Synergistic effects of Metformin and Captopril on C. elegans

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Summary
There are over 400 million patients suffering from type 2 diabetes and over 1 billion suffering from hypertension. Pharmaceuticals commonly used by these patients include Metformin for type 2 diabetes and Captopril for hypertension. Considering that the two diseases are interrelated, and can both be seen in an individual, Metformin and Captopril take part in combination therapies for many people. However, the synergistic effects of these active ingredients have not been previously documented. This study examines the effects of Metformin and Captopril using model organisms called Caenorhabditis elegans nematode worms. Concentrations of Metformin were 25mM, 50mM and 100mM, while those of Captopril were 2mM, 4mM, 6mM and 20mM. The combination group contained Metformin and Captopril synchronised as 25mM-2mM, 50mM-4mM, and 100mM-6mM, respectively. The independent variable is the concentration of active ingredients, whereas the dependent variable is the change in body length of C. elegans nematodes. The experiment involved the picking of the worms from the containing petri dishes under a stereomicroscope and measuring their body lengths with the software of a digital microscope. The results showed up to 25% decrease in average body lengths of the worms exposed to Metformin solely. Captopril caused 5% decrease in average body length of the worms. When Metformin and Captopril were combined, an increase of up to 11% in average body length was recorded. C. elegans body size is regulated by the TGF-β signal pathway, and the possible outcomes of the manipulation of body length by Metformin and Captopril are further discussed.

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Introduction
According to the latest data of the World Health Organisation, there are over 400 million people suffering from type 2 diabetes (1). In addition, as of year 2000, there exist approximately 1 billion people with hypertension, and this alarming number is projected to increase to almost 1.5 billion by 2025 (2). These diseases are known to share common metabolic pathways (3). As expected, there are numerous drugs aimed to improve the prognosis of individuals with these diseases. For type 2 diabetes and hypertension, the widely used active therapies are Metformin and Captopril, respectively. These active ingredients, apart from targeting these specific diseases, may have other effects on model organisms that can shed light on possible human related changes. Metformin, for example, was observed to decrease the average body lengths of Caenorhabditis elegans nematode worms (4). Such effects observed on C. elegans are indicators of several changes that may occur in the human body. The reliability of the connection between C. elegans and H. sapiens is further supported by the fact that the genomes of the two organisms are 40% homologous (5). C. elegans is widely used as a model organism in research (Figure 1), as it is a unique organism that allows the expression of particular results in relationship with human systems. It is suggested that the reduction in the average body lengths of C. elegans nematode worms is directly related to Metformin’s cancer- and tumour-suppressing features (4). Thus, the changes in body length of C. elegans worms treated with Metformin suggest that body length may be an appropriate readout for learning about drugs’ effect on cancer.

This study focuses on the effects these active...
ingredients exhibit on *C. elegans*, to propose assumptions regarding possible effects on humans. At the molecular level, the changes in body length of *C. elegans* worms is regulated by the TGF-β signal pathway (6, 7). In humans, Metformin suppresses the TGF-β1 pathway (8). Considering the two aforementioned sources, it can be stated that Metformin reduces the average body lengths of *C. elegans* worms by targeting and suppressing the TGF-β pathway. TGF-β1 is an isofrom of TGF-β, so comparisons between *C. elegans* and *H. sapiens* can be considered appropriate. Malfunctions in the TGF-β signal pathway also lead to the development of various types of cancers (9). In gastric cancer cases, human TGF-β1 outputs increased activity (9). Moreover, TGF-β signals also trigger metastasis in cancer cases (10). In light of all the information, we can conclude that the TGF-β signal pathway regulates *C. elegans* body size, that Metformin may suppress this signal pathway, and that the cancer- and tumour-suppressing effect of Metformin may be attributed to its function of suppressing TGF-β activity. In addition, Captopril, the other active ingredient that has been tested in this research project, was found to increase TGF-β1 signal pathway activity unlike Metformin (11). Therefore, Metformin and Captopril are shown to have antagonistic effects.

The individual effects of Metformin and Captopril on *C. elegans* have been laid out by various papers, but the effect these active ingredients will have on *C. elegans* when combined has not been documented. Patients suffering from type 2 diabetes are highly prone to also developing hypertension, so it is very likely that these individuals will be prescribed multiple drugs that contain Metformin and Captopril. In this case, will the cancer- and tumour-suppressing effect of Metformin be negated by the antagonistic Captopril?

We hypothesized that if Metformin and Captopril are applied together on *C. elegans*, then the body length-decreasing effect of Metformin on *C. elegans* will be negated. Thus, the purpose of this research project can be listed as follows: 1) To examine the effects of separate administration of Captopril and Metformin on the average body size of *C. elegans* nematodes, and 2) to examine the effects of combined therapies of Metformin and Captopril on the average body size of *C. elegans*.

**Results**

We measured the body lengths of the 10 nematodes from each experimental group (Figure 2). The group

![Figure 2. Average body lengths of the nematodes in the control group to different concentrations of Metformin and Captopril.](image)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Metformin 25mM</th>
<th>Metformin 50mM</th>
<th>Metformin 100mM</th>
<th>Captopril 2mM</th>
<th>Captopril 4mM</th>
<th>Captopril 6mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.240 ± 0.03668</td>
<td>1.064 ± 0.02458</td>
<td>1.049 ± 0.03416</td>
<td>1.028 ± 0.04158</td>
<td>1.063 ± 0.02683</td>
<td>1.179 ± 0.03291</td>
</tr>
</tbody>
</table>

*p = 0.0008* for Metformin 25mM; *p = 0.0013* for Metformin 50mM; *p < 0.0001* for Metformin 100mM; *p = 0.0011* for Captopril 2mM; *p = 0.2273* for Captopril 4mM; *p = 0.1324* for Captopril 6mM.
that contained Captopril at 20mM concentration unexpectedly had no nematode worms in the petri dishes; some possible reasons will be discussed later.

Metformin decreased the average body lengths of the worms (Figure 2A-C). This result is comparable to that of Wu and colleagues (4). All tested concentrations of Metformin yielded statistically significant results, as calculated by t-test ($p < 0.05$). On the other hand, Captopril decreased the average body lengths at the lowest concentration (2mM, Figure 2D), but as the concentration increased, average body lengths of the worms also increased. There was a decrease of 0.006 millimetres going from 4mM to 6mM (Figure 2E), indicating that the higher concentration led to decreased average body lengths. However, the only statistically significant result in groups of Captopril was obtained in 2mM administration (Figure 2D). The results related to 4mM and 6mM administrations were not statistically significant ($p > 0.05$).

We next treated groups with both Metformin and Captopril (Figure 3). The average body length decreased in the first group (Metformin 25mM + Captopril 2mM) compared to the control group, but the other two groups reflected an increase of up to 11% in average body length compared to the control group (Figure 3A). In the second experimental group (Metformin 50mM + Captopril 4mM), average body lengths of the worms increased by 5%, and in the third experimental group (Metformin 100mM + Captopril 6mM, Figure 3C), average body lengths of the worms increased by 11%. Only the third experimental group (Metformin 100mM + Captopril 6mM, Figure 3C) yielded statistically significant results ($p$-value < 0.05). The results obtained from the other two experimental groups were not statistically significant. However, the significant increase of body length in the 100mM Metformin and 6mM Captopril group may suggest a combined effect of Metformin and Captopril (Figure 4).

**Discussion**

For all three concentrations of Metformin administration, the results were comparable to those in reference articles: Metformin exhibits reductions in *C. elegans* body lengths. As also mentioned in the introduction, *C. elegans* body length is regulated by the TGF-β signal pathway, and Metformin is known to suppress TGF-β signal pathway activity (8). TGF-β signal pathway is an important factor to take into consideration regarding cancer development in humans. Therefore, these results may also point out to an anti-cancer effect Metformin may exhibit with reference to TGF-β signal pathway.

Captopril decreased the average body lengths of the nematodes, but as the concentration increased, the average body lengths also increased. There were no worms present in the 20mM administration of Captopril, probably due to the fact that 20mM is too high of a concentration for the worms to successfully maintain homeostasis and metabolism. Furthermore, Captopril has been shown to increase TGF-β1 (an isoform of TGF-β) activity (11). The results from this study, therefore, are comparable to those of Paolo et al. (11). If Captopril exhibited increased average body length in the 2mM administration, the results would match completely.

The third part of this research project consisted of combined administrations of Metformin and Captopril. In the first group (Metformin 25mM + Captopril 2mM), the average body lengths of the worms experienced a decrease of 6.5% compared to the control group. In the second group, (Metformin 50mM + Captopril 4mM), there was a considerable increase in the average body length of 5.5% compared to the control group. In the final group, (Metformin 100mM + Captopril 6mM), a significant

<table>
<thead>
<tr>
<th>Control</th>
<th>Metformin 25mM + Captopril 2mM</th>
<th>Metformin 50mM + Captopril 4mM</th>
<th>Metformin 100mM + Captopril 6mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.240 ± 0.03668</td>
<td>1.165 ± 0.0263</td>
<td>1.306 ± 0.04379</td>
<td>1.379 ± 0.02676</td>
</tr>
</tbody>
</table>

Figure 3. Average body lengths of the nematodes in combined administration groups of Metformin and Captopril. A) Control vs Metformin 25mM + Captopril 2mM; $p = 0.1139$. B) Control vs Metformin 50mM + Captopril 4mM; $p = 0.2623$. C) Control vs Metformin 100mM + Captopril 6mM; $p = 0.0069$. 
increase in average body length of 11.1% compared to the control group was observed. Thus, when the two active ingredients are combined, Metformin’s length-decreasing effect was reversed. On the other hand, Captopril’s partial length-increasing effect increased considerably when combined with Metformin. The overall significant increase in average body lengths of the nematodes in the final group (Metformin 100mM + Captopril 6mM) may be attributed to an increase in the TGF-β activity. An increase in TGF-β activity was shown to promote cancer development, but further connections should be analysed for definite conclusions.

In order to further elaborate on this topic, Metformin and Captopril’s effect on the C. elegans TGF-β signal pathway can be examined at the molecular level. Furthermore, other active ingredients of drugs that target other diseases can be tested on C. elegans and their TGF-β interactions can be researched. Moreover, the combined administrations of Metformin and Captopril can be tested at different concentrations (such as Metformin 25mM + Captopril 6mM instead of Metformin 25mM + Captopril 2mM).

Materials and Methods

The subjects of the experiments were C. elegans nematodes. The independent variables were the concentrations of Metformin and Captopril. The dependent variable was the average body size of the C. elegans nematodes. The standard C. elegans methods were followed (12). For each different concentration of Metformin and Captopril, three experimental setups were prepared, and the results were compared. The details of the materials are listed below.

Nematode Growth Medium (NGM) Preparation

Three different concentrations of Metformin were tested: 25mM, 50mM, and 100mM. These concentration values are the same as those suggested by Wu and colleagues (4). A 10 mL 1M stock solution was prepared by dissolving powdered Metformin in distilled water. 1.6562 g of Metformin was gathered and poured into a 10 mL falcon tube along with 10 mL distilled water. Therefore, a 10 mL 1M stock solution was prepared. In order to integrate the active ingredient into NGM, the amounts of 1M stock solution corresponding to the desired concentrations were mixed with 7 mL NGM in 6 cm petri dishes. For each individual concentration, three separate setups were prepared, so the desired amounts of 1M stock solution was calculated in accordance to 21 mL (7 mL for each petri dish). 525 μL of 1M stock solution was used to prepare Metformin of 25mM concentration. For 50mM and 100mM concentrations, the amount was doubled and quadrupled, respectively.

Captopril, on the other hand, was tested in four different concentrations: 2mM, 4mM, 6mM, and 20mM (13). 2.1729 g of Captopril was gathered and placed into a 10 mL falcon tube. Contrary to Metformin, Captopril did not dissolve in 10 mL distilled water, so the amount of distilled water was doubled, making it 20 mL. The stock solution, therefore was 0.5M instead of 1M. Additionally, the falcon tube was heated to 37 degrees Celsius to ease the dissolution. In order to establish a
concentration of 2mM, 84 µL of 0.5M stock solution was used. The same ration was maintained when preparing 4mM, 6mM and 20mM concentrations. The appropriate amounts of solutions were added into separate falcon tubes containing 21 mL NGM. Afterwards, the liquids in the falcon tubes were mixed with a Vortex mixer. Into each petri dish, 7 mL of NGM mixed with the desired amounts of active ingredients was poured. Micropipettes were used in the course of these processes to ensure accuracy.

Gathering of Nematodes from Stock and Solution Preparation for Nematode Synchronization

C. elegans strains received from Abdullah Gül University were stored in a petri dish for stocking. The worms were detached and transferred to falcon tubes from the broth present in the petri dish with M9 Buffer solution. To prepare 250 mL M9 Buffer solution, 1.45 g Na₂HPO₄·7H₂O, 0.75 g KH₂PO₄, 1.25 g NaCl, 0.0625 g MgSO₄·7H₂O were placed into a 250 mL glass beaker. Distilled water was added to the beaker to raise the solution up to 250 mL. The solution was then filtered with a 0.22µm syringe filter and a syringe. The synchronisation of the nematodes was conducted using the bleach solution, which was freshly-prepared before use. In order to prepare 20% alkaline bleach solution, 7.5 mL 1M NaOH (prepared by mixing 3 g of solid NaOH and 7.5 mL distilled water), 6 mL household bleach, and 16.5 mL distilled water were mixed in a glass beaker. The solution was then separated into two falcon tubes with 15 mL capacity.

Egg Preparation

During the course of the experiments, the nematodes were grown from the egg stage. Therefore, the aim was to retrieve only the eggs of the nematodes from the initial stock of worms. First, 5 mL M9 Buffer solution was added by a micropipette to the petri dishes containing the initial nematode stocks. The petri dishes were mixed gently on a horizontal axis, allowing both the adult worms and the eggs to be released from the broth to the M9 Buffer solution. This solution now containing the adult worms and the eggs was transferred with a micropipette into two 15 mL falcon tubes. The tubes were centrifuged at 2500RCF for 90 seconds, so the adult worms and the eggs settled to the bottom of the tubes. The extra M9 Buffer solution in the tubes was discarded after centrifuge. This process was repeated twice to ensure that the bleach solution was washed away completely off the eggs. Lastly, 7 mL of M9 Buffer was added to the tubes containing the eggs and the tubes were shaken to dislodge the eggs into the solution. To each petri dish that contained NGM and active ingredients, 300 µL of this M9 Buffer and nematode egg suspension was added.

Data Collection

Four days after the planting of the eggs, living nematodes in the petri dishes were picked and placed on microscope slides using a stereomicroscope and platinum wire. The platinum wire was soldered into the broken tip of a glass Pasteur pipette. The end of the wire was burnt in flame before use. The body length of each living nematode was measured using the Motic Educator software (Figure 5). The data collected was analysed with GraphPad Prism 7 software, conducting t-tests.

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References