Pharmacophore

ISSN-2229-5402

Journal home page: http://www.pharmacophorejournal.com

Pharmacophore
an International Journal
ISSN 2229-5402

ANTITUMOR ACTIVITY OF SILVER NANOPARTICLES AND ALPHA-LIPOIC ACID COMBINATIONS IN COLORECTAL CANCER INDUCED EXPERIMENTALLY

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ARTICLE INFO

Received:
18th Oct 2017
Received in revised form:
08th Mar 2018
Accepted:
15th Mar 2018
Available online:
28th Apr 2018

Keywords: *Colorectal cancer, Silver nanoparticales, Alpha lipoic acid, Inflammatio*

ABSTRACT

The present study was conducted to evaluate the antitumor effect of silver nanoparticles and Alpha-lipoic acid and their combination against 1,2-dimethylhydrazine (DMH-) induced colorectal carcinogenesis in experimental rats. Fifty Male wistar albino rats were divided into five groups. Group (1): negative control group, group (2) colorectal cancer induced rats (CRC) (positive control), group (3): CRC group treated with silver nanoparticles (CRC+AgNPs), group (4): CRC induced rats treated with Alpha-lipoic acid (CRC+ALA) and group (5): CRC induced rats treated with combinations (CRC+ AgNPs + ALA). The obtained biochemical results revealed significant reduction in serum transforming growth factor-β (TGF-β), Tumor necrosis factor α (TNF-α), C – Reactive Protein (CRP), carcinoembryonic antigen (CEA) and colon cancer specific antigen-4 (CCSA-4) levels in CRC induced group treated either with silver nanoparticles or alpha-lipoic acid and their combinations compared to CRC induced group. Regarding gene expression, we found significant down regulation in K-RAS gene expression with treatment with either AgNps or ALA or their combinations comparing to untreated group. Histopathological study greatly supports these results. In Conclusion; the present study highlighted the antitumor effect of AgNPs, ALA and their Combination in ameliorating CRC induced experimentally through its potential anti-inflammatory effect.

To Cite This Article: Amal Hamza, Fatma AlSolami, (2018), "Antitumor activity of silver nanoparticles and alpha-lipoic acid combinations in colorectal cancer induced experimentally", ***Pharmacophore, 9(2),*** *45-51.*

Introduction

Cancer is a national and international health problem. There are endogenous and exogenous factors causing the sequential accumulation of genetic alterations, a scenario named by multistep oncogenesis [1]. Among these cancers, Colorectal carcinoma ranked the third most common cancer worldwide and the fourth most common cause of death [2], which represents nearly 9% of all cancer- related deaths [3]. According to Saudi National Cancer Registry data in 2013, colon cancer considered first among males accounting for 13.9% and third among females (10.2%) preceded only by breast and thyroid cancers [4]. Family history, lifestyle and genetic predisposition are major risk factors for this cancer [5].

Colon cancer begins in the inner lining of the colon or rectum as a growth of tissue called a polyp growing slowly. The result of a progressive accumulation of genetic and epigenetic changes causes uncontrollable growth of colonocytes [6]. Colorectal cancer can be graded clinically as 4 stages, with the highest grade and mortality associated with mainly liver [7].

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Current therapies for colorectal cancer include surgical intervention, radiation and chemotherapeutic drugs enriching the therapeutic effect, but toxicity and side effects associated with these therapies are obstructing the clinical utility. Hence, it is necessary to identify possible alternative therapeutic approaches to reduce the mortality rate of this devastating disease [8].

Alpha-lipoic acid (ALA) also known as the thioctic acid, is a naturally occurring dithiol compound, which is essential for the function of different enzymes that take part in mitochondria's oxidative metabolism. Alpha-Lipoic acid possesses strong antioxidant activity, directly scavenge a number of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and chelates transition metal ions [9]. Also, Alpha lipoic acid increases the production of glutathione, an antioxidant that plays a role in the detoxification and elimination of potential carcinogens and toxins [10]. It has been confirmed that ALA shows anti-inflammatory effects, antiproliferative activity and anti-apoptotic properties [11]. Several epidemiological studies have reported that Alpha lipoic acid inhibits growth of a variety of cancer cells *in vitro* including human colon cancer (HT-29) cells, acute T-cell leukemia. The principal mechanisms involved in these *in vitro* effects were antioxidant activity and induction of apoptosis [12-14]. Research has also shown that it has the possibility to outperform chemotherapy in its ability to reduce cancer cell formation; with little to no side-effects or collateral damage.

The use of nano particles with an optimum size, surface characteristics, and dosage could enhance the solubility of lipophilic drugs and lead to the enhanced permeability and retention (EPR) effect for passive targeting and reduce side effects [15]. Among many types of nanoparticles NPs, silver nano particles (AgNPs) have gained increasing interest in the field of nanomedicine due to their unique properties and obvious therapeutic potential in treating a variety of diseases. AgNPs have been mostly studied, and their broad antimicrobial activity has been described for major pathogenic species of bacteria, viruses and other eukaryotic microorganisms [16]. Also, AgNPs were reported to possess a wide range of biological effects, including antioxidant, anti-angiogenic and anti-inflammatory effeccts. Silver NPs have been shown to induce the apoptotic pathway *in vitro* through free oxygen radical generation [17, 18]. Silver nanoparticles exhibited antitumor properties in human colon cancer cells. Study results suggest that AgNPs reduced the growth and viability of colon cancer cells (HCT116) and increased apoptosis. Therefore, AgNPs might be considered as anticancer agents [19].

Based on the aforementioned evidence, we selected AgNPs as another active substitute molecule to test the effect of anticancer activity in the combined treatment with ALA.

Material and Methods

Chemicals

Silver nanoparticles 60 nm, (±)-α-Lipoic acid and 1,2 -Dimethyl hydrazine dihydrochloride (DMH) were purchased from Sigma Aldrich company, St. Louis, MO, USA.

Animals

Fifty adult male albino wister rats (mean weight 120-130 g) were used in this study. Rats were obtained from the animal house of the King Fahd Medical Research Center (KFMRC), Jeddah, Saudi Arabia. The animals were acclimatized to laboratory conditions for one week prior to initiation of experiments. The animals were grouped and housed in cages environmentally controlled (25°C, 12-h light /12-h dark cycle). A commercial balanced diet and tap water were given throughout the experimental period *ad libitum*. This study was approved by the ethics committee of Faculty of Medicine at King Abdulaziz University.

Experimental Design

Animals were divided into 5 groups, 10 rats in each group treated as follows: **Group (1):** Set as healthy control group that received 1 ml of oral saline daily. **Group (2):** Set as colorectal cancer induced group (Positive control) CRC. 1,2 -Dimethylhydrazine dihydrochloride (DMH) was intrarectally injected in a dose of (20 mg/kg BW) single dose weekly for 6 weeks [20]. **Group (3):** Set as (CRC+AgNps) rats received silver nano-particles daily in a dose of (0.5mg/ml) orally [21] for 2 weeks after colon cancer induction. **Group (4):** Set as (CRC+ALA) rats received orally alpha lipoic acid (60 mg/kg body weight) daily [22] for 2 weeks after colon cancer induction. **Group (5):** Set as (CRC+A LA + AgNps) rats received combination of silver nano particles and ALA daily orally for 2 weeks after colon cancer induction. At the end of the experimental period, the rats were fasted overnight. The next day, the rats were anesthetized by general volatile anesthesia using ether. Blood samples were immediately withdrawn by capillary micro-tubes from retro-orbital venous plexus into plain tube with gel. The blood sample were separated into serum by centrifuged at 3000 rpm for 15 minutes at 4ºC and used for biochemical analysis.

Biochemical analyses

Serum colon cancer specific antigen-4 (CCSA-4) and Serum carcinoembryonic antigen (CEA) levels were estimated by enzyme linked immunosorbent assay (ELIZA) technique using CCSA-4 and CEA assay kit purchased from INOVA Biotech co., Ltd, Beijing, China according to the manufacture instructions. Serum Transforming Growth Factors β1 (TGF-β1) and Tumor necrosis factor α (TNF-α) were measured according to the manufacture instructions of INOVA Biotech Co., Ltd, Beijing, China. The kits use a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to determine the level

of TGF-β1 and TNF-α in serum samples. Serum C – Reactive Protein (CRP) level was determined by enzyme linked immunosorbent assay (ELISA) technique using CRP assay kit purchased from Immunospec Corporation, California, Canoga Park, USA according to the instructions provided with CRP assay kit.

Molecular genetic analysis (expression of KRAS):

Isolation of total RNA

Total RNA was isolated from colon tissue of rats in the different studied groups using a Qiagen RNease miniKit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After extraction and purification, the RNA yield and purity was determined by measuring absorbance at 260 nm/280 nm on a Nanodrop spectrophotometer (Thermo Fisher Scientific). The complete RNA isolated from male rat colon tissues was reverse transcribed into cDNA using ImProm-II™ Reverse Transcription System from Promega (Madison, USA) in accordance with the manufacturer's instructions. Reactions were performed in a final volume of 5µl of the treated samples; then they were added to 15µl of reaction mix, containing nuclease-free water, ImProm-II™ 5x reaction buffer, $MgCl_2$ solution for reaction enhancement, dNTP mix as a source of oligonucleotides for cDNA synthesis and ImProm-II™ Reverse Transcriptase for reaction catalysis. Reverse transcription was performed in a thermo cycler (Gene Amp 9700, Applied Bio systems) under the following reaction conditions: 25°C for 5 min, 42°C for 90 min and 70°C for 15 min. Obtained cDNA samples were stored at -20°C, ready to be used as PCR templates. Real-time RT-PCR was performed using the Quanti Fast SYBR Green PCR Kit (QIAGEN) according to manufacture instructions. The sequences of target genes are illustrated in the following table (A).

Table A: The sequences of specific primer of the genes used are listed

Target genes	Forward (F) and reverse (R) Primers (5'→3')
KRAS-RAT	F: ' 5-GAG TAC AGT GCA ATG AG -3'
	R: '5-CTA GAA CAG TAG ACA CG -3'
GAPDH-RAT	F: '5 -CAC GAG AAA TAT GAC AAC TC – 3'
	R: '5 - ACT CAG AAG ACT GTG GAT G -3'

Histopathological examination of colon tissue

After fixation of colon tissues in formalin saline (10%) for 24 hours, the colon tissues of rats in the different groups were washed in tap water. For dehydration, several alcohol dilutions were applied (methyl, ethyl and absolute ethyl). Paraffin bees wax tissue blocks were prepared for sectioning at 4 µm thicknes by sledge microtome. The obtained tissue sections were collected, deparaffinized and stained by hematoxylin and eosin stain and examined by light microscopy and histopathological assessments made.

Statistical Analysis

The obtained results were statistically analyzed by comparing the values of investigated groups with the values of individual normal ones. Results were expressed as mean ± S.E. Significant differences among groups were calculated using analysis of variance ONE WAY ANOVA coupled with Statistical Package for the Social Science (SPSS) program. ANOVA at $p < 0.05$ was considered significant.

Results

Table 1. Comparison of serum levels of colon cancer – specific antigen -4 and carcinoembryonic antigen between differen studied groups

Experimental groups	**CCSA-4 (ng/ml)**	**CEA (Pg/ml)**
Control (- ve)	82.23±1.83	0.85±0.03
Colorectal cancer(CRC)	111.56±7.86 [a]	1.46±0.18 [a]
CRC + AgNPS	86.00±3.86 [b]	0.92±0.09 [b]
CRC + ALA	94.68±5.01 [b]	0.98±0.09 [b]
CRC (AgNPS + ALA)	90.91±3.25 [b]	0.93±0.09 [b]
Significance between groups	0.001	0.002

Data are expressed as mean +/- standard error. a: significance versus control group; b: significance versus colorectal cancer group using one way ANOVA test.

Table 2. Comparison of serum levels of C-reactive protein, transforming growth factor- β and tumor necrosis factor –α between different studied groups

Experimental groups	CRP (ng/ml)	TGF-β1 (pg/ml)	TNF-α (pg/ml)
Control (- ve)	57.95±7.97	27.76±1.50	0.57±0.03
Colorectal cancer (CRC)	135.12±23.41[a]	40.04±0.99[a]	1.03±0.08[a]
CRC + AgNPS	61.85±8.49[b]	33.75±5.44	0.66±0.01[a,b]
CRC + ALA	65.83±4.05[b]	37.58±6.97	0.71±0.04[b]
CRC (AgNPS + ALA)	63.92±1.92[b]	35.09±1.62	0.69±0.04[b]
Significance between groups	0.0001	0.293	0.0001

Data are expressed as mean +/- standard error. a: significance versus control group; b: significance versus colorectal cancer group using one way ANOVA test.

Table 3. Correlation between measured parameters in all studied groups

Measured parameters	CRP (ng/ml)	CCSA-4 (ng/ml)	CEA (Pg/ml)	TGF-β1 (pg/ml)
CCSA-4 (ng/ml)	0.122 (0.400)	-	-	-
CEA (Pg/ml)	0.188 (0.192)	**0.298* (0.035)**	-	-
TGF-β1 (pg/ml)	0.162 (0.261)	0.174 (0.228)	0.114 (0.431)	-
TNF-α (pg/ml)	**0.346* (0.261)**	**0.470** (0.001)**	**0.495** (0.0001)**	0.142 (0.325)

Data was expressed as correlation (r) and significance. Correlation between parameters was made using Pearson correlations

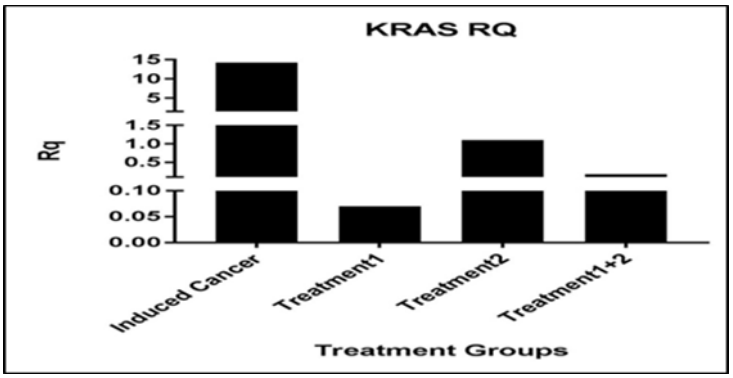

Figure. 1. Expression pattern of KRAS gene at induced and treatment conditions

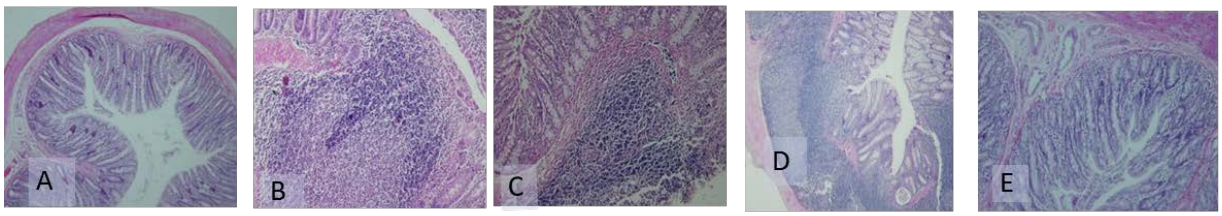

Figure. 2. Photomicrographs showing H & E stained colon histopathological sections in DMH-induced colorectal tumorigenesis in rats followed by treatment with silver nanoparticles and lipoic acid: (A) photomicrograph of healthy control group characterized by normal architecture (X10), (B) photomicrograph of colorectal cancer induced group displaying differentiated carcinoma and massive inflammatory (X20), (C) photomicrograph of treated group with AgNps showed inflammatory features with necrosis. Normal colonic epithelium is also shown (X20), (D) Photomicrograph of treated group with ALA, showed moderate inflammation with poorly differentiated carcinoma in sub mucosal.Normal colonic epithelium is also shown (X10), (E) Photomicrograph of treated group with A LA + AgNps complex displaying normal colonic epithelium characterized by simple columnar epithelial cell X20. The section was cut parallel to the muscle layer.

Data in Table (1) revealed that serum levels of CEA and CCSA-4 showed significant increase in colorectal cancer induced group as compared to the control group. Moreover, CRC treated with silver nanoparticles, Alpha-lipoic acid and their combination showed significant decline in CEA and CCSA-4 as compared to CRC induced group ($P \leq 0.05$).

As Illustrated in Table (2) colorectal cancer group displayed significant elevation in serum CRP, TGF-β and TNF-α relative to the control group. Conversely, treatment with silver nanoparticles, Alpha-lipoic acid and their combination showed significant reduction in serum CRP, TGF-β and TNF-α comparing with CRC induced group ($p< 0.05$).

Table (3) showed the correlation between the serum CCSA-4 ,CEA CRP, TNF –α and TGF-β in all experimental groups. Our results demonstrated a positive significant correlation between carcinoembryonic antigen and colon cancer specific antigen-4 (r= 0.298, P =0.035). Also, positive significant correlation was found between tumor necrosis factor-α and C- reactive protein (r= 0.346, P =0.014), colon cancer specific antigen -4 (r= 0.40, P =0.001) and carcinoembryonic antigen (r= 0.495, P =0.0001).

The results in Figure (1) showed the gene expression levels assessment of K-RAS in colon tissue of the different studied groups. Data revealed that K-RAS gene was significantly upregulated in colon tissue of untreated cancer induced rats. However, the expression level of K-RAS gene was significantly down regulated in rats treated with silver nanoparticls, lipoic acid and the combinations as compared to untreated cancer induced group. While, cancer induced group treated with alpha-lipoic acid exhibited significant up-regulation in the expression levels of K-RAS genes compared with silver nanoparticles treated group.

Colon tissue histopathology

Colon tissue sections of healthy control group showed normal histological structure of the mucosa, submucosa and muscularis layers. While sections in colon tissue of colorectal cancer induced group showed poorly differentiated carcinoma with massive inflammatory reaction. Histological investigation of colon tissue sections of silver nanoparticles treated group showed normal colonic epithelium and massive inflammatory reaction in the sub mucosal with necrosis. Microscopic examination of colon tissue section of group treated with alpha lipoic acid (ALA) showed massive inflammation in the sub mucosal with poorly differentiated carcinoma and the mucosa layer is almost destroyed and invaded by cancer cells. Histological examination of colon tissue section of group treated with ALA and silver nanoparticle showed formation of distinct and intact cellular structures and goblet cells.

Discussion

Colorectal disease (CRC) is the most widely recognized and broadly investigated gastrointestinal tumors in modernized nations. Although there is a growing awareness about hazard factors and pathologic mechanisms, it considered the third highest cause of cancer mortality [23]. Despite of many cancer drugs have been used to decrease the size of tumors dramatically, most cancers eventually relapse, posing a critical problem to overcome. Hence, it is important to find possible alternative therapeutic ways to reduce the mortality rate of this devastating disease [24].

In the current study, Colorectal tumor model was induced in Wistar rats by intrarectally injection of DMH (20 mg/kg) once a week for 6 weeks. DMH is used to induce Colorectal cancer, which is metabolized into the carcinogenic metabolite without previous metabolism by other tissues or colon bacteria in rodents [25, 26]. The carcinogenic metabolite of DMH is responsible for DNA methylation in various organs, including epithelial cells in the proliferative compartment of the crypts, which results in a great loss of colonic cells by apoptosis, an increase in proliferation, and an apparent increase in mutations of colonic epithelial cells [27]. This was clear through the significant elevation in CEA and CCSA-4 and inflammatory markers (TNF-α ,CRP and TGF-β) in our study, which may be due to induction of cancer by DMH. The high levels of TNF-α and TGF- β 1 can lead to a chronic inflammatory state and may be implicated in tumor initiation and promotion [28]. In addition, serum levels of CRP have been associated with increased risk of colorectal cancer [29].

On administering silver nanoparticles, alpha lipoic acid and thier combination, colon cancer biomarkers CEA and CCSA4 levels steadily returned to near normal. CRC rats were treated with silver nanoparticles, alpha lipoic acid and their combination showed a significant reduction in TNF α, TGF β and CRP. The silver nanoparticles had a pronounced effect on inhibited pro inflammatory cytokine. These results are conforming to the result of [30]. Activation of NF-κB lead to the elevation of serum levels of inflammatory cytokine [31]. In the present study, ALA-induced reduction in the levels of inflammatory markers in the colon of rats with CRC might be due to its ability to decrease the expression of NF-κB. These results are in agreement with those of [32].

The present study revealed significant upregulation in the gene expression level of K-RAS in colon tissue of colorectal cancer induced rats. This finding is in agreement with that in the previous studies of [33], [34] and [35]. Mutations in the k-RAS gene are responsible for activation of the k-RAS pathway which is implicated in colon carcinogenesis in humans and rats [36]. Functional studies in cell culture and mouse models support the specific role for K-RAS mutation in colorectal cancer progression and maintenance [37].

Treatment with silver nanoparticles in colorectal cancer induced rats led to significant reduction in KRAS gene expression level in colon tissue as shown in the current data. The suggested mechanism for the inhibition of k-RAS gene expression level

due to treatment with silver nanoparticles in colon cancer-induced rats is the ability of silver nanoparticles to suppress the activation of Akt and Erk [38]. KRAS mutations occur frequently and drive MEK–ERK mitogenic pathway activation. Lipoic acid appears to protects against chemical-induced DNA damage that can lead to cancerous transformation [39]. Lipoic acid may help prevent metastatic cancer spread by reducing activity of enzymes that tumors use to invade tissues [40]. lipoic acid has been found to induce apoptosis in hepatoma cells via the targeting PTEN/Akt pathwa [41]. The histopathological results greatly support the present biochmical analysis.

Conclusions

Based on our results, it could be concluded that silver nanoparticles plus ALA has a promising therapeutic role against colorectal cancer induced by 1,2 dimethylhydrazine as indicated by the observed improvement in the measured histological, molecular and biochemical markers. These effects achieved through the powerful anti-inflammatory properties. These results represented good therapeutic approaches for intervention against progressive of colorectal cancer with special reference to the inflammatory response.

Conflict of interest:

The authors declared no conflict of interest.

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