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Teaching an old dog new tricks: The case of Fenbendazole

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ABSTRACT

The objective of this study is the assessment of the cytotoxic effect of fenbendazole and its commercially available formulation, which is used for its antihelmintic properties. The formulation was tested for its efficacy as well as the determination of the ingredients with proliferation assays and analytical techniques. HPLC, LC-MS and NMR confirmed the stated amount of active ingredient on the label. Dissolution studies were performed to simulate the ability of fenbendazole to dissolve adequately in the fluids of the Gastrointestinal tract, be absorbed in the circulation and reach certain areas of the human body. However, dissolution studies showed that both brands possess issues in their distribution. The in vitro drug screening exhibited potential cytotoxic effect in different types of human cancer cell lines and MDA-MB-231 human breast adenocarcinoma cells appeared to be the most sensitive with IC_{50} value lower than 10 μM.

Introduction

Benzimidazoles are heterocyclic, aromatic compounds, which are composed of a six-membered benzene fused to a five-membered imidazole moiety. Benzimidazole analogs have exhibited various pharmacological properties such as antiparasitic, antiviral, antimicrobial, antihistamine, antidiabetic, analgesics, anticancer and the results of their use in biological and clinical studies has been promising [1–8].

Fenbendazole, methyl [5-(phenylsulfanyl)−1H-benzimidazol-2-yl] carbamate, is a broad spectrum anthelmintic used for the treatment of gastrointestinal parasites such as pinworms, tapeworms, hookworms and other parasites in animals and people [9–11]. Fenbendazole has the ability to bind to beta-tubulin [12], disrupt the formation of microtubules in the parasites, leading to their death without causing significant damage to the host [13–15].

A similar mechanism of action is also displayed by some anticancer drugs (vinblastine, vincristine, vinorelbine, paclitaxel, docetaxel), which proposes that fenbendazole could have anticancer activity [16]. Studies on the antitumor effect of fenbendazole have supported this theory [17–19].

The aim of this work was to assess the potential cytotoxic effect of fenbendazole and its most widely used commercial formulation against a panel of human cancer cell lines representing the most common types of cancer. Dissolution and analytical studies were performed in order to identify and quantify the content in active ingredient and determine the availability and the effectiveness of the formulation.

Material and methods

Instrumentation

Dissolution studies

The instrument used for dissolution studies is a USP Apparatus 2, Model RCZ-8B Type Medicine Dissolving Instrument (LTPM, China) attached with paddles. The paddle was rotating at 100 rpm, at 37 °C ± 1 for 90 min.

NMR analysis

The instrument used for NMR analysis is a 400 MHz Bruker Avance spectrometer (AV-III-HD, 400, Rheinstetten, Germany) with the following parameters set: Pulse angle, 30°; pulse width, 41.6 μs; data points 96,152; number of scans, 64; acquisition time (AQ), 3.999 s; spectral width, 12,019.23 Hz. A line-broadening factor of 0.1 Hz was applied to FIDs before Fourier transformation, and the repetition delay was 60 s. Phase and baseline distortions were also applied to automatically correct all spectra, using TOPSPIN (version 3.5pl5, Bruker Biospin, Spring, TX, USA). For DOSY ^{1}H NMR, stimulated echo bipolar gradient pulse experiments were used with a pulse delay of 5 ms after each

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gradient, a pulse-field gradient length of 1 ms and a diffusion delay of 100 ms.

All data were processed with TOPSPIN (version 3.5pl5, Bruker Biospin, Spring, TX, USA) software. The processing parameters were 1024 points along the Laplace spectrum diffusion axis and 20,000 MaxEnt iterations. In the DOSY spectra the horizontal axis represents the chemical shifts (ppm) and the vertical axis the diffusion coefficients ($\mu m^2 s^{-1}$).

HPLC and LC-MS analysis

The instrument used for HPLC and LC-MS analysis is an Agilent 1260 Infinity Series equipped with a MWD detector and a 6120 Series Quadrupole System (Agilent Technologies Inc., Richardson, TX, USA). The mass spectrometry source was electrospray ionization (ESI) with the following parameters: nitrogen as drying gas with a flow rate of 12 L/min at 350ºC, nebulizer pressure at 35 psi and capillary voltage at 3 kV, negative mode operation, scan analysis and a 200–2000 *m/z* scan range.

OpenLAB Chemstation was used to process the chromatograms (version M8301AA, Revision C.01.07 Agilent Technologies Inc., Richardson, TX, USA).

Dissolution test

In order to test in vitro the release profile of the drug, dissolution studies should take place. The effectiveness of a specific pharmaceutical form depends upon its intrinsic ability to dissolve in the fluids of the Gastrointestinal (GI) track. For this study we contacted two different experiments, one in Phosphate Buffer Solution (PBS) pH 6.8 and one in Sodium Dodecyl Sulfate (SDS) Solution 2% in order to compare the dissolution profile.

Samples were collected at 30, 45 and 90 min. The sample size was 0.5 g of the powder, which based on the label claim, is equal to 111 mg of Fenbendazole. Each sample was collected at its time point with a 10 mL syringe and immediately filtered with 0.45 μm PTFE filters. After each sample collection, the solution in the vessel was immediately filled with 10 mL of plain buffer in order to remain the balance within.

NMR analysis

5 mg of fenbendazole were added (analytical standard and commercial formulations) to 0.5 ml of deuterated solvent (DMSO-d^6, Eurisotop, MA, U.S.A.). 5 mm NMR tubes were used.

HPLC and LC-MS analysis

Fenbendazole analytical standard was purchased from Sigma-Aldrich (Louis, MO, USA). Sodium dihydrogen phosphate dehydrate and hydrochloric acid were also obtained from Sigma-Aldrich (Taufkirchen, Germany), methanol for HPLC was purchased from Macron Fine Chemicals (Arnhem, The Netherlands). LC-MS grade water and acetonitrile were purchased from Fisher Scientific (Pittsburgh, USA). Packets of two commercial brands of fenbendazole powder were purchased in order to proceed with the analysis (Fig. 1 and Fig. 2).

For HPLC and LC-MS analysis, fenbendazole analytical standard and samples of three different LOT numbers from the two brands of fenbendazole powder were dissolved in hydrochloric methanol (0.4% v/v HCl) and filtrated when necessary through a 0.45 μm nylon filter.

All samples were analysed by using a column (Zorbax Eclipse RP C18 reverse-phase, 250 mm x 4.6 mm I.D., 5 μm, Agilent, Santa Clara, CA, USA) at 20 °C. Mobile phase (A) was 0.01 M sodium phosphate dihydrate, and mobile phase (B) acetonitrile. (A) and (B) were mixed at a 30:70 (v/v) ratio at the start of the gradient and switching at 10 min to a 70:30 (v/v) ratio. Flow rate was 1.0 ml/min, injection volume was 10 μL, the autosampler temperature was maintained at 15 °C and ultraviolet (UV) detection was carried out at 298 nm. The time period of analysis was 10 min.

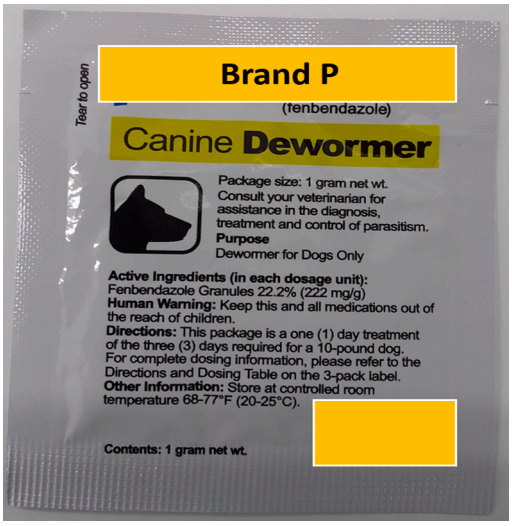

Fig. 1. Fenbendazole powder, commercial Brand P (panacur®C).

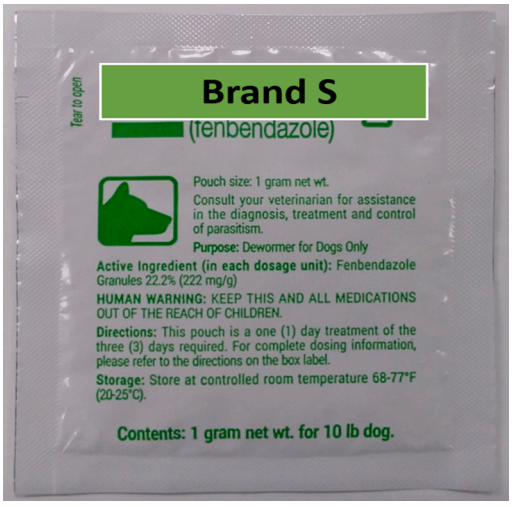

Fig. 2. Fenbendazole powder, commercial Brand S (safe-guard®4).

Cell culture

A panel of seven different commercial cancer cell lines was used in the present study that were purchased from ECACC (European Collection of Authenticated Cell Cultures) (Salisbury, UK) and ATCC (American Type Culture Collection) (Manassas, VA, USA). MCF7 human breast adenocarcinoma cells (luminal type) (ECACC 86,012,803), MDA-MB-231 human breast adenocarcinoma cells (triple negative) (ECACC 92,020,424), HCT116 human colorectal carcinoma cells (ECACC 91,091,005), COLO699N human lung cancer cells (ECACC 93,052,608), PC3 human prostate adenocarcinoma cells (ECACC 90,112,714), A375 human malignant melanoma cells (ECACC 88,113,005) and HuNS1 human multiple myeloma cells (ATCC CRL-8644) were cultured in RPMI 1640 supplemented with 10% FBS and 2% L-Glutamine. RPMI 1640 Catalog#R0883 and L-glutamine Catalog#G7513–100ML were obtained from Sigma-Aldrich, Darmstadt, Germany and FBS Catalog#FB-1001/500 was obtained from BioSera, Nuaille, France. All cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere and passaged when cells reached 80% confluence.

Exposure to increasing concentrations of samples tested

Viable cells were seeded at a density of 2×10^4 (200μl/well) in 96-

well plate and incubated at 37°C and 5% CO_2 for 24 h to form a cell monolayer. After 24 h of incubation, supernatant on the monolayer was discarded and 200μl of culture medium and varying concentrations (0.001 μM, 0.01 μM, 0.1 μM, 1 μM, 10 μM) of the analytical standard of fenbendazole and the two commercial powders (Brand P and Brand S) were added and incubated for 24, 48 and 72 h time points. Drugs were diluted in Dimethyl sulfoxide (DMSO) for the preparation of stock solutions before their dilution at the predetermined concentrations in culture media.

Cell proliferation assay

AlamarBlue assay was performed to assess cell proliferation. After the specific time points that cells were treated with the various concentrations of the three samples, 20 μl of 0,15 mg/ml resazurin sodium salt Catalog#199,303 (Sigma-Aldrich, Darmstadt, Germany) in PBS was added to each well and incubated for 3 h at 37 °C and 5% CO_2. Fluorescence was measured at 560 nm excitation and 590 nm emission wavelength. The experiment was performed in triplicates. The average fluorescence was calculated for each triplicate.

Statistical analysis

One sample *t*-test was used to determine differences in the mean by comparing the treated samples with the untreated controls. P values < 0.05 were considered to indicate a statistically significant difference. Results were calculated using the Microsoft Excel 2016. The measurements from the proliferation assay were used to determine the IC_{50} values by using the Microsoft Excel 2016.

Results

Dissolution test

The initial attempt was conducted by using Phosphate Buffer Solution. The powder was dispersed almost immediately at the beginning of the experiment but due to its electrostatic nature, it was partially attached to the paddle. The buffer in the vessel was a white color during the whole procedure and a remarkable quantity was left undiluted. This combined with the fact that the sample size significant lower of the recommended, gave a prediction of poor dissolution profile from a visual perspective. The samples were analyzed and quantified through HPLC and gave low results of dissolution for fenbendazole. Six different samples were tested in total, three batches from Brand P and three batches from Brand S. Phosphate Buffer Saline was used as reference solution.

Sodium Dodecyl Sulfate (SDS) Solution 2% is a solution often used in cases where dissolution appears to be rather weak. The critical micelle concentration (CMC) of SDS in RT conditions is 8.2 mM and in our case the SDS concertation is significantly higher than this. However, this solution did not appear to be helpful adequately. Visually, the solution seemed to be cloudy and non-clear through the whole experiment but more homogenous comparing to the previous conditions. Based on the calculated results, it is observed that dissolution was done in stages, since at the first minutes of the dissolution the quantities were lower and then started to increase slightly. However, the percent of the dissoluted compound was low even after 90 min.

To be more specific, Table 1 describes the observed results from the PBS Dissolution experiment and Table 2 from 2% Sodium Dodecyl Sulfate (SDS) Solution.

NMR analysis

Deuterated DMSO was chosen to run a ^1H and DOSY NMR on fenbendazole analytical standard and commercial brands in order to observe better the solubility and content of the samples. The chemical shifts, as well as the spectrum are presented below (Fig. 3).

^1H NMR (DMSO-d^6): δ 3.76 (s, 3H, $COOCH_3$), 7.11, 7.13, 7.14 (m, 2H, 2′-H and 6′-H), 7.20, 7.18, 7.16 (m 2H, 4′-H and 6–H), 7.26, 7.28, 7.30 (m, 2H, 3′-H and 5′-H), 7.44, 7.46 (d, 1H, 7-H), 7.52 (s, 1H, 4-H), 11.73 (bs, 2H, N—H, 1-H and 1″-H deuterium oxide exchangeable).

The DOSY spectra reveal that both commercial products contain, apart from the active ingredient fenbendazole, other ingredients, possibly excipients that usually appear at the 1–6 ppm region (S1-S3).

HPLC and LC-MS analysis

The method used for HPLC and LC-MS analysis was validated according to ICH guidelines and the validation parameters are presented in the table below (Table 3).

Three different LOT numbers from each commercial brand were analysed by the validated HPLC method and fenbendazole was identified and quantified (Table 4). Its molecular weight was confirmed by LC-MS (S4-S6 and S7-S9).

Effects of various concentrations of fenbendazole analytical standard, Brand P and Brand S on the proliferation of cancer cell lines

To examine the effect of the three samples (analytical standard, Brand P and Brand S) on cellular proliferation, the mitochondrial activity was measured with AlamarBlue assay. According to the results from the specific assay, IC_{50} values were calculated for each human cancer cell line (Table 5).

Discussion

The need for effective anticancer drugs is vital and for this reason drug repurposing has gained ground the recent years [20]. In the present work, two commercial brands of fenbendazole were tested in order to confirm the presence and concentration of this active ingredient in the formulation and its possible cytotoxic activity on seven types of cancer cell lines.

Dissolution test is one of the most vital tests for oral dosage forms. Due to different formulation steps, the addition of various ingredients and agents, the release of a drug can be significantly altered. Dissolution is a powerful tool for scientists in order to evaluate the amount of the released drug in vitro and predict its behavior within the body. The most usual way to examine if a drug meets or not the established standards, is

Table 1
Percentage of the dissolved fenbendazole from each sample, for the three different time points. Phosphate Buffer Saline pH 6.8 was used as Dissolution Media.

Time Point	Brand P			Brand S		
	LOT A515A01	LOT A495A01	LOT 458A01	LOT A522A01	LOT A481A02	LOT A503A01
30	83.3%	86.4%	44.3%	79.2%	74.2%	67.0%
45	77.4%	74.7%	49.4%	76.2%	51.8%	57.5%
90	55.0%	60.7%	38.9%	58.6%	28.9%	52.3%
RSD%*	30min	21.25				
	45min	20.16				
	90min	25.49				

*between Brand P & Brand S.

Table 2
Percentage of the dissolved fenbendazole from each sample, for the three different time points. Sodium Dodecyl Sulfate (SDS) Solution 2% was used as Dissolution Media.

Time Point	Brand P			Brand S		
	LOT A515A01	LOT A495A01	LOT 458A01	LOT A522A01	LOT A481A02	LOT A503A01
30	12.0%	12.0%	10.3%	10.5%	11.5%	11.4%
45	14.7%	20.4%	13.6%	20.2%	14.5%	14.9%
90	15.7%	26.5%	16.1%	26.6%	15.4%	15.3%
RSD%*	30min	6.47				
	45min	18.72				
	90min	29.32				

*between Brand P & Brand S.

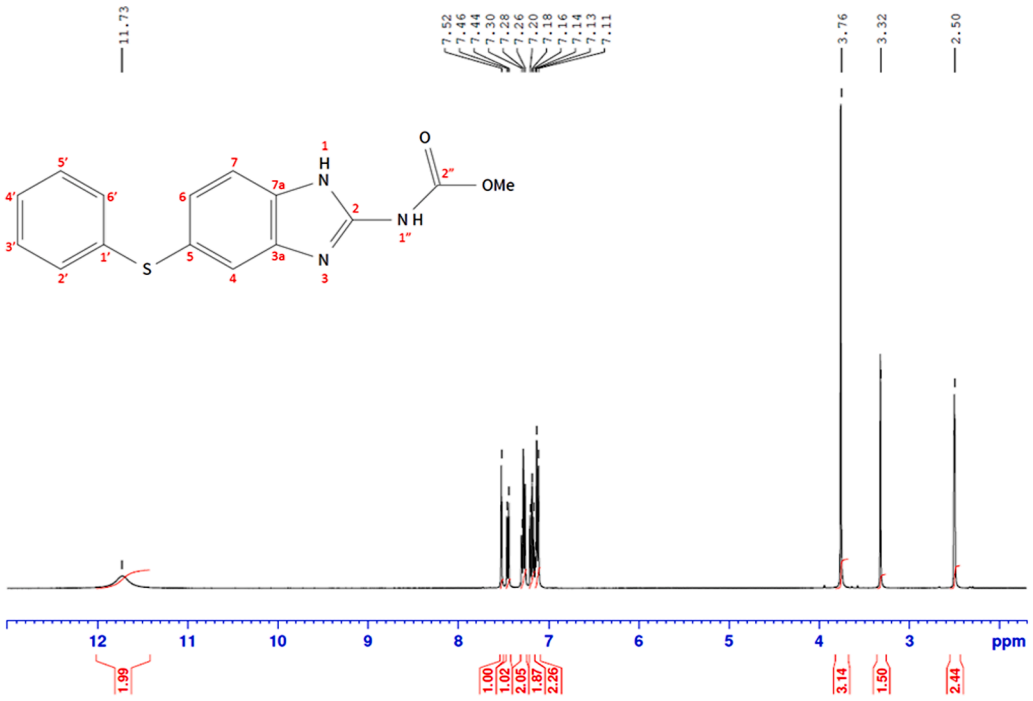

Fig. 3. ^{1}H NMR spectrum of fenbendazole in DMSO-d_6.

Table 3
Validation parameters for the determination of fenbendazole by HPLC.

	HPLC		
System suitability			
Theoretical plates	11,758		
Symmetry factor	0.84		
%RSD	0.60		
Linearity			
Slope	26.5		
Intercept	−7.5		
R^2	0.9998		
Concentration range (ppm)	6.25–18.75		
Robustness	RT	Plate count	Peak symmetry
Column temperature (18 & 22°C)	4.64	11,340	0.80
	4.60	11,949	0.82
Flow rate (0.5 & 1.5 ml/min)	11.01	8354	0.89
	2.91	8520	0.87
Precision	Intra-day		Inter-day
Mean peak area	309.97		318.03
%RSD	0.50		0.59
Accuracy			
Number of solutions analyzed	18		
Concentration range (ppm)	6.25–18.75		
Mean recovery ±%RSD	100.02 ± 0.30		
LOD (ppm)	0.2		
LOQ (ppm)	0.6		

Table 4
Quantity of fenbendazole in commercial formulations.

Brand Name	LOT Number	Quantity on the label (mg in 1 g powder)	Quantity found (mg)
Brand P	A515A01	222	216.8
	A495A01	222	216.4
	A458A01	222	217.2
Brand S	A522A01	222	217.8
	A481A02	222	217.2
	A503A01	222	218.8
RSD%*		–	0.39

*between Brand P and Brand S.

to compare if the results of the dissolved compounds agree with the monograph requirements. Unfortunately, in this case, in the European Pharmacopoeia monograph for fenbendazole for veterinary use there is not a reference for dissolution conditions, thus we compare the drug with some general accepted values [21].

We observed that between the two different brands examined, some significant points were standing out. In the case of PBS, the maximum amount was released initially at the first 30 min for both brands. However, the results from the next time points show that dissolution is not homogenous and that the solution has undissolved pieces. Although the two brands present significant differences, both oppose weaknesses.

Table 5
IC_{50} values with AlamarBlue assay of fenbendazole analytical standard, Brand P and Brand S against MCF7, MDA-MB-231, HCT116, PC3 and A375 at 24 h, 48 h and 72 h ("NS" - No Significant, no effect on COLO699N and HuNS1).

AlamarBlue		Analytical Standard Fenbendazole	Brand P	Brand S
24h	**MCF7**	12,93 μM ($p = 003$)	13,67 μM ($p=<0.0001$)	12,67 μM ($p=<0.0001$)
48h		9,41 μM ($p = 0.005$)	9,39 μM ($p = 0.001$)	9,32 μM ($p = 0.004$)
72h		8,05 μM ($p = 0.007$)	7,95 μM ($p = 0.01$)	7,78 μM ($p = 0.01$)
24h	**MDA-MB-**	NS	NS	NS
48h	**231**	8,71 μM ($p = 0.01$)	8,76 μM ($p = 0.01$)	8,73 μM ($p = 0.01$)
72h		6,87 μM ($p = 0.005$)	6,74 μM ($p = 0.004$)	6,32 μM ($p = 005$)
24h	**HCT116**	15,69 μM ($p = 0.0006$)	15,18 μM ($p = 0.0001$)	14,73 μM ($p = 0.001$)
48h		12,89 μM ($p = 0.02$)	11,95 μM ($p = 0.02$)	13,39 μM ($p = 0.02$)
72h		11,86 μM ($p = 0.01$)	11,49 μM ($p = 0.007$)	11,56 μM ($p = 0.01$)
24h	**PC3**	22,62 μM ($p = 0.004$)	24,62 μM ($p = 0.002$)	20,86 μM ($p = 0.0005$)
48h		15,31 μM ($p = 0.009$)	14,95 μM ($p = 0.006$)	14,29 μM ($p = 0.01$)
72h		12,9 μM ($p = 0.002$)	13,61 μM ($p = 0.003$)	11,83 μM ($p = 0.002$)
24h	**A375**	18,41 μM ($p = 0.01$)	18,28 μM ($p = 0.009$)	20,96 μM ($p = 0.01$)
48h		12,72 μM ($p = 0.03$)	12,60 μM ($p = 0.03$)	12,88 μM ($p = 0.02$)
72h		14,03 μM ($p = 0.01$)	14,27 μM ($p = 0.009$)	11,31 μM ($p = 0.009$)

This is why a second buffer was used in order to compare the results. Since fenbendazole is hard to dissolve, the addition of a surfactant would enhance its solubility. However, it was proved that SDS solution was not helpful. The release of the compound was increasing after each time point; however, it hardly meets a percentage over 20%. Between the two brands, the difference is not so significant especially at the initial stages and in both cases; the amount of the released drug is particularly low even after several minutes. It is vital to mention at this point, that the products used for this study were veterinary medicines and not for human use.

Qualitative analysis by NMR provided useful information on the solubility and content of the two commercial brands, Brand P and Brand S. The ^{1}H and DOSY NMR experiments show that fenbendazole is soluble in dimethyl sulfoxide and that the brands contain this active ingredient as well as other, possibly excipients, although they are not mentioned on the products labels.

To calculate the quantity of fenbendazole in samples taken from three different batches from each brand, a quick, simple, robust, time and cost effective HPLC method was developed and validated. The results showed that the quantity measured was in agreement with the quantity mentioned on the labels. Mass analysis confirmed the presence of the active ingredient in the formulation.

The in vitro AlamarBlue assay results have exhibited that both commercial Brand P and Brand S had similar effect on the proliferation of the various human cancer cell lines. The IC_{50} values had in all cases been reduced with the duration of the experiments, highest IC_{50} values at 24 h and lowest IC_{50} values at 72 h. Two types of cells, the COLO699N human lung adenocarcinoma and HuNS1 human multiple myeloma appeared to be highly resistant, and the three samples (fenbendazole, Brand P and Brand S) had no statistically significant effect against them. Three types, HCT116 human colorectal carcinoma cells, PC3 human prostate adenocarcinoma cells and A375 human malignant melanoma cells exhibited similar IC_{50} value for all samples and it was around 11 μM. The two human breast adenocarcinoma cell lines, MCF7 (luminal b) and MDA-MB-231 (triple negative), appeared to be more sensitive against the three samples and the triple negative MDA-MB-231 cell line had the lowest IC_{50} value that was around 6 μM, while for the luminal b MCF7 cell line was around 8 μM.

Interestingly, human breast adenocarcinoma cell lines appeared to be the most sensitive among all the different cancer cell types that had been tested. The most significant effect was observed against MDA-MB-231 cell line that was a triple negative human breast adenocarcinoma cell line, while the MCF7 human breast adenocarcinoma cell line was positive for estrogen (ER) and progesterone (PR) receptors. MDA-MB-231 cells were more Warburg type relying on glycolysis of ATP under normoxic and hypoxic conditions. MCF7 cells were more Pasteur type relying on ATP production from oxidative phosphorylation at normoxic conditions and increased their glycolytic activity under hypoxia. Phenotypically, MDA-MB-231 cells were more mesenchymal and multidrug resistant while MCF7 cells were more epithelial [22]. It was not completely understood but there was a specific feature at the phenotypic, genotypic or metabolic differences between the two cell lines, which made MDA-MB-231 cells more sensitive to fenbendazole.

It was clear that depending on the cancer type, cells were more or less sensitive to the three samples. When the comparison was made between the compounds, commercial Brand S exhibited greater cytotoxic effect against most of the human cancer cell lines. The presence of the active substance (fenbendazole) was confirmed with the analytical techniques for both commercial Brand P and Brand S products, but when tested for human use against human cancer cells, the result was that Brand S had a more significant effect than Brand P.

Conclusion

The present study reveals that fenbendazole and its commercial formulation, which is used as an antihelmintic drug, although having formulation issues that hinder the distribution in the human body, exhibit cytotoxic effect against different types of human cancer cell lines. The results from all of our tests suggest that further research needs to be carried out on this active ingredient that could possibly lead to the development of a potential anticancer drug.

Disclosure

The authors report no conflicts of interest in this work.

The samples of the two commercial brands of fenbendazole (panacur®C and safe-guard®4) were purchased to perform the study.

Consent

No studies on patients or volunteers have been performed.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ctarc.2022.100601.

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