The Potential of Pasak Bumi (*Eurycoma longifolia* Jack) Root Extract Nanoparticles as Anti-Prostate Cancer Agent against Cell Cycle Regulation, Apoptosis, and Senescence based on In Vitro Tests

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Abstract. Prostate cancer poses a major health challenge worldwide, with increasing incidence rates and limited current treatment modalities, which highlights the urgent need for innovative therapeutic alternatives. Utilizing nanoparticles derived from plant extracts is emerging as a promising solution, offering a superior drug delivery system. *Eurycoma longifolia* Jack roots, or *Pasak Bumi*, which are widely used in ethnobotany, are known to contain the compound eurycomanone, which exhibits pro-apoptotic activity against malignant cells. This investigation sought to assess the efficacy of *Pasak Bumi* root-derived nanoparticles (PBN) in inhibiting the proliferation of prostate carcinoma cells (PC-3), through an in vitro approach. A nanoparticle sample was obtained through the addition of chitosan and 0.4% Na-TPP to *Pasak Bumi* root extract. Flow cytometric analysis was employed to evaluate cell cycle distribution, while genes expression of Casp-3, Casp-8, and HAX-1 were quantified via qRT-PCR. The senescence cell detection test was performed using associated-β-galactosidase staining. PBN treatment supressed PC-3 cell

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proliferation through G0/G1 phase arrest and downregulation of HAX-1 genes expression, while promoting apoptosis via upregulation of Casp-3 and Casp-8 transcripts. PBN also increased the percentage of PC-3 senescence cells. This study demonstrates the potential of PBN to inhibit prostate cancer cells.

Keywords: *anticancer prostate, apoptosis, cell cycle, Eurycoma longifolia* Jack*, nanoparticles, senescence.*

1 Introduction

Prostate cancer, which constitutes 7.1% of all male malignancies and is the second most prevalent cancer among men, represents a significant concern for global public health [1,2]. In Indonesia, prostate cancer is prevalent at 14.8 per 100,000 population, accounting for 7.4% of all men's cancers [3]; 95% of prostate cancer cases are adenocarcinoma, while other types are less frequent [4]. This type of cancer is characterized as malignant due to its aggressive nature and rapid spread beyond the prostate's confines [5]. A similar set of challenges has been observed in other malignancies, such as leukemia, which is ranked eleventh among cancers in terms of global cancer-related mortality. The drawbacks associated with standard chemotherapeutic approaches, encompassing significant toxicity and the emergence of drug resistance, have necessitated the exploration of innovative alternatives. Such alternatives include plant-derived nanoparticles and secretome-based therapies [18].

Prostate cancer development involves genes and cellular processes, with Caspase (Casp)-3 and Casp-8 being crucial for apoptosis, the programmed cell death process. Casp-8 is involved in apoptotic following death receptor fusion, while Casp-3 is involved in the apoptosis execution stage [6]. These genes are essential for regulating apoptosis and suppressing aberrant proliferation of cancer cells. Furthermore, HCLS1 Associated Protein X-1 (HAX-1) is a gene known to be implicated in prostate cancer. This gene modulates cell cycle progression, thereby sustaining the survival and expansion of malignant cells [7]. Furthermore, dysregulation of cell cycle and senescence are linked to prostate cancer development. The interplay of these genes and cellular processes underscores the complexity of prostate cancer and highlights potential targets for therapeutic interventions and further research.

Prostate cancer treatment typically involves surgery, radiation therapy, hormone therapy, chemotherapy, and immunotherapy, which are considered less effective due to non-specific targeting of cancer cells [8]. Nanoparticles offer a promising avenue for developing therapies targeting cancer cells with minimal leakage, improving drug delivery systems' stability, bioavailability, and absorption [9]. Nanoparticles can be synthesized from ethanol extract using plant-based

methods. Nanoparticle plant extracts can effectively combine the properties of nanomaterials with various ingredients present in the extract. In addition, it can minimize the negative effects of the synthesis pathway related to dangerous or toxic chemical reagents [10]. The synthesis of nanoparticles from diverse parts of plants, including the roots of *Eurycoma longifolia* Jack, is a subject of significant research interest.

Belonging to the Simaroubaceae family, *E. longifolia* Jack is a tropical species indigenous to the Southeast Asian region. It is also called *Pasak Bumi* in Indonesia [11]. *E. longifolia* Jack, a renowned medicinal plant, has been found to possess cytotoxic and antiproliferative properties against various types of human cancer cells [12]. *E. longifolia* Jack is rich in numerous bioactive compounds, including squalene derivatives, alkaloids of the canthin-6-one and β-carboline classes, quassinoids, eurycolactone, eurycomalactone, laurycolactone, biphenyl neolignans, as well as biologically active steroids [13]. One of the quassinoid molecules, namely eurycomanone, has been discovered in *E. longifolia* Jack. The anticancer activity of *E. longifolia* Jack was demonstrated in vitro through chromatin condensation, inducing cytotoxicity and causing cancer cells to undergo apoptosis [14]. However, the effects of *E. longifolia* nanoparticles on prostate cancer-associated cell cycle regulation, apoptosis-related genes, and cellular senescence have yet to be fully elucidated. The present investigation sought to test whether *E. longifolia* Jack root extract nanoparticles, also known as *Pasak Bumi* (PBN), demonstrate potential in vitro anticancer activity for prostate cancer, specifically to determine the potential of PBN in cellular processes of cell cycle regulation, targeting genes such as Casp-3, Casp-8, and HAX-1, as well as senescence being involved in prostatic malignancy progression.

2 Materials and Methods

2.1 Nanoparticle Formation

Nanoparticles were synthesized by combining 60 mL of a solvent mixture (consisting of 20 mL of propylene glycol, 20 mL of 70% ethanol, and 20 mL of 10% DMSO) with 15 mL of ethanol extract from *E. longifolia* Jack. To this mixture, a 1% chitosan dilution in acetic acid was added, after which it was swirled for 10 minutes using a magnetic stirrer. Subsequently, 20 mL of Na-TPP 0.4% was meticulously introduced into the extract solution at a rate of one drop every second. The solution was then subjected to magnetic stirring for a further 15 minutes. The successful formation of nanoparticles was confirmed by the emergence of sediment and turbidity [15,16]. Physicochemical characterization of PBN was performed through particle size analysis (PSA),

zeta potential measurement (ZPA), and transmission electron microscopy (TEM) imaging.

2.2 Cell Culture

PC-3 cells (ATCC CRL-1435), a human prostate cancer cell line, were propagated in accordance with standard protocols. These cells were obtained from the Aretha Medika Utama Biomolecular and Biomedical Research Center in Bandung, Indonesia. The cells were cultivated in Dulbecco's Modified Eagle Medium High Glucose (DMEM-HG, Biowest L0104) medium, which was enriched with 20% fetal bovine serum (FBS). The FBS was supplied by Biowest (S1520). These cell cultures were stored in T25 flasks at 37 °C with 5% CO_2. Every 2 to 3 days, the culture medium was replaced, and subculture was carried out when cell confluence reached approximately 70 to 80% [17-19].

2.3 Cell Cycle Analysis

PC-3 cells were inoculated into culture flasks to achieve a seeding density of 5 × 10^5 cells/25 cm^2 and subsequently treated. The research group consisted of a negative control (NC), which was not treated; a DMSO control (DMSO), namely a group treated with 10% DMSO; and a group treated with PBN at various concentrations (37.5; 75; 150) µg/mL. Cell cycle assessment was conducted using a Cell Cycle Assay Kit with Red Fluorescence (Elabscience, E-CK-A351), with all steps executed as per the guidelines outlined by the manufacturer. The analysis was conducted using a flow cytometry method (MACSQuant Analyzer 10, Miltenyi Biotec) [20,21].

2.4 Gene Expression Measurement with qRT-PCR

Using the Direct-zol RNA Miniprep Plus kit (R2073), cellular RNA was isolated in strict accordance with the manufacturer's recommended protocol. RNA purity and concentration were then assessed via microdrop plates and spectrophotometric analysis using the Multiskan GO system (Thermo Scientific, 51119300). Next, cDNA synthesis was completed using the SensiFAST cDNA synthesis kit (Meridian Bioscience, BIO-65054). The AriaMx Real-Time qPCR System (Agilent, G8830A) served as the platform for quantitative gene expression analysis [18-19, 22]. Primary sequences of apoptosis and cell proliferation genes were Casp-3 (F: 5'-AGAACTGGACTGTGGCATTG-3'; R: 5'-GCTTGTCGGCATACTGTTTCAG-3'), Casp-8 (F: 5'-AGGCCAGATCTTCACTGTCC-3'; R: 5'-GGTCACTTGAACCTTGGGAA-3'), and HAX-1 (F: 5'-GAGGAGGGATACGTTTCCACGA-3'; R: 5'-GTCTCTGACTCAGGACCTGGAA-3'). GAPDH (F: 5'-GAAGGTGAAGGTCGGAGTC-3'; R: 5'-GAAGATGGTGATGGGATTTC-3') was used as a housekeeping gene.

2.5 Senescence Detection Assay

Senescent cells were analyzed according to Priyandoko et al. with modifications [18,21,23]. For the histochemical detection of senescent cells, the Senescence Cell Histochemical Staining Kit (Sigma-Aldrich, CS0030-1KT) served as the primary reagent, applied strictly based on the manufacturer's recommended protocol. An inverted microscope (Olympus, SKX41-F32FL) was used to view cell senescence, and senescent cells were counted manually.

2.6 Statistical Analysis

All datasets underwent statistical processing with SPSS version 26.0, with results reported as mean ± SD. Normally distributed data ($p > 0.05$) were analyzed by one-way ANOVA with Tukey HSD or Dunnett post-hoc comparisons, while non-normal distributions ($p < 0.05$) were assessed via Kruskal-Wallis analysis with Mann-Whitney post-hoc testing [23-25].

3 Results and Discussion

Ranked as the second most common malignancy in men, prostate cancer is characterized by dysregulation of cell cycle progression, senescence, and apoptotic pathways. The primary quassinoid of *Pasak Bumi* root extract, eurycomanone, has demonstrated efficacy in suppressing the expansion of tumor cells, triggering programmed cell death in breast carcinoma cells (MCF-7) [26], as well as small (A549) and large (H460) lung carcinoma cells, and inducing cell cycle arrest [27]. To maximize its potential, a new drug delivery system using PBN production is recommended. It aims to improve solubility, bioavailability, pharmacology, stability, and distribution to tissue macrophages, prevent toxicity, and sustainable delivery as well as protect against physical and chemical degradation [28].

Cellular cycle dysregulation in cancer cells is a common feature that can occur. This dysregulation has been shown to result in increased proliferation of cancer cells due to an increase in the G0/G1 phase of the cell cycle [29]. The effect of PBN on the cell cycle phases (G0/G1, S, and G2/M) can be seen in Table 3. These data reveal that cells subjected to PBN treatment exhibit G0/G1 phase suppression compared to the NC group. This observation suggests that PBN exhibits antiproliferative activity against PC-3 cells. The most effective concentration was PBN 37.5 μg/mL. Dot blot PBN on the PC-3 cell cycle is shown in Figure 1. Such findings corroborate the work of Okba et al., which demonstrated that *Pasak Bumi* has the capacity to halt the G0/G1 phase [27]. The results further revealed that PBN treatment resulted in a notable elevation in the percentage of cell populations residing within the S and G2/M phases

compared to NC. The present data indicate that the compounds present in PBN function by impeding the initial checkpoint of the cell cycle, in contrast to the blockade of genomic replication occurring during the S phase and the suppression of DNA repair processes in the G2/M phase [30]. Apart from that, this is also related to the temporal distribution of cell cycle phases in rapidly dividing human cells (cancer cells), with the G1 phase lasting approximately 11 hours, the S phase lasting roughly 8 hours, and the M phase persisting for approximately 1 hour [31]. Treatment administered 24 hours after seeding prompts cells to advance into subsequent stages of the replication cycle, as both the S and G2/M checkpoints are considerably shorter in duration than the G0/G1 stage. It is important to note that different responses may also occur in different cell types. For example, Moses et al. found that freeze-dried leaf extract from *Pasak Bumi* affects the G2/M phase cell cycle in breast cancer cells (MCF-7) while affecting the S phase in epithelial-like cells (MDA-MB-231) [32]. The suppression of the G0/G1 phase by *Pasak Bumi* is achieved through a mechanism associated with Cyclin-dependent kinases (CDK)-2, -4, D1, D3, and protein (p)-21. This process is further regulated by cyclin-dependent kinase inhibitor 1A (CDKN1A) [33].

Table 1 Effect of various PBN concentrations toward PC-3 cell cycle levels.

Samples	Cell Cycle		
	G0/G1 Phase	S Phase	G2/M Phase
Untreated PC-3 cells (NC)	92.63 ± 1.30	5.91 ± 1.12	0.40 ± 0.19
PC-3 + DMSO 1% (DMSO)	92.23 ± 2.65	6.35 ± 2.04	0.46 ± 0.25
PC-3 + PBN 37.5 µg/mL	91.08 ± 0.73	6.73 ± 0.90	0.75 ± 0.16
PC-3 + PBN 75 µg/mL	90.82 ± 1.80	7.30 ± 1.63	0.79 ± 0.26
PC-3 + PBN 150 µg/mL	90.04 ± 0.13	7.22 ± 0.37	1.15 ± 0.63

*Values are expressed as mean ± SD.

Apart from cell cycle dysregulation, apoptosis dysregulation also indicates problems due to symptoms of various diseases. Generally, in cancer there is uncontrolled growth, inhibition of apoptosis, and angiogenesis [34-36]. Apoptosis is regulated by caspases, one of which is the initiator caspase (Casp-8) and the executive caspase (Casp-3) [36], while HAX-1 is responsible for cancer cell proliferation that results in uncontrolled cell growth, which is indirectly linked to cell cycle progression. The impact of PBN on the transcriptional levels of Casp-3, Casp-8, and HAX-1 genes in PC-3 cells is depicted in Figure 2. PBN treatment produced a statistically notable ($p < 0.05$) upregulation of Casp-3 and Casp-8 transcripts, alongside a concurrent downregulation of HAX-1, with 150 µg/mL demonstrating the greatest efficacy. A study on freeze-dried *Pasak Bumi* extract was reported to induce apoptosis by increasing Casp-3 within MDA-MB-231 breast cancer cells, along with Casp-8 upregulation in MCF-7 cells [32]. These results support previous research,

which stated that eurycomanone, the main quassinoid compound found in *Pasak Bumi*, functions as an anticancer compound. It plays a role in modulating key molecular pathways that govern cell proliferation, programmed cell death, and inflammatory responses [37].

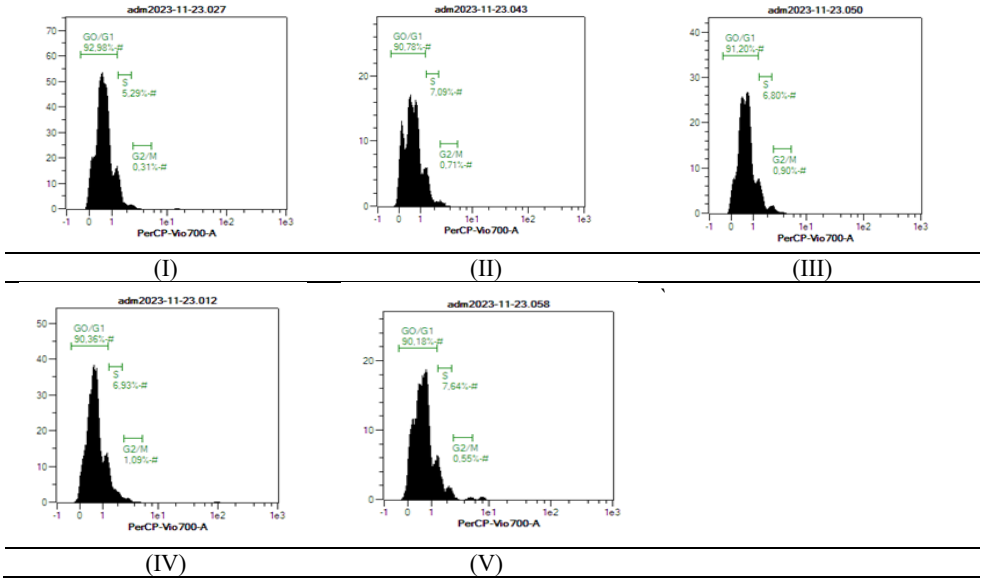

Figure 1 Dot blot representation of various PBN concentrations on the PC-3 cell cycle.

*All values are reported as mean ± standard deviation (SD). I: NC is defined as the untreated cells, II: PC-3 cells +DMSO1%, III: PC-3 cells + PBN 37.5 µg/mL, IV: PC-3 cells + PBN 75 µg/mL, V: PC-3 cells + PBN 150 µg/mL.

Cellular senescence, closely linked to cell cycle dynamics and apoptotic pathways, represents a permanent halt in cell cycle activity occurring in a steady state. It is characterized by multiple macromolecular changes as well as a hypersecretory, pro-inflammatory phenotype triggered by a range of stimuli, such as telomere attrition, genomic damage, and aberrant oncogene signaling [38,39]. Anticancer therapy often causes tumor cell senescence, which likely contributes to treatment success [40]. Figure 3 shows a visual representation of cell senescence (red arrow) after staining with associated-β-galactosidase. Figure 4 displays the quantitative results of senescence cells, showing that the effect of PBN treatment on PC-3 senescence cells is concentration-dependent. Among the concentrations tested, PBN at 150 µg/mL demonstrated the highest efficacy in promoting senescence in PC-3 cells (41.49%), markedly exceeding that observed in the NC group (25.93%).

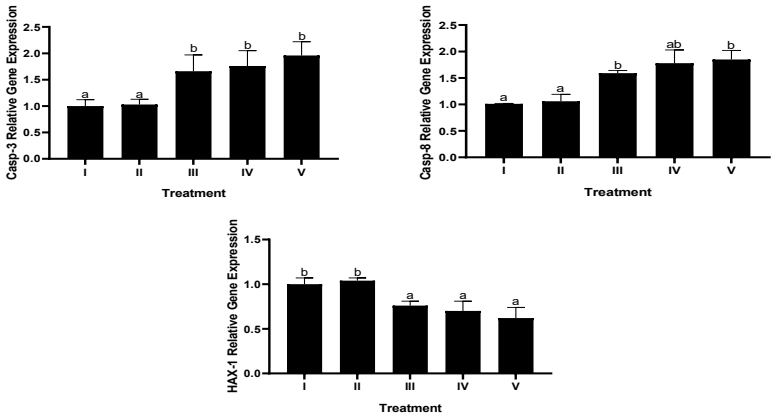

Figure 2 Effect of various PBN concentrations toward Casp-3, Casp-8, and HAX-1 gene expression in PC-3 cells.

*All values are reported as mean ± standard deviation (SD). (I) NC; (II) DMSO; (III) PBN 37.5 µg/mL; (IV) PBN 75 µg/mL; (V) PBN 150 µg/mL. Different superscript marks indicate significant differences ($p < 0.05$) Tukey HSD test.

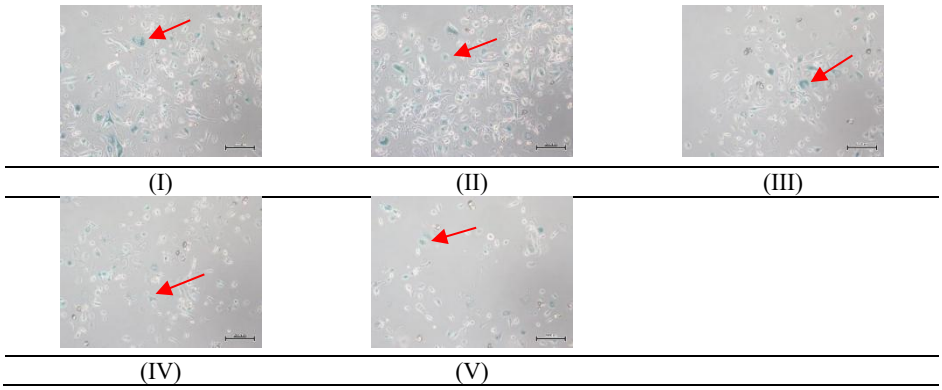

Figure 3 Effect of various PBN concentrations toward the morphology of senescent PC-3 cell.

*10x magnification PC-3 senescent cells (red arrow). I: NC (untreated cells), II: PC-3 cells + DMSO1%, III: PC-3 cells + PBN 37.5 µg/mL, IV: PC-3 cells + PBN 75 µg/mL, V: PC-3 cells + PBN 150 µg/mL.

PBN has anticancer potential in inhibiting the spread of cancer cells through suppressing cell growth using the senescence mechanism. This may be because eurycomanone in PBN can cause activation of specific cellular pathways such as cell cycle and apoptosis, inhibition of telomerase, regulation of p53/p21 and p16INK4a/Rb pathways as well as interactions with specific target molecules.

This has been explained by one of the studies on anticancer compounds in HepG2 cells. The induction of senescence in cells can inhibit cell survival due to the activity of inhibitors of the cyclin-dependent kinase (p16INK4a)-retinoblastoma (Rb) and p14 Alternative Reading Frame (p14ARF)-p53 pathways [41]. However, further investigation needs to be done.

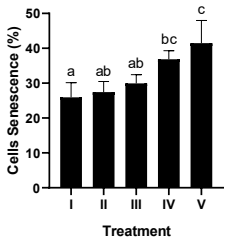

Figure 4 Effect of various PBN concentrations toward senescent PC-3 cells.

*All values are reported as mean ± standard deviation (SD). (I) NC; (II) DMSO; (III) PBN 37.5 µg/mL; (IV) PBN 75 µg/mL; (V) PBN 150 µg/mL Distinct superscript marks denote statistically significant distinctions as determined by Tukey HSD test ($p < 0.05$).

Figure 5 illustrates a proposed mechanism underlying PBN's effects on cell cycle regulation, apoptosis, and senescence. The apoptotic pathway is initiated through Fas-Associated Death Domain (FADD) alongside p53, both of which subsequently stimulate Casp-8 upregulation, leading to elevated Casp-3 expression together with activated caspase-9 activity, while the cell cycle and senescence pathways are governed by p16 INK4a/RB signaling and the p53/p21 axis. INK4a regulated by p16 leads to inhibition of CDKs. Unphosphorylated RB subsequently triggers cell cycle arrest, manifested as an elevation in G0/G1 phase accumulation, ultimately promoting increased senescence. On the other hand, cell cycle arrest indirectly inhibits cancer cell proliferation by decreasing HAX-1, MDM2 (Mouse Double Minute 2), and Bcl-2 (B-cell lymphoma 2) expression.

This study's limitations include the absence of standard chemotherapy controls, normal-cell toxicity evaluation, protein-level validation, and in vivo analysis. Subsequent studies should entail the incorporation of comparative chemotherapy controls, the evaluation of normal cell toxicity, the utilization of multiple prostate cancer cell lines, protein-based mechanistic analyses, as well as in vivo experimentation. These additional investigations are warranted to further substantiate the therapeutic potential alongside the safety profile of PBN.

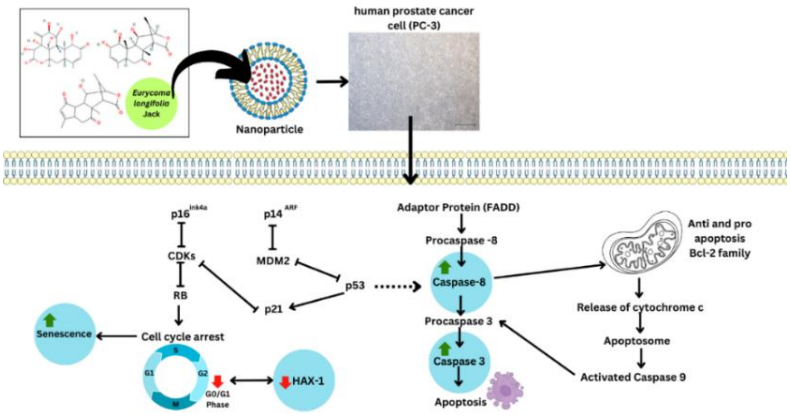

Figure 5 Schematic overview of the proposed mechanism of PBN in combating prostatic malignancy, targeting cell cycle progression, apoptosis, and senescence.

4 Conclusion

PBN demonstrated antiproliferative, pro-apoptotic, and senescence-inducing effects against PC-3 cells by reducing HAX-1 expression, inhibiting the G0/G1 phase, and increasing Casp-3 and Casp-8 expression. However, further studies are needed to validate its therapeutic potential and safety.

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