated, in the majority of the cases, by means of upstream signaling of the pro-inflammatory cytokines such as IL1beta and TNF-alpha, or as a response to extracellular stimuli from the tumoral microenvironment (Karin et al. 2002). In addition, NFκB could interact with other important transcription factors involved in the neoplastic process development such as STAT3 (Grivennikov & Karin 2010).

Also, studies showed TNF-alpha to be an important inflammatory mediator, acting in both lesion and tissue repair processes. When this cytokine is chronically produced, it acts as an endogenous promoter of tumor growth, creating a favorable microenvironment for development and dissemination of tumoral cell (Balkwill & Mantovani 2001). Other studies have shown the crucial role of TNF-alpha during the first step of carcinogenesis, acting both in cells predisposed to cancer development and in inflammatory cells in the stroma of different murine models (Balkwill 2006). Orlikova et al. (2013) indicated the GTN beneficial effect on the TNF-alpha pathways in leukemic cell lineage, while GTN treatment was able to decrease the TNF-alpha level, inhibiting NFκB activation, without showing toxicity to other healthy blood cells. Thus, the results pointed to GTN treatment effectiveness due to the decrease of the inflammatory process, particularly in the immediate response, in relation to TNF-alpha, IL1beta, and NFκB pathways.

Finally, the chemoprevention effects of GTN, by means of significant preneoplastic and neoplastic lesion reduction and cancer progression delay, contributed to a prostatic microenvironment balance. The immediate molecular biological response to GTN and also celecoxib treatments highlighted the inflammation as an important promoter and also intensifier process for prostatic cancer. In addition, celecoxib showed itself to be efficient in the COX2-level regulation in the TRAMP mice model, even in advanced disease grade. Also, we concluded that the inflammatory process control in the first prostatic cancer grades was crucial for the downregulation of the signaling pathways involved in the proliferative processes in advanced cancer grade.

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**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-15-0540

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**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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