Effect of metformin on clastogenic and biochemical changes induced by adriamycin in Swiss albino mice


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Abstract

Diabetes mellitus (DM) is a chronic disease that is characterized by deteriorating glycemic control. The disease is known to be caused by imbalance between reactive oxygen species (ROS) and antioxidant defense systems. Hyperglycemia is commonly observed in a wide variety of diseases, including cancer. Although, therapy against glycemic control, is used in all these diseases, the diabetic cancer patients are on additional therapy with anticancer drugs. The objective of present study was to study if Glucophage (metformin), a very popular antidiabetic agent can avert the mutagenicity and lipid peroxidation caused by adriamycin (ADR), which is a commonly used cytotoxic drug. The experimental protocol included oral treatment of mice with different doses (62.5, 125 and 250 mg/kg day) of metformin for 7 days. Some mice in each group were injected i.p. with ADR (15 mg/kg). In each case animals were killed, 30 or 24, 48 and 72 h after the last treatment and femurs were excised for cytological studies by micronucleus test. Additional experiments on estimation of glutathione (GSH) and malondialdehyde (MDA) were undertaken in blood and serum, respectively. Twenty-four hour after the treatment, blood from each mouse was collected from heart and preserved for analysis. The results obtained revealed that pretreatment with metformin: (i) reduced the ADR-induced frequency of micronuclei without any alteration in its cytotoxicity and (ii) protected against the ADR-induced increase and decrease of MDA and GSH, respectively. The exact mechanism of action is not known, however, the inhibition of ADR-induced clastogenicity and lipid peroxidation by metformin may be attributed to the antioxidant action of the latter. Our results demonstrate that metformin might be useful to avert secondary tumor risk by decreasing the accumulation of free radicals and inhibition of mutagenicity.

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Keywords: Metformin; Adriamycin; Micronucleus inhibition; Lipid peroxidation; Mice

1. Introduction

Hyperglycemia is a condition of an imbalance between ROS and antioxidant defense systems, which cause deterioration of glycemic control. In addition to diabetes mellitus (DM), hyperglycemia is commonly observed in a wide variety of diseases and is a major risk factor for a number of cancers (lymphocytic leukemia, β-cell lymphoma, breast, pancrease, liver, colon, bladder, prostate and oral cavity) [1–4]. Elevated serum glucose has been reported to provoke deliberation of ROS [4]. ROS play a central role in formation of advance glycation end products (AGE) involved in the pathogenesis of secondary complications of DM due to oxidative stress [4–6]. It is the genesis of ROS that also potentiate hyperglycemia [7] and cancer due to direct
oxidative injury to DNA resulting in adducts and strand breaks [8,9]. Furthermore, the cytotoxic treatment regimen (cyclophosphamide, vincristine, doxorubicin and dexamethasone) is also known to cause hyperglycemia and a high incidence of mortality [1].

Adriamycin (ADR), an anthracycline antibiotic is one of the many cytotoxic drugs used in the clinical management of a wide variety of cancers (breast and oesophageal carcinomas, osteosarcoma, Kaposi’s sarcoma, soft-tissue sarcomas, and Hodgkin’s and non-Hodgkin’s lymphomas, gastric, liver, bile-duct, pancreatic and endometrial carcinomas) [10,11] which are also known to be associated with DM. The antitumor effects of ADR involve the production of free radicals [10,12] cause induction of sister-chromatid exchanges, chromosome aberrations, micronuclei and recurrence of cancer [13–15].

A wide variety of natural foods, herbal products and synthetic compounds have been reported to be chemopreventive against induced genotoxicity and oxidative stress [16–22]. Nevertheless, very few of these products have multiple biological action to simultaneously antagonize hyperglycemia, lipid peroxidation, genotoxicity and avert secondary malignancies. Most of the antidiabetic agents (both natural and synthetic) have been known to be the scavengers of ROS [23–25]. However there is a paucity of an agent which can control blood glucose and inhibit lipid peroxidation, while reverting the induced genotoxicity caused by a cytotoxic drug. Search of such agents is thought to be beneficial to diabetic patients, who have cancer too and are on cytotoxic therapeutic regimen.

Literature reports suggest that some antidiabetic agents, including thiazolidinediones and biguanides have been known to possess anticarcinogenic effects. Caraci et al. [26] have shown phenformin to inhibit proliferation in various cell lines (SH-SY5Y neuroblastoma and LNCaP prostate adenocarcinoma) and cause antitumoral activity in different animal models. Recent studies on thiazolidinediones, have shown rosiglitazone to protect human neuroblastoma SH-SY5Y cells against acetaldehyde-induced cytotoxicity [27] and against MPP+-induced cytotoxicity [28]. Yamamoto et al. [29] found troglitazone to cause cytotoxicity and apoptosis in human hepatoma cells. Since it is well known that most of the anticancer and cytotoxic drugs are oxidants, clastogens and cause recurrence of cancer, it is imperative to find anticarcinogenic and antidiabetic agents that possess both antioxidant and antigenotoxic potentials. Among the antidiabetic agents metformin (1-(diaminomethylidene)-3,3-dimethyl-guanidine) is the most commonly prescribed oral anti-hyperglycemic drug, used against the management of type 2 diabetes. It is reported to have several properties, including (i) reducing formation of AGE that are involved in the pathogenesis of the secondary complications of diabetes [5], (ii) replenishing the deficient levels of glutathione in DM [30] and (iii) potentiate the antioxidant defense [31]. In addition metformin has been found to inhibit the development of a number of cancers, including mammary adenocarcinomas and pancreatic carcinogenesis in hamsters [32,33]. Nevertheless, there is paucity of literature on the protective activity of metformin against clastogenic and oxidant activity of anticanccer drugs.

ADR is commonly used in diabetic patients suffering from cancer and has itself potential to increase hyperglycemia, besides causing genotoxicity and recurrence of cancer by increasing ROS. Hence, it was found worthwhile to study the protective effects of metformin against genotoxicity and lipid peroxidation induced by ADR, in order to analyze the relevance of metformin to avert the recurrence of cancer.

2. Materials and methods

2.1. Chemicals

Adriamycin was obtained from Farmitalia Carlo Erba, Italy. Metformin was purchased from Merck Sante, France. The foetal calf serum, laboratory reagents and stains were obtained from Sigma Chemical Company, St. Louis, MO, USA.

2.2. Animals

Swiss albino male mice (SWR, home bred), aged 6–8 weeks and weighing 26–30 g, obtained from Experimental Animal Care Center, College of Pharmacy, King Saud University, were used in the experiment. All experimental mice were provided with Purina chow and free access to water. The animals were maintained under controlled conditions of temperature, humidity and light.

2.3. Dose selection, preparation of drugs and route and duration of administration

The dose of ADM was based on an earlier study our laboratory [21]. The dose range of metformin used was based on some studies in the literature [34,35] and human therapeutic dose with reference to surface area rule. The doses of metformin selected in the present study were 62.5, 125 and 250 mg/kg day. The prescribed dose of this drug for an average weight of human being is 500 mg/day. According to the rule of surface area ratio of mice (20 g) and man (60 kg), the ratio was calculated to be 0.0026 and the dose of metformin per kg mice would be (0.0026 × 500 × 50 = 65 mg/kg). The lower dose used in the present study was 62.5 mg/kg day and
the medium dose (125 mg/kg day) was double and the high
dose (250 mg/kg day) was quadruple. The increased medium
and high doses were used in view of the fact that the metabolic
rate in mice is more as compared to human beings [36]. Sing-
le dose of ADM was soluble in water and hence the solution
of ADM was given intraperitoneally in tap water (0.1 ml/10 g
body weight of mice). Aqueous suspension of metformin was
administered (24 h apart for 7 days) by gavage in tap water
(0.1 ml/10 g body weight of mice). The animals in the control
group were administered the vehicle by gavage (0.1 ml/10 g
body weight of mice).

2.4. Experimental groups

A total of 160 male mice were used in different control and
treatment groups of the micronucleus test (five mice in each
group) of the two separate experiments (single sampling: 30 h
and multiple samplings: 24, 48 and 72 h) carried out in the
study. Another 160 animals were used to conduct additional
experiments on biochemical changes in MDA and GSH (20 mice in each group, blood were pooled from 2 mice to consti-
tute as 1). These experiments were conducted in 24 h group.
The experimental groups of mice consisted of (1) untreated
control (tap water); (2) metformin 62.5 mg/kg day; (3) met-
formin 125 mg/kg day; (4) metformin 250 mg/kg day; (5) ADR
15 mg/kg i.p.; (6) metformin 62.5 mg/kg day pretreatment
(7 days) + ADR 15 mg/kg i.p.; (7) metformin 125 mg/kg day
pretreatment (7 days) + ADR 15 mg/kg i.p.; (8) metformin
250 mg/kg day pretreatment (7 days) + ADR 15 mg/kg i.p.
Metformin dissolved in water was administered orally to
groups 2, 3, 4, 6, 7 and 8 for 7 days. ADR (aqueous solu-
tion) was injected (15 mg/kg i.p., group 4) 30 or 24, 48 and
72 h before sacrifice. In each case animals were killed either
30 or 24, 48 and 72 h after the last treatment, femurs excised
for cytological studies by micronucleus test. Additional exper-
iments were undertaken in the 24 h group. Twenty-four hour
after the last, blood from each mouse was collected from heart
and preserved for analysis of GSH and MDA estimation.

2.5. Micronucleus test

The procedure described by Schmid [37] was used. From the
excised femurs, cells were collected in foetal calf serum.
After centrifugation the cells were spread on slides and air-
dried. Coded slides were fixed in methanol and stained with
May-Gruenwald solution followed by Giemsa staining. The
polychromatic erythrocytes (PCE) were screened for micronu-
clei and the normochromatic erythrocytes (NCE) in order to
obtain a PCE/NCE ratio to analyze the mitodepression (bone
marrow depression). Mitodepression, is a term which denotes
the reduction of the ratio of PCE/NCE due to inhibition of PCE.

2.6. Estimation of MDA level in serum

The level of MDA in serum was used as an index of in
vivo lipid peroxidation. It was determined as per the method
described by Satoh [38] as a thiobarbituric acid reactive sub-
stance (TBARS). The concentration of TBARS (expressed as
nmol of MDA per milliliter of serum) was calculated from a
standard curve that was obtained from freshly prepared stan-
dard solution of 1,1,3,3-tetramethoxypropane. The absorbance
was read at 532 nm.

2.7. Determination of GSH in blood

The protocol described by Tietze [39] was used. GSH con-
tent in blood was spectrophotometrically measured by using
Ellman’s reagent (DTNB). The concentration of blood GSH
(expressed as µmol/ml) was calculated from a standard curve
that was obtained from freshly prepared standard solution of
GSH. The absorbance was read at 412 nm.

2.8. Statistical analysis

The different parameters studied were subjected to
statistical analysis with one-way ANOVA and post hoc
Tukey–Kramer multiple comparison test was done.

3. Results

3.1. Effect of metformin on ADR-induced
micronuclei in PCE and the ratio of PCE/NCE

The results showing the effect of metformin on
micronucleated polychromatic erythrocytes (M-PCE) and the ratio of polychromatic to normochromatic cells
(PCE/NCE) indicating mitodepression after 30 or 24,
48 and 72 h of metformin treatment are presented in
Tables 1 and 2. The frequency of M-PCE observed
in untreated mice (group 1) and treatment with met-
formin at 62.5, 125 and 250 mg/kg day (groups 2–4)
were found to be in the same range except the high dose
(group 4) of metformin at 48 and 72 h where the values
were slightly less; however, the difference was negligi-
ble when compared to mitodepression induced by ADR.
ADR treatment (group 5) was found to induce a signifi-
cant increase in the frequency of micronuclei after 30 h
(P < 0.01) and 24, 48 and 72 h (P < 0.001). The ratio of
PCE/NCE was suppressed after 24 h (P < 0.01), 30, 48
and 72 h (P < 0.001) treatment with ADR as compared to
controls.

The pretreatment with metformin at low dose (group
6) failed to prevent the ADR-induced frequency of
micronuclei and bone marrow depression after 30, 24,
48 and 72 h, as compared to ADR treatment (group 5).
The frequency of micronuclei induced by ADM was
inhibited in mice pretreated with medium dose (group
Table 1
Effect of metformin on adriamycin-induced reduction in the frequency of micronuclei and the ratio of femoral erythrocytes in male Swiss albino mice, after 24, 48 and 72 h after treatment

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment and dose (mg/kg body weight)</th>
<th>Duration of treatment</th>
<th>Polychromatic erythrocytes (PCE) screened (mean ± S.E.)</th>
<th>Micronucleated PCE (%)</th>
<th>Normochromatic erythrocytes (NCE) screened (mean ± S.E.)</th>
<th>PCE/NCE ratio (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (tap water, 0.3 ml/mouse)</td>
<td></td>
<td>5600 6200 4920</td>
<td>0.29 ± 0.05</td>
<td>5420 6250 4641</td>
<td>1.03 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>Metformin (62.5 mg/kg day)</td>
<td></td>
<td>5213 5100 5300</td>
<td>0.32 ± 0.03</td>
<td>5317 4600 5350</td>
<td>0.98 ± 0.03</td>
</tr>
<tr>
<td>3</td>
<td>Metformin (125 mg/kg day)</td>
<td></td>
<td>5136 5100 5434</td>
<td>0.35 ± 0.05</td>
<td>5350 5000 5230</td>
<td>0.97 ± 0.04</td>
</tr>
<tr>
<td>4</td>
<td>Metformin (250 mg/kg day)</td>
<td></td>
<td>5153 5287 4812</td>
<td>0.41 ± 0.06</td>
<td>5550 6220 5419</td>
<td>0.94 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>Adriamycin (15 mg/kg)</td>
<td></td>
<td>4300 3900 3500</td>
<td>3.12 ± 0.32**</td>
<td>7500 9200 9730</td>
<td>0.63 ± 0.09**</td>
</tr>
<tr>
<td>6</td>
<td>Metformin (62.5 mg/kg day) + adriamycin (15 mg/kg)</td>
<td></td>
<td>4200 4000 3800</td>
<td>2.58 ± 0.49</td>
<td>7500 5155 10,000</td>
<td>0.57 ± 0.04</td>
</tr>
<tr>
<td>7</td>
<td>Metformin (125 mg/kg day) + adriamycin (15 mg/kg)</td>
<td></td>
<td>4000 3500 3000</td>
<td>1.87 ± 0.36*</td>
<td>8700 7300 7895</td>
<td>0.48 ± 0.04</td>
</tr>
<tr>
<td>8</td>
<td>Metformin (250 mg/kg day) + adriamycin (15 mg/kg)</td>
<td></td>
<td>3100 3400 2900</td>
<td>1.66 ± 0.31**</td>
<td>6200 9444 10,000</td>
<td>0.52 ± 0.05</td>
</tr>
</tbody>
</table>

Fifteen mice were used in each group; groups 2–4 were statistically compared with group 1 and groups 6–8 were statistically compared with group 5; *P<0.05; **P<0.01; ***P<0.001 (one-way ANOVA and post hoc Tukey–Kramer multiple comparison test was done).
Table 2
Effect of metformin on adriamycin-induced reduction in the frequency of micronuclei and the ratio of femoral erythrocytes in male Swiss albino mice

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment and dose (mg/kg body weight)</th>
<th>Polychromatic erythrocytes (PCE) screened</th>
<th>Micronucleated PCE (%) (mean ± S.E.)</th>
<th>Normochromatic erythrocytes (NCE) screened</th>
<th>PCE/NCE ratio (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (tap water, 0.3 ml/mouse)</td>
<td>5200</td>
<td>0.28 ± 0.05</td>
<td>5130</td>
<td>1.02 ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>Metformin (62.5 mg/kg day)</td>
<td>5323</td>
<td>0.34 ± 0.06</td>
<td>5200</td>
<td>1.03 ± 0.07</td>
</tr>
<tr>
<td>3</td>
<td>Metformin (125 mg/kg day)</td>
<td>5430</td>
<td>0.35 ± 0.06</td>
<td>5360</td>
<td>1.03 ± 0.06</td>
</tr>
<tr>
<td>4</td>
<td>Metformin (250 mg/kg day)</td>
<td>5000</td>
<td>0.40 ± 0.05</td>
<td>5260</td>
<td>0.98 ± 0.10</td>
</tr>
<tr>
<td>5</td>
<td>Adriamycin (15 mg/kg)</td>
<td>4700</td>
<td>3.81 ± 0.90**</td>
<td>8300</td>
<td>0.56 ± 0.04***</td>
</tr>
<tr>
<td>6</td>
<td>Metformin (62.5 mg/kg day) + adriamycin (15 mg/kg)</td>
<td>4500</td>
<td>2.14 ± 0.34</td>
<td>7500</td>
<td>0.66 ± 0.12</td>
</tr>
<tr>
<td>7</td>
<td>Metformin (125 mg/kg day) + adriamycin (15 mg/kg)</td>
<td>4200</td>
<td>1.61 ± 0.37*</td>
<td>8200</td>
<td>0.61 ± 0.14</td>
</tr>
<tr>
<td>8</td>
<td>Metformin (250 mg/kg day) + adriamycin (15 mg/kg)</td>
<td>3000</td>
<td>1.45 ± 0.26*</td>
<td>6500</td>
<td>0.48 ± 0.05</td>
</tr>
</tbody>
</table>

Five mice were used in each group; groups 2–4 were statistically compared with group 1 and groups 6–8 were statistically compared with group 5; *P < 0.05; **P < 0.01; ***P < 0.001 (one-way ANOVA and post hoc Tukey–Kramer multiple comparison test was done).

7) after 24, 30 and 48 h (P < 0.05) and high dose (group 8) of metformin after 24 and 48 h (P < 0.01) and 30 and 72 h (P < 0.05). The PCE/NCE ratio was not significantly altered in any of the pretreatment groups (6, 7 and 8) when compared to mitodepression induced by ADR (group 5).

3.2. Effect of metformin on ADR-induced lipid peroxidation

The treatment with metformin failed to induce any significant changes in the serum levels of MDA at the lower doses (groups 2 and 3), while the high dose (group 4) decreased the concentration of MDA as compared to the values obtained in the control (group 1). ADR treatment (group 5) increased the MDA levels significantly (P < 0.001) as compared to group 1. The pretreatment with metformin was found to significantly reduce the levels of MDA (P < 0.05, group 7) and (P < 0.01, group 8) (Table 3).

3.3. Effect of metformin on ADR-induced glutathione

The blood levels of GSH were not affected by the treatment with metformin at the lower doses (groups 2 and 3), whereas the higher dose (group 4) was found to significantly (P < 0.05) increase the levels of GSH. ADR treatment (group 5) significantly (P < 0.001) decreased these concentrations. Pretreatment with metformin was found to significantly (P < 0.05) increase the GSH concentrations at the higher doses (groups 7 and 8) as

Table 3
Effect of metformin on adriamycin-induced changes in the MDA and GSG levels of male Swiss albino mice

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment and dose (mg/kg body weight)</th>
<th>Serum levels of MDA (nmol/ml) (mean ± S.E.)</th>
<th>Blood levels of GSH (µmol/ml) (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (tap water, 0.3 ml/mouse)</td>
<td>2.54 ± 0.28</td>
<td>207.24 ± 11.63</td>
</tr>
<tr>
<td>2</td>
<td>Metformin (62.5 mg/kg day)</td>
<td>2.24 ± 0.29</td>
<td>222.47 ± 20.98</td>
</tr>
<tr>
<td>3</td>
<td>Metformin (125.0 mg/kg day)</td>
<td>1.91 ± 0.10</td>
<td>255.38 ± 29.83</td>
</tr>
<tr>
<td>4</td>
<td>Metformin (250 mg/kg day)</td>
<td>1.68 ± 0.19**</td>
<td>281.08 ± 30.54*</td>
</tr>
<tr>
<td>5</td>
<td>ADR (15 mg/kg)</td>
<td>6.96 ± 0.56***</td>
<td>92.48 ± 18.75***</td>
</tr>
<tr>
<td>6</td>
<td>ADR (15 mg/kg) + metformin (62.5 mg/kg day)</td>
<td>5.25 ± 1.06</td>
<td>122.60 ± 19.75</td>
</tr>
<tr>
<td>7</td>
<td>ADR (15 mg/kg) + metformin (125.0 mg/kg day)</td>
<td>4.26 ± 0.70*</td>
<td>156.82 ± 21.28*</td>
</tr>
<tr>
<td>8</td>
<td>ADR (15 mg/kg) + metformin (250.0 mg/kg day)</td>
<td>3.56 ± 0.61**</td>
<td>182.35 ± 26.15*</td>
</tr>
</tbody>
</table>

Twenty mice were used in each group; groups 2–4 were statistically compared with group 1 and groups 6–8 were statistically compared with group 5; *P < 0.05; **P < 0.01; ***P < 0.001 (one-way ANOVA and post hoc Tukey–Kramer multiple comparison test was done).
compared to the values obtained after treatment with ADR (Table 3).

4. Discussion

The results obtained in the present investigation showed that metformin treatment does not induce micronuclei and has little or no bone marrow suppression up to 3 days after 7 days of treatment. These results clearly indicate lack of any clastogenic and/or cytotoxic activity caused by metformin. Our data on induction of micronuclei and bone marrow suppression caused by ADR is supported by the literature reports on the proven genotoxicity and cytotoxicity of ADR [13–15]. The exact mode of formation of micronuclei is not known, however, there is experimental evidence to show that induction of clastogenicity and/or cytotoxicity might be related to the formation of adducts or breaks of DNA [9] and/or inhibition of its synthesis [12], under the influence of topoisomerase II activity [40]. We have not studied the effect of ADR and/or metformin directly on DNA and hence we cannot be definite about the specific mechanism of these compounds on DNA. Nevertheless, our experimental studies on the effect of ADR on MDA and GSH show the involvement of free radical species in the formation of micronuclei and mitodepression, presumably due to damage to DNA. Our observation support reports in the literature [15,31,41] which described lipid peroxidation as the major cause of ADR-induced DNA fragmentation, clastogenicity and cell damage.

The exact mechanism of the involvement of lipid peroxidation in the genotoxicity caused by ADR is not known. Nevertheless, it is widely accepted that the action of ADR involves the generation of free radicals, mitochondrial dysfunction, formation of AGEs, activation of nuclear factor-κB (NF-κB) and apoptosis [6,44]. Previous studies have also shown metformin to decrease the cellular oxidative reactions, reduce the rate of formation of AGE and activation of NF-κB [6,49]. Moreover, the intracellular GSH is also known to play a key role in regulating the activation of NF-κB [30]. It is possible that the influence of metformin on GSH might have been instrumental in regulating the NF-κB. These reports support our finding on metformin-induced decrease and increase of the endogenous levels of MDA and GSH, respectively, showing the impact of metformin on increasing the antioxidant defense. There are no parallel experiments conducted on other antidiabetic agents, however, rosiglitazone has been found to reverse the acetaldehyde [27] and MPP+ [28] induced apoptosis in human neuroblastoma SH-SY5Y cells. These studies have shown the involvement of rosiglitazone-induced expression of antioxidant enzymes.

Most antioxidants are known to have mutagenic potential [50], however, the treatment with metformin, in the present study was found to increase antioxidant defense and was devoid of any genotoxicity and/or cytotoxicity in mice. Pretreatment with metformin was found to protect against ADR-induced increase of lipid peroxidation and micronuclei formation. The intensity of bone marrow suppression caused by ADR was not potentiated. Our study demonstrate that metformin might be useful to reduce the accumulation of oxidative stress and mutagenicity and might be useful to avert secondary
tumor risk in patients who are exposed to cytotoxic and/or anticancer drugs. Further studies are suggested on antidiabetic agents (thiazolidinediones and biguanides) that are known to possess anticarcinogenic potentials, in order to find compounds which can be simultaneously beneficial for glycemic control and inhibition of mutagenicity and lipid peroxidation.

References


[29] Y. Yamamoto, S. Yamagishi, C.C. Hsu, H. Yamamoto, Advanced glycation end products-receptor interactions stimulate the growth of human pancreatic cancer cells through the induction of platelet-


