Research article

Halofuginone: a novel oral and intravesical agent for the treatment of non-muscle invasive bladder cancer

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https://doi.org/10.31083/j.jmcm.2018.04.407

Abstract

Background: Non-muscle invasive form (NMIBC) is a chronic disease with a high recurrence rate and requires lifelong surveillance. Various intravesical agents were shown to reduce tumor recurrence but unfortunately, none of these agents proved to be of benefit in long-term prevention of local recurrence or disease progression. Aim of Research: Previous studies have shown that Halofuginone (HF), an antiprotozoal agent, exerts anti-neoplastic activity in various cancer models. Our aim was to evaluate the in vivo activity of oral and intravesical HF treatment in an experimental mouse model harboring NMIBC. Methods: Initially, 60 mice were divided into six treatment groups to evaluate the toxicity of this anti-parasitic agent on the bladder mucosa. The second stage included 126 mice which underwent intravesical implantation with Mouse Bladder Tumor cells (MBT-2): Group 1 (n = 30) received no treatment, group 2 (n = 32) received 6 intravesical instillations of PBS, group 3 (n = 32) received 6 doses of 250 μg oral HF, whereas group 4 (n = 32) received 6 intravesical instillations of 250 μg HF. Results: The average weight of bladders, which reflects the anti-neoplastic activity, differed significantly between the control and treated groups: 88.8 mg $\pm$ 15.58 SEM and 81.2 mg $\pm$ 13.79 SEM for untreated and PBS-treated mice, respectively, versus 38.0 mg $\pm$ 4.02 SEM and 39.6 mg $\pm$ 5.97 SEM for animals treated with oral and intravesical HF, respectively. Conclusions: HF exerted a significant anti-neoplastic activity in mice bearing NMIBC upon oral as well as intravesical administration. These results may constitute the basis for the maintenance of oral treatment with HF in patients with NMIBC.

Submitted: November 12, 2018; **Revised:** November 24, 2018; **Accepted:** November 25, 2018

1. Introduction

Urothelial cell carcinoma (UCC) of the bladder is the 10th most common cancer worldwide. The morbidity and mortality associated with bladder cancer are relatively high with more than 160,000 deaths per year reported worldwide. With the expected increased life expectancy of the general population, this chronic malignancy carries a significant economic burden with an annual expenditure of 4.6 billion dollars in 2017 in the US only [1].

NMIBC accounts for $\sim$ 80% of bladder cancers and has a high propensity for recurrence after transurethral resection (up to 75%). In addition, it is also at high risk for progression to the muscle invasion form (15%-25%) [2–4]. Various intravesical agents have been shown to reduce tumor recurrence rate when used in conjunction with transurethral tumor resection. Unfortunately, none of these agents proved to be of benefit in long-term prevention of local recurrence or disease progression [5].

Bacillus Calmette-Guerin (BCG) immunotherapy is considered the gold standard therapeutic intravesical agent especially in the high-risk group [6]. However, BCG treatment is not infrequently associated with serious side effects ranging from dysuria and hematuria to BCG sepsis and systemic tuberculosis [7]. The pivotal study of the South Western Oncology Group (SWOG) [8] demonstrated that long duration of treatment results in better outcome. Unfortunately, only 16% of the participants of this study were able to tolerate the full dose-schedule of BCG maintenance regimen due to substantial toxicity. In addition, about 20-40% of patients treated primarily with BCG will show recurrent disease at the first follow up evaluation 3 months after instillation [9–11]. These findings emphasize the need for new effective agents with a reduced toxicity profile that will allow continuous and long-term administration.

Halofuginone (HF) is a low molecular weight, synthetic halogenated derivative of febrifugine, a natural quinazolinone alkaloid isolated from the Chinese herb *Dichroa febrifuga.* HF reduces collagen type α1 (I) gene expression and extracellular matrix deposition [12–15]. Moreover, HF was shown to inhibit matrix metalloproteinase-2 (MMP-2) expression, angiogenesis, stromal support and tumor growth *in vitro* and in animal model of bladder cancer metastasis [16].

The aim of our present study was to evaluate the anti-neoplastic activity of HF administered both topically and orally in a mouse bladder tumor (MBT-2) model, that was used to assess the efficacy of HF treatment for NMIBC.

2. Materials and Methods

As previously described in detail [18], we have used the MBT-2 mouse model to assess the efficacy of HF as a possible anti-neoplastic agent.

2.1. Animals

Inbred 8-10-week old female C3H/eb mice, obtained from the animal facility, Sackler School of Medicine, Tel-Aviv, Israel, were

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housed at a temperature of 22-24°C in 50-70% humidity with a 1014hr dark/light cycle.

2.2. Tumor

The MBT-2 was originally derived from Urothelial cell carcinoma of the bladder induced by oral administration of N-{4-(5-nitro-2-furyl)-2-thiazolyl} formamide (FANFT), a potent urinary bladder carcinogen [18]. The tumor was maintained *in vivo* by serial subcutaneous implantations into the backs of C3H/eb mice, as well as in tissue culture in RPMI-1640 medium containing10% fetal calf serum supplemented with penicillin (100 units/mL) and L-glutamine (300 mg/L). Following serial implantations, the original T5 tumor became more aggressive and was designated T50 [20].

2.3. Tumor cell implantation

Preparation of single cell suspensions from subcutaneous tumors was performed by mincing the fresh tumor under aseptic conditions and adding RPMI-1640 medium to the minced tissue. This was followed by filtration through a 200 μm nylon mesh in order to obtain single cells. The number of viable cells was determined by Trypan blue exclusion. The procedure was basically performed as described by Soloway and Masters [21] with minor modifications. For implantation of tumor cells, mice were anaesthetized with subcutaneous injection of sodium pentobarbital (70 mg/kg body weight). A 24 gauge Teflon IV catheter was introduced into the bladder transurethrally. A total of 5×10^6 viable tumor cells in 0.05-0.1 mL were delivered into the bladder through the catheter (there was no need for thermal cauterization). The mice remained anesthetized for another 45-60 minutes to prevent voiding of tumor cells.

2.4. Drug

HF, a small molecule halogenated quinazolinone alkaloid, was kindly provided by Collgard Biopharmaceuticals Ltd. (Petah Tikva, Israel). Vials of HF were supplied as a drug solution containing 50 mg HF dissolved in phosphate-buffered saline (PBS).

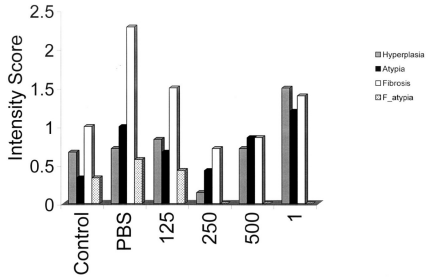

Fig. 1. Type and extent of histologic changes of the mice bladder wall according to the treatment group.

2.5. Study design

After obtaining approval of the ethical committee of the animal's facility (Approval number: 11-02-48) the study was undertaken in two phases (Fig. 2):

Phase I: Assessment of HF toxicity to normal urothelium; 60 mice were divided into 6 treatment groups each consisting of 10 animals: five groups were treated by six intravesical administrations of the following agents: PBS-(group 1), HF 125 μg-(group 2), HF 250 μg-(group 3), HF 500 μg-(group 4), HF 1000 μg-(group 5). The last group received no treatment at all (group 6).

Evaluation of local toxicity was carried out in a blind fashion and included the following variables: epithelial hyperplasia, mucosal atypia, submucosal fibrosis and fibrosis/atypia (mucosal erosion with submucosal inflammatory response). Each one of these variables was graded 0 or 1 for absence or presence, respectively, and whenever present, on a 3 grades scale depending on the severity of the histologic findings.

Phase II: In the second stage we evaluated animals which were implanted with MBT-2 cells. Animals were divided into four treatment groups. Group I ($n = 30$) received no treatment and served as the control. Group II ($n = 32$) was treated with six intravesical instillations of PBS every other day starting two days after tumor cell implantation. Group III ($n = 32$) received 6 doses of 250 μg oral HF every other day as a supplement to the drinking water. Group 4 ($n = 32$) was treated with 6 intravesical instillations of 250 μg HF.

All treatments were carried out under light pentobarbital anesthesia and the various agents were inserted into the bladder via a 24-gauge Teflon catheter. Upon completion of the treatment, the animals were sacrificed via IV KCl injection; the bladders were removed and weighed, and processed for histology. The slides were examined in a blind fashion by the study pathologist.

2.6. Statistical analysis

Data are presented as mean values ± standard error of the mean (SEM). Our findings are based only on results obtained from animals in which successful tumor implantation was observed during histologic examination. Statistical analysis was performed according to unequal variance unpaired t-test. P-values ≤ 0.05 were considered statistically significant.

3. Results

Phase I: Assessment of HF toxicity: This phase included 50 mice treated with intravesical instillations of PBS or HF. All treated animals did not show any clinically demonstrable side effects. Mild reactive urothelial changes were observed on microscopic examination of the harvested bladders with no significant differences between the various groups. The detailed histological changes of the bladders subjected to the various treatment regimens are summarized in Fig. 1. It is evident that the control group had a similar degree of reactive changes compared with the drug treated animals which had mild to moderate drug effect on the bladder wall. The bladder weight of the animals was not affected by the treatment and remained similar to the untreated control group.

Phase II: Evaluation of HF anti-tumor activity. This phase included 126 C3H/eb female mice divided into 4 treatment groups. As shown in Table 1, the average weight of bladders, which reflects the anti-neoplastic activity of the drug, was significantly lower in mice treated with HF (orally or intravesically) compared with animals that were managed with PBS or received no treatment.

Oral administration of HF resulted in somewhat lower, but statistically insignificant mean bladder weight compared with intravesical instillations: 38.06 mg ± 4.02 (SEM) vs 39.65 mg ± 5.97, respectively ($P = 0.819$).

Animals of the control and PBS groups had higher mean bladder weight of 88.86 mg ± 15.58 and 81.28 mg ± 13.79, respectively.

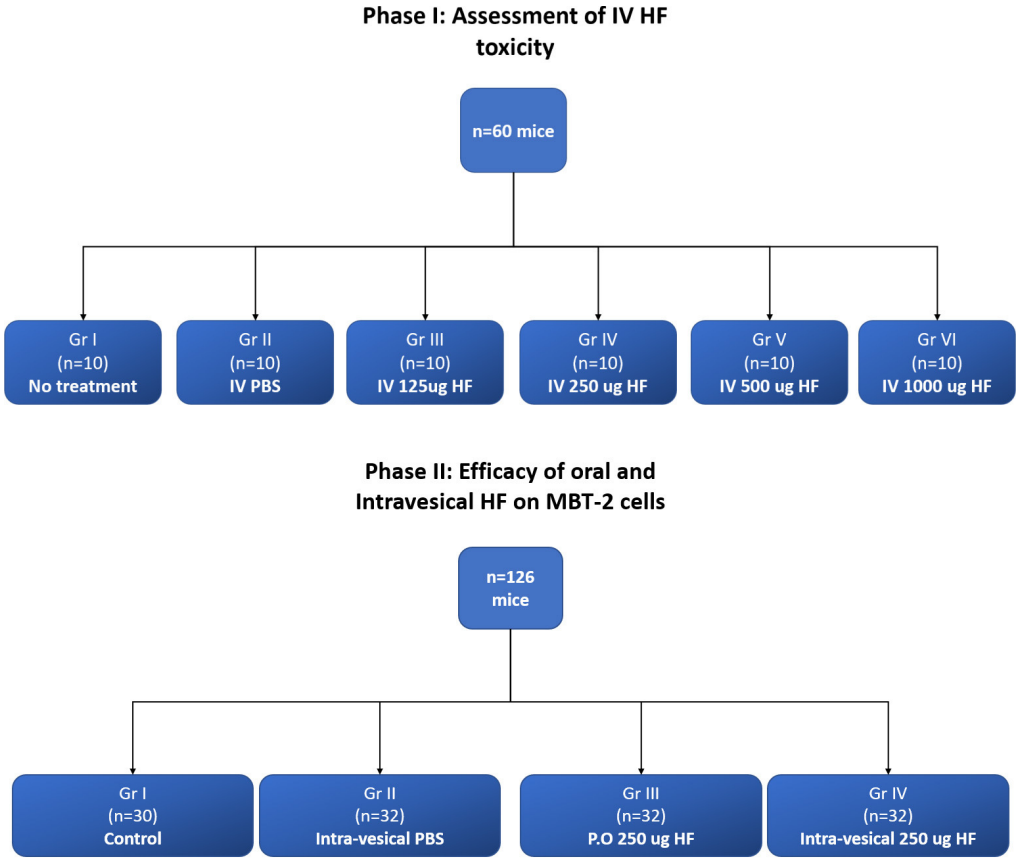

Fig. 2. Summary of the experimental design and treatment groups.

Table 1. Effect of Halofuginone on MBT-2 tumor weight in C3H/eb female mice following intravesical and oral administration

Tx. Group	No. mice	Mean bladder weight	*P* value compared to control
Control	30	88.86 mg (± 15.58 SEM)	1
PBS	32	81.28 mg (± 13.79 SEM)	0.75
Oral – HF	32	38.06 mg (± 4.02 SEM)	0.007*
IV – HF	32	39.65 mg (± 5.97 SEM)	0.003*

These differences were found to be statistically significant with P values of 0.003 and 0.007 for oral and intravesical installation treatment, respectively. When considering the untreated animals, those treated with intravesical and oral HF had statistically significant lower mean bladder weight (Δ 49.21 mg and Δ 50.8 mg, respectively).

4. Discussion

The natural history of NMIBC is characterized by high recurrence rate even after maintenance intravesical therapy, with up to 80% recurrence and 20% progression of the disease [3]. The high recurrence rate reflects the chronic nature of this malignancy and one cannot expect to manage it successfully by episodic treatments. Like similar chronic medical conditions (e.g. hypertension or diabetes) that require daily medications, we believe that bladder cancer patients may benefit from continuous long-term drug exposure.

The standard intravesical route is inconvenient and expensive which emphasizes the need to search for alternative modalities of treatment which will overcome these limitations. The ideal mode of treatment for patients with NMIBC would be daily oral administration of a potent anti-neoplastic agent with minimal side effects, limited drug interactions and adequate urinary concentration. Apart from the inconvenience of intravesical therapy, oral administration has several additional advantages including continuous and not intermittent effect on the target tissue, it affects the entire urinary tract and not only the bladder, anti-neoplastic bioactivity can take place systemically and not just on bladder mucosa and submucosal layers which is beneficial in all stages of bladder cancer. Additionally, an orally administered drug may be given immediately prior to and after an excisional procedure to reduce the undesired phenomenon of post-operative tumor cell implantation. HF hydrobromide is a small molecule quinazolinone hydrobromide salt (TempostatinTM) derived from Febrifugine, an alkaloid originally isolated from the plant, *Dichroa febrifuga*. HF is used worldwide as an antiprotozoal agent that acts against *Coccidia* parasites predominantly in commercial poultry production [22].

Previous *in vitro* studies have demonstrated that HF displays a potent anti-neoplastic activity against bladder cancer and other malignancies. This has been carried out by several mechanisms

including anti-angiogenic, anti-invasive and anti-proliferative activity [16, 24–27]. The mechanism by which HF suppresses urothelial cell carcinoma (UCC) invasion and tumor progression was studied by Elkin at al., who reported a significant inhibition of matrix metalloproteinase-2 (MMP-2) gene expression at the transcriptional level by HF [16]. MMP-2 (Collagenase type IV) is an extracellular matrix degrading enzyme which plays a critical role in tumor invasion and metastasis [17]. Furthermore, in the study of Elkin et al., it was shown that HF-treated bladder carcinoma cells (5637) failed to invade through reconstituted basement membrane (Matrigel)-coated filters. Another finding in that study was the ability of HF to reduce the number of lung metastases of MBT2 cells. This was noted following HF-treatment as compared with the high metastatic activity exhibited in animals managed by control untreated cells [16].

In a different study Elkin *et al.*, investigated the effect of HF on transplantable and chemically induced mouse bladder carcinoma [23]. In both models, oral administration of HF resulted in a profound anticancer activity. Histological examination of the tumor tissue revealed a marked decrease in blood vessel density (reduction in the angiogenesis) and in both collagen $\alpha 1$ and H19 gene expression. No systemic toxicity was observed in mice receiving HF.

We have previously used the MBT-2 model to evaluate the safety and efficacy of repeated intravesical administration of various agents in NMIBC, with promising results and minimal toxicity [15]. This model is used very often as an initial step for evaluation, tolerability, toxicity and efficacy of various anti-neoplastic agents. Using this model, we were able to demonstrate a statistically significant reduction in tumor weight both in the intravesically and orally administrated HF.

The first aim of our study was to assess the local toxicity of HF to the bladder wall. As shown in Table 1, administration of increasing doses of HF did not result in a significant damage to the bladder wall as demonstrated in the histologic examination for the treated bladders.

In the second stage of the current study we were able to show that both oral and intravesical treatment with HF results in a statistically significant reduction in the mass of the bladder tumors. These results demonstrate that oral drug administration can exert similar anticancer activity when compared with the intravesical route.

Oral treatment with HF was used in a phase I clinical trial reported by De Junge and colleagues. This agent was given orally once or twice daily to patients with advanced solid tumors. Dose-limiting toxicity was nausea, vomiting and fatigue which was controlled by treatment with anti-emetics of the $5HT_3$ receptor antagonist family. Less frequent reported side effects included Gastrointestinal bleeding events which necessitate caution. Pharmacokinetics studies revealed a relatively long half-life of HF of approximately 30h, which results in accumulation of effective levels of HF in the tissue [26].

Based on the data presented and other initial reports in early clinical trials it seems that HF may be considered for oral administration in urinary tract malignancies.

In conclusion, intravesical and oral administration of HF is tolerable and efficient for the treatment of bladder cancer in a mouse MBT-2 model. These results may be the basis for future studies on human NMIBC managed by long term daily oral administration.

Acknowledgements

The authors acknowledge the institutional support from the Department of Cell Biology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel.

Conflict of Interest

None of the contributing authors have any conflict of interest, including specific financial interests or relationships and affiliations relevant to the subject matter or materials discussed in the case report.

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