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# Azo-hydrazone analogue BT-1F, induces anti-inflammatory activity in inflammatory *in-vitro* and *in-vivo* models

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**Abstract**

Inflammation is a crucial process involved in the progression of multiple forms of cancers and accepted as major hallmark. Numerous inflammation prone agents are elevated in neoplastic milieu.These perceptions are fostering novel anti-inflammatory therapeutic strategies to cancer progression. As an approach, anti-angiogenic azo-hydrazone analogue, BT-1F was investigated to evaluate the anti-inflammatory activity in *in-vitro* and *in-vivo* non- tumorigenic & tumorigenic inflammatory animal models. Reports revealed that, BT-1F showed noticeable anti-inflammatory activity by protecting human red blood cells (HRBCs) membrane and by inhibiting the activity of both inflammatory enzymes (COX2 & 5-LOX) significantly in *in-vitro*. The *in-vivo* paw edema anti-inflammatory activity suggested that inhibition of carrageenan induced inflammation. Further, BT-1F exhibited the remarkable tumour reduction in an ascites inflammatory neoplastic EAC model through normalizing the inflammatory biomarkers. In conclusive, BT-1F is an anti-inflammatory pharmacophore which could be developed into therapeutic molecule in future.

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Inflammation is a biological reaction of the immune system required for defensive against injuries or certain deleterious inflammogens[7]. Inflammatory mechanism represents a sequence of ordered, influential response including both vascular and cellular events[1]. The inflammatory response is regulated through several mediators and some signaling nexus in the complicated network[27]. The prolonged period of inflammation could leads to the progression of severe disease including cancer. Inflammation can induce neoplastic progression, whereas cancer can promote inflammatory response[14]. The possible association of inflammation and cancer was first hypothesized in the 17th century, on the basis of morphological examination[5] and nowadays it is well known about cytokines cells, enzymes and physiological mechanism, which are the midways between inflammation and cancer[21]. However it is now clear that, in spite of cell proliferation, inflammatory cells, activated stroma, growth factors, and DNA mutation promoting agents are the critical risk factors for the progression of neoplasia[11]. A crucial role of inflammation in the development of carcinogens is now become widely accepted phenomenon and established as tumour hallmark. Moreover, approximately 20% of cancers including ascites cancer are related to chronic inflammation[2]. The inflammation promoting factors are rich in tumour ascites, whereas an inflammatory melieu like ascites exhibits drug resistance. Elevated number of pro-inflammatory mediators such as chemokines, cytokines and growth factors are noticed in cancer ascites[19]. Hence, targeting the tumour promoting inflammation is the promising therapeutic strategy for cancer, which is the main focus of the study.

The hydrazone constituent plays a critical role in heterocyclic chemistry and their derivatives have a numerous pharmacological attributes including anti-inflammatory and anticancer[25]. While, azo molecules also showed a broad pharmacological properties including anti-inflammatory, anti-tumour attributes[8,24]. Based on these research investigation, azocoupled hydrazone analogues have been designed, which is an important strategy towards treatment of many disease including inflammatory related cancer treatment. Earlier we have recorded that newel azo-hydrazone analogue "2-Methyl-quinolin-8-yloxy)- acetic acid [(5-chloro-2-nitro-phenyl)-(2-chloro-phenylazo)- methylene]-hydrazone" or BT-1F (Fig. 1A) has better antioxidant and anti-angiogenic mediated anti-neoplastic property[32]. Based on these evidences, we have extended the investigation to study the anti-inflammatory activity and its related cancer inhibition property in *in-vitro* and *in-vivo* anti-inflammatory and *in-vivo* anticancer model.

*Materials :*

Cyclooxygenase-2 (COX2) and 5-lipoxygenase (5-LOX) inhibitors assay kit from Cayman Chemical, Ann Arbor, MI (USA). Mouse interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF- α) ELISA kit were obtained from Sigma Aldrich (USA). All the other chemicals utilized were of analytical grade and were procured from Hi-Media, Mumbai (India). All the experimental assays were performed at minimum three

independent times and analyzed. Photograph of all the images were taken using Canon power shot Sx500 IS camera.

*In-vitro anti-inflammatory activity of BT-1F:*

*In-vitro HRBCs membrane stabilization assay:*

HRBC membrane stabilization assay was used for the examination of anti-inflammatory activity of BT-1F as described earlier[13]. Equal volume of sterilized alsevers solution and human blood were mixed. The mixture was centrifuged at 3000 rpm to isolate the packed cells, then washed and made a 10% v/v cell suspension with isosaline. Various concentrations of BT-1F (1, 5, 10, 15 and 20 µg/ml) were prepared and each concentration was mixed with 2mL of hyposaline, 0.5 mL of HRBC suspension and 1mL of phosphate buffer. All the reaction components were incubated at 37°C for 30 minutes and proceeded for centrifugation at 3000 rpm. The hemoglobin level in the supernatant was evaluated spectrophotometrically at 560 nm. The percentage of hemolysis was calculated by using the formula.

Percentage of protection= 100 - [(OD of treated sample/ OD control) × 100]

*Cyclooxygenase-2 (COX2) inhibition assay:*

The enzymatic assay was carried out by using colorimetric COX2 inhibitor screening assay kit. In brief, the assay mixtures contained different concentrations of BT-1F and other chemicals were added according to the manufacturer's instructions. The plate was shaken for few seconds and incubated at 25°C for 5 minutes. The reaction was initiated by adding TMPD (20µL) and arachidonic acid (20µl) to each well. Again the plate was shaken and incubated at 25°C for 5 minutes. The micro plate reader were used to read the absorbance at 590 nm. Percentage inhibition of enzyme was determined by following formula.

% inhibition = untreated blank-treated / untreated blank x 100

*The 5-lipoxygenase (5-LOX) Inhibition Assay:*

The 5-LOX assay was performed by using colorimetric 5-LOX inhibitor screening assay kit. In brief, in 96-well microtiter plate, different concentrations of BT-1F were added and other relevant chemicals were incorporated according to the manufacturer instructions. The plate was incubated at room temperature for 5 minutes. To the assay mixture, 10µL of linoleic acid was added and incubated for 10min. Absorbance was read at 490nm using microtiter plate reader. Percentage inhibition of enzyme was determined by following formula.

% inhibition= untreated blank-treated/ untreated blank x 100

*Animals and ethics:*

The BALB/C mice (27–30 g) were used throughout the study and were maintained under CPCSEA guidelines with ethical clearance (Ref No. KSHEMA/IAEC/01/2020 Date: 04.06.2020). Acute toxicity studies of

BT-1F was done previously and dosage was fixed[6].

*In-vivo anti-inflammatory activity of BT-1F:*

*Carrageenan-induced paw edema model in mice:*

The mice were used to determine the anti-inflammatory activity of BT-1F against carrageenan induced paw edema as reported earlier[33]. Animals were divided into four groups (n=6).

Group I: Normal

Group II: Carrageenan control

Group III: Positive Control (Indomethacin)

Group IV: Animals treated with BT-1F

Prior to experiment saline, indomethacin and BT-1F (20 and 40 mg/kgbw) were injected to group II, group III and group IV animals respectively and left the animals for 2h. After 2h, 100µl of 2% carrageenan were injected to all group animals right paw except normal group. All the experimental animals right paw were considered for observation at different time intervals (1h, 2h, 3h, 4h and 5h) and paw volume were measured by using digital plethysmometer.

*In-vivo Ehrlich ascites carcinoma (EAC) model development, treatment and analysis of pro-inflammatory biomarkers :*

Murine ascites tumor model was developed to investigate the *in-vivo* anti-tumour efficacy of the BT-1F as per earlier reported protocol[15]. In brief, EAC tumor is regularly maintained in mice peritoneum cavity. EAC cells ($2.5\times10^5$) from donor mice were collected and re-injected to the mice peritoneum (*i.p*). Animals were divided into three groups (n=6). On fourth day of tumor induction, three doses of BT-1F (20 &40 mg/kg bw) were administered to group-II & III animals intra-peritoneally on every alternate days and group-I mice served as control. After third dose, all the animals were sacrificed to isolate the blood samples and tumor-growth parameters including tumor volume, ascites secretion, cell count and survivability were noticed and documented. From the blood samples serum was collected and analyzed the pro-inflammatory markers such as, IL-6, IL-1β and TNF-α through ELISA kit method[26].

*Statistical analysis :*

The data were represented as mean ± standard deviation (SD) using Graph Pad prism version 8.0. All experiments were repeated three independent times. The results were analyzed using one-way ANOVA. Statistical significance were expressed in terms * $p< 0.05$ to ** $p< 0.01$.

*BT-1F exhibits potent anti-inflammatory activity in-vitro :*

The HRBCs membrane stabilization assay has been used to study the *in-vitro* anti-inflammatory activity. The percentage of membrane stabilization increased with the increasing drug concentration. At 20µg/ml concentration, BT-1F showed 88.12% erythrocyte membrane stabilization by protecting against hemolysis which is highly notable protection near to the standard drug (Fig. 1B). Additionally, the anti-inflammatory activity was again

assessed by examining the activity of inflammatory marker enzymes such as COX2 and 5-LOX. The enzyme activity inhibition improved with the increased concentration of BT-1F that effectively inhibit the activity of COX2 up to 91% and it was near to the inhibition of standard drug (Fig. 1C). Similarly the activity of 5-LOX enzyme was decreased with the concentration dependent manner and finally declined up to 87%, which is one of the notable inhibitory activity of the BT-1F (Fig. 1D). Overall, the outcome implied that BT-1F had excellent anti-inflammatory activity.

*BT-1F remarkably reduced the inflamed paw :*

*In-vitro* anti-inflammatory outcome was re-validated through *in-vivo* inflammatory paw edema model. The BT-1F (20 & 40 mg/ kg bw) was administered to the animals prior to experiment, after few hours of carrageenan induction paw volume was recorded. Results exhibited that BT-1F treated group animals showed remarkable inhibition in the inflamed paw volume approx. 4 fold compared to untreated control group, which are on almost par with the standard drug used in this study (Fig. 2). Overall, BT-1F effectively showed the potent anti-inflammatory activity in *in-vivo* also.

*BT-1F diminishes inflammatory tumour growth by declining the pro-inflammatory markers :*

In ascites model system, BT-1F was administered to the tumour bearing mice in a dose dependent manner. Results show that BT-1F decline the tumour proliferation as significantly as compared with control (Fig. 3B). Declined tumour proliferation correspondence to the reduced final ascites secretion was 6.2 & 4.4 ml at 20 & 40 (mg/kg bw) (Fig. 3C) respectively and reduced number of cells upto 1.7 fold (Fig. 3D) in BT-1F treated animals in a dose dependent manner. In addition, BT-1F expands the period of life span (Fig. 3E) significantly in treated animals. The fact underlying this was evaluated by measuring the inflammatory markers. Results revealed that BT-1F significantly decreased the level of serum biomarkers like IL-6, IL-1β and TNF-α approx 2 folds (Fig. 3) and thereby exhibiting anti-tumour effect.

When the inflammation sustained for too long it becomes chronic, which leads to the evolution of many harmful diseases including cancer. The role of inflammation prone chemokines, cytokines, inflammatory enzymes and adhesion molecules has been closely associated with chronic inflammation[3]. These inflammatory conditions have been involved in several steps of carcinogenesis[20]. There are many extensive research outcomes demonstrated that inflammation plays a chief role in the establishment, advancement and/or aggressive growth of various cancers. Given its myriad tumour prone effects, inflammation became a target for tumour prevention and treatment. In supportive, anti-inflammatory agents could effectually decline the risk of progressive cancers[22]. A number of small molecule NSAIDs were developed for the treatment of various malignancies including ascites tumour[9]. In the present, we have used small molecule inhibitor azo-hydrazone analogue (BT-1F) (Fig. 1A) to investigate the anti-inflammatory and its mediated cancer

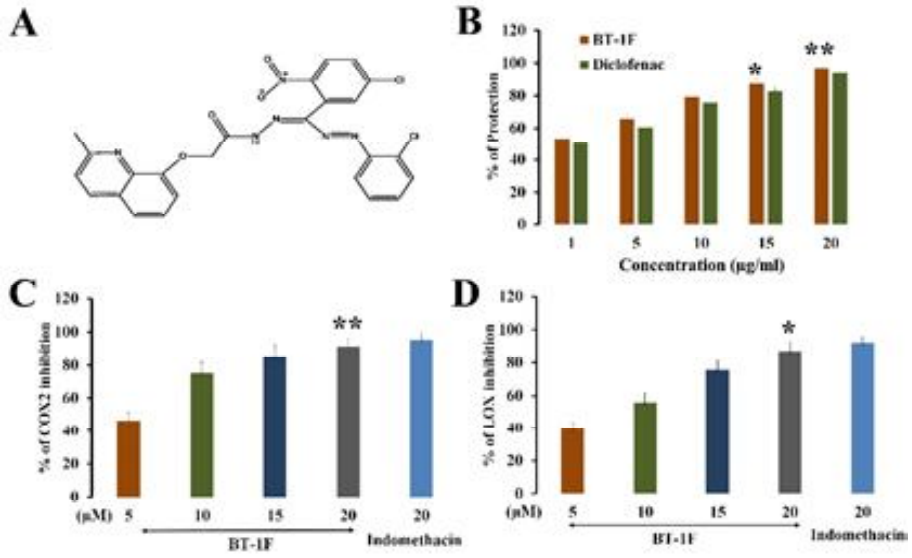



**Fig. 1: BT-1F exerts effective anti-inflammatory activity in *in-vitro*:** The human blood and sterilized alsevers solution were mixed equally. The mixture was centrifuged at 3000 rpm, to this, different concentrations of BT-1F (1, 5, 10, 15 and 20 µg/ml), hyposaline and phosphate buffer were added. All the components were incubated and centrifuged at 3000 rpm. The membrane protection was calculated: A) Structure of BT-1F. B) Effect of BT-1F on hyposaline induced hemolysis on RBCs membrane. The enzyme inhibition assay was performed by using COX2 and 5-LOX inhibitor assay kit. To the assay mixture, varied concentrations of BT-1F (5, 10, 15 & 20µM) were added and carried out as per manufacturer's protocol: C) BT-1F notably decreased the activity of COX2 enzyme. D) Inhibition of 5-LOX enzyme activity on concentration dependent manner.

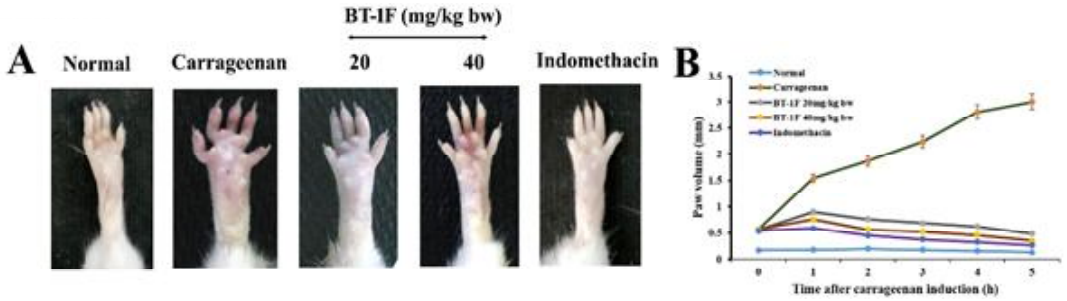



**Fig. 2: Inflammation inhibition effect of BT-1F on paw edema model:** BT-1F (20&40mg/kg bw) were administered to the animals prior to carrageenan induction and saline was incorporated to control group instead of drug. The impact of BT-1F on inflammation was examined by measuring A) Swelling and redness of the soft paw tissue. B) Inflammatory paw volume of control and treated animals.

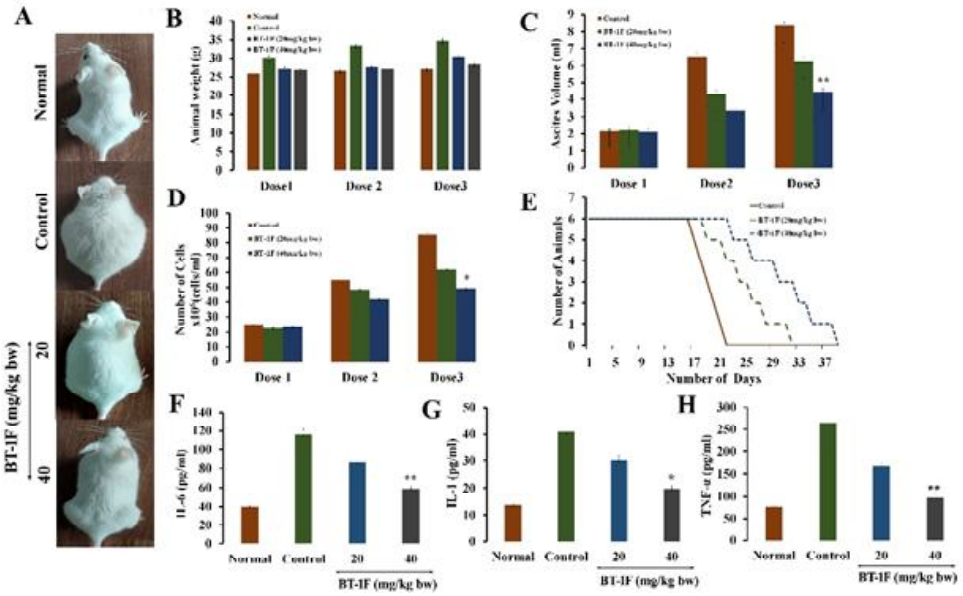
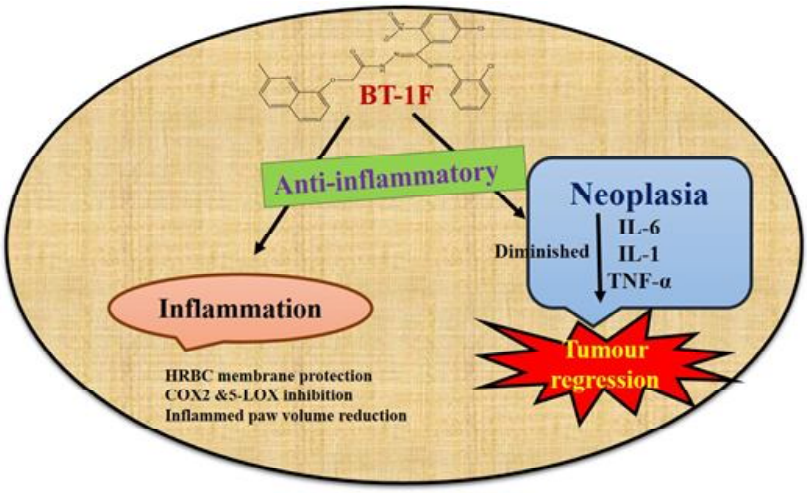



**Fig. 3: BT-1F regress the proliferation of inflammatory murine ascites (EAC) tumor and normalized the inflammatory biomarkers.** Ascites tumor was developed by inducing EAC cells in mice (*i.p*). After four days of tumor growth, BT-1F (0, 20 & 40 mg/kg bw) was administered (3 doses) on every alternate day. At last, serum was collected for inflammatory markers analysis: (A) physical morphology of all group animals. B) Reduced body weight of BT-1F treated mice on dose dependent manner. (C) Reduction in ascites secretion. (D) Decrease in number of cells after BT-1F treatment. (E) Kaplane-Meier graph representing the prolonged survivability. Decreased level of inflammation prone biomarkers in treated serum: F) IL-6. G) IL-1β. H) TNF-α.

## Graphical Abstract



inhibition. The hydrazone moiety had multiple pharmacological functions like anti-cancer and anti-inflammatory attributes[29]. With respect to inflammatory associated cancer research, the azo molecules have been recorded to be potent anti-inflammatory and anti-neoplastic drug[4,17]. Previously we have reported that azo-hydrazone analogue "2-Methyl-quinolin-8-yloxy)- acetic acid [(5-chloro-2-nitro-phenyl)-(2-chloro-phenylazo)- methylene]-hydrazone" (BT-1F) showed notable antioxidant and anti-angiogenic mediated cancer inhibition[32]. Since there is direct relevance between ROS, inflammation and neoplasia, we have extended research investigation to evaluate the cancer inhibition effect of BT-1F through anti-inflammatory activity[23]. Based on these informations, initially we have examined the anti-inflammatory activity of BT-1F by *in-vitro* HRBCs membrane stabilization assay. The membrane of RBCs is analogous to the membrane of lysosome, so for erythrocyte membrane stabilization was done by the drug may as well protect the lysosomal memb-ranes[18]. The lysosomal membrane stabilization is important in decreasing the inflammatory process by deterring the release of lysosomal components of activated-neutrophil like proteases and enzymes, which causes further damage and inflammation in tissue after extra cellular constituents'release[31]. On the basis of this assay results, BT-1F showed remarkable anti-inflammatory function as compared to untreated control (Fig. 1B). Arachidonic acid (AA) is chiefly metabolized through COX and LOX enzyme pathways and a number of studies on the inflammation associated neoplasia have focused on these pathways. The COX-2 and 5-LOX plays a critical role in tumour-related inflammation and cancer progression[12,30]. The COX2 and 5-LOX is up regulated in most of human cancers including ascites tumour. This up-regulation leads to poor prognosis and reduced survival rates in cancer patients. The importance of both COX2 and 5-LOX in inflammatory cancer progression, they served as a main therapeutic target of inflammation associated cancers[10]. On basis of this, we have evaluated the anti-inflammatory activity in *in-vitro* by enzyme inhibition assay, as a result, BT-1F significantly decreased the activity of both the enzymes that are almost on par with the standard drug (Fig. 1C & D). Carrageenan mediated acute-inflammation is one of the highly recommendable experimental protocol to screen the anti-inflammatory drugs and is trusted to be biphasic[28]. So far, *in-vitro* outcomes was further validated via *in-vivo* carrageenan induced paw edema model to study the anti-inflammatory effect. Results showed that BT-1F significantly declined the inflammatory paw volume as compared with the control (Fig. 2). Inflammation is one of the major pitfall for the evolution of all types of neoplasia including ascetic cancer. Ascites is associated with inflammatory environ rich in pro-inflammatory agents, which exerts drug resistance[16]. Hence, we used the EAC model to evaluate the anti-inflammatory mediated tumour inhibition. Ascites fluid is one of the chief nutrition source for neoplastic cells and upraised level of ascites was noticed in the swiftly developing cancer, which will meet the metabolic needs of growing cells in ascites. Therefore, declined ascites fluids signifies the tumour inhibition rendering it an acceptable tumor model for *in-vivo* studies[15].Our findings implied that there was noticeable decrease in tumour growth (Fig. 3B) on dose dependent manner, which will reflect in reduced volume

of ascites secretion (Fig. 3C) and viable EAC cell count (Fig. 3D). Moreover, BT-1F extended the survivality of treated animals compared to untreated control (Fig. 3E). Furthermore, BT-1F normalized the pro-inflammatory marker cytokines in serum (Fig. 3). In an inflammatory neoplastic condition there are several pro-inflammatory mediators' like cytokines such as IL-6, IL-1β and TNF-α are elevated[26]. Overall, BT-1F significantly down regulated the growth of tumour through anti-inflammatory activity was proved in *in-vitro* and *in-vivo* model.

Inflammation is the major culprit for the evolution of many cancers, hence anti-inflammatory agents are the powerful strategy for cancer treatment. Current study was carried out to examine the anti-neoplastic effect of BT-1F via anti-inflammatory activity. Our pre-clinical investigation results exhibited that BT-1F has impressive anti-inflammatory activity both in *in-vitro* and *in-vivo*. Besides BT-1F undermine the ascites tumour growth by reducing the level of inflammatory cytokines. Overall, BT-1F is a potent anti-inflammatory drug, which effectually inhibited the inflammatory ascites cancer. Thus, BT-1F is one of the hopeful candidate in inflammatory related cancers therapeutics.

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*Conflict of interest :*

The authors exhibit that there are no conficts of interest.

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