Reduction by aspirin of death rate due to acute coronary syndrome in breast cancer by the normalization of dermcidin isoform 2: A randomized, parallel group trial

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# Equal contribution

Abstract

Background: As environmentally induced stresses has been reported to promote breast cancer in females, the association of dermcidin isoform 2 (DCN-2), an environmentally induced major atherosclerotic risk factor in female breast cancer (fBC) subjects was investigated. Since the treatment of leukocytes with 15 µM aspirin inhibited DCN-2 synthesis, the effect of oral administration of 14 mg aspirin/70 kg body weight to fBC subjects was performed to find out the reduction if any, of death rate due to acute coronary syndrome (ACS).

Methods: Synthesis of DCN-2 in leukocytes was determined by in-vitro translation of mRNA and quantitated by Enzyme Linked Immunosorbent Assay. Nitric oxide was determined by methemoglobin method. fBC (n = 1140) were asked to ingest of aspirin everyday for 2years and the plasma dermcidin level was determined. The death rate due to ACS was determined by Z-test.

Results: DCN-2 in fBC patients was found to be increased to 36.75 ± 0.85 nM from 15.50 ± 0.64 nM (p<0.0001). After oral ingestion of 14 mg/70 kg body weight aspirin (n=1140), the death rate due to ACS in fBC decreased to 10.43% from ≈50% as determined by Z-test (Z-score=5.89, p<0.0001).

Conclusion: The increased incidence of ACS in fBC could be related to the increase of plasma DCN-2 level, a major risk factor for atherosclerosis and oral administration of aspirin could be helpful to reduce the death rate due to ACS in breast cancer by reducing systemic DCN-2 synthesis.

Introduction

The death rate due to the occurrence of acute coronary syndrome (ACS) or Acute Myocardial Infarction (AMI) in subjects with breast cancer (BC) is reported to be significantly higher than that in general female population at large [1]. In this context, it has been reported before that while the death due to ACS or AMI in female population was 34%, the occurrence of BC in the victims was found to increase the incidences of death due to ACS or AMI by ~50% [1]. It should be mentioned here that the increased death rate due to ACS or AMI in BC victims was reported fallaciously, at least in part, due to the therapeutic uses of chemotherapy and radiation [1] to save the lives of the victims.

The aggregation of platelets by various aggregating agents including ADP, l-epinephrine, collagen or thrombin is an essential physiologic event for the life saving process of blood coagulation [2]. In contrast, excessive platelet aggregation particularly by ADP on the site of atherosclerotic plaque rupture or fissuring has been reported to cause the formation of thrombus (a micro aggregate of platelets embedded in fibrin mass) on the site of injury on coronary artery wall [3,4]. Thrombus thus formed on the coronary artery blocked the normal circulation of blood essential for physiologic functions of the heart muscles that may in consequence lead to ACS or even to AMI which are known to be the major killers of human race [3,4]. As the atherosclerotic plaque rupture is the leading cause of coronary thrombosis [4], the development of atherosclerotic plaque is usually equated with the prothrombotic condition leading to ACS or AMI. Although the mechanism of development of atherosclerosis remains obscure, both diabetes mellitus (type I and type II) and hypertension have been reported to be the two major risk factors for atherosclerosis [5]. Currently, the development of atherosclerosis could only be controlled by the control of diabetes mellitus and hypertension [6].

We have reported before the appearance of an environmentally induced stress protein identified to be dermcidin isoform 2 (DCN-2), an 11kDa molecular weight protein that was found to be simultaneously a hypertensive and a diabetogenic protein as well as a potent platelet aggregating agent through enhanced synthesis of thromboxane A2 (TXA2) [7]. There are numerous reports that suggest the role of environmentally induced stresses like smoking, alcohol consumption...
which may help both the development and promotion of BC [8]. In this context, we have recently reported that tobacco smoke was capable of inducing systemic DCN-2 synthesis [9].

It has been reported before that oral administration of acetyl salicylic acid (aspirin) in subjects with ACS or AMI resulted in the reduction of systemic synthesis of DCN-2 through the stimulation of nitric oxide (NO) synthases [7] that resulted in the control of hyperglycemia due to the hepatic synthesis of insulin [7].

Although the use of aspirin has been reported to reduce the development of ACS or AMI through its ability to inhibit platelet aggregation [10], it should be mentioned here, that the inhibition of platelet aggregation does not result in the reduction of atherosclerosis which is known to lead to ACS or AMI due to the development of prothrombotic condition [3,4]. In other words, the inhibition of cyclooxygenase cannot reduce atherosclerosis, and as described above, the control of diabetes mellitus and hypertension which are known to be the major risk factor for atherosclerosis could be attained through the stimulation of the systemic NO synthesis for the control of the both atherosclerotic risk factors [6].

We herein describe, that the chronic use of 14 mg aspirin/70 kg body weight might reduce the occurrence of ACS or AMI in BC patients through the neutralization of systemic DCN-2 synthesis as reported in the cases of victims affected by ACS or AMI [7] not only through hypertension, but also through the increase of the systemic insulin synthesis in BC victims.

Materials and methods

Ethics statement

The research project, "The reduction by aspirin of death rate due to acute coronary syndrome in breast cancer by the normalization of dermcidin isoform 2: A Randomized, Parallel Group Trial" required nominal amount of blood (2 mL) from patients with breast cancer. The Institutional Review Board, Human and Animal Research Ethics Committee, Sinha Institute of Medical Science and Technology, Kolkata, India approved the study on the condition that followed the approved Human Ethics Protocol strictly in accordance with 1964 Helsinki declaration and no deviation in the study was allowed without the prior written permission of the board. This study involved the participation of female breast cancer patients in the age group of 25-55 years as well as equal number of age matched normal female volunteers. No mentally retarded, pregnant women or prisoner took part in the study. All the volunteers signed an informed consent form prior to their participation in the study. It was ensured that the Breast Cancer patients had no other life-threatening infection. Care was taken to see that none of the volunteers were hospitalized for any condition within the last 6 months. Patients with Breast Cancer were selected for the study only under the strict supervision of an oncologist. Nominal amount of blood samples were drawn under the supervision of the attending physician and nurses. Seepage of blood after withdrawn, if any, was controlled by appropriate technique. Written consent was obtained from each of the patients.

The study also requested BC patients to ingest 14 mg of aspirin/70 kg body weight once daily for 2 years. The participants with BC were asked to swallow aspirin with water only after having a full meal containing bread, fat and proteins (meat, fish and cheese). No volunteers with cancer were allowed to participate in the study without written consent (judicial affidavit) as signed by a magistrate and when the participants were selected, they were asked to sign an informed consent form in the presence of a witness.

This study also used healthy white New Zealand Rabbit after being examined by a certified veterinarian (according to animal protocol no 14B of the institute) to raise antibody against DCN-2. A standard diet and sterile water were given ad libitum. Appropriate permission was obtained from the Institutional Review Board, Human & Animal Research Ethics Committee, Sinha Institute of Medical Science & Technology, Kolkata, India. The committee inspected the progress and problems of the current investigation routinely. Care was taken to ensure that no animals were unnecessarily harmed or were subjected to pain during the study and the studies were performed only in the presence of a member belonging to the Animal Right Group.

Selection of BC patients

Only female BC patients 25 to 55 years of age (n=1140) participated in the study (Figure 1). The BC was diagnosed by mammogram followed by biopsy and was categorized by TNM classification at presentation. As there is no medication known to prevent AMI in BC, and the use of the therapies themselves is reported to cause AMI. To delineate the effect of aspirin in the prevention of AMI in BC and to keep the study well focused those patients who were undergoing therapies including chemotherapy, radiation and even surgery was not included in the study. Incidentally, it should be noted here, in parallel but independent study, use of SNP patch has been reported to improve the pathological problems associated with different cancers including BC [11]. These volunteers were given appropriate legal counseling in the presence of their family members and legal counselors. All selected volunteers were asked to obtain judicial affidavit from the court of law and signed informed consent form. They were also advised to discontinue aspirin anytime they wanted and they were at liberty to begin use of any therapy including chemotherapy, radiation or surgery for their condition without any consent from the investigators at any time. The details of the characteristics of the BC patients are provided in table 1. The study was carried out in a random and double-blind fashion, i.e. neither the subject nor the investigators was aware of the treatment a subject receives. The identity of the investigational products was blinded by a numerical code.

Exclusion criteria for female breast cancer (BC)

None of the subjects had a history of diabetes mellitus, systemic hypertension, severe infections, or life-threatening cardiovascular or cerebrovascular conditions. The subjects who had bundle branch block or left ventricular hypertrophy as shown in EKG were excluded from the study. Before the withdrawal of peripheral blood from the BC subjects no cardiac therapy was initiated.

Selection of normal female volunteers

Age and gender matched normal volunteers also participated in the study. These volunteers had never taken any contraceptives nor had any history of diabetes mellitus and systemic hypertension. All volunteers were asked to stop taking any medication including aspirin for at least 2 weeks before participating in the study.

Diagnosis of the occurrence of ACS or AMI in BC subjects

At hospitalization, the subjects affected by ACS or AMI had typical chest pain, lasting more than 30 min, the characteristic of ACS or AMI. The occurrence of ACS or AMI was confirmed by the characteristics of EKG such as ST elevation and by the determination of CK-MB.
isoenzyme within 6h of the hospitalization. Death due to ACS or AMI was certified by appropriate hospital personnel after consultation with the attending physicians.

Preparation of leukocyte suspension from the peripheral blood

Venous blood was withdrawn by using 19-gauge siliconized needle and was collected in plastic vial. The blood sample was anticoagulated by adding 1vol of sodium citrate to 9vol of blood as described before [12]. Leukocytes were isolated from the buffy coat and purified by ficoll histopaque gradient [13]. The leukocyte preparation was suspended in Tyrode’s buffer pH 7.4 and was used as soon as possible.

Oral administration of aspirin

This randomized trial was conducted at the Department of Medicine, Sinha Institute of Medical Science & Technology (registered with the Clinical Trial Registration India (CTRI), trial registration no: CTRI/2014/12/005235). The period of recruitment was from January 7, 2009 to February 12, 2014 and the last follow-up date was March 27, 2014. The protocol was approved (Protocol No. S.I.M.S.T. 912 of 2008/D, dated: November 7, 2008) by the Institutional Review Board, Sinha Institute of Medical Science & Technology before enrollment of participants had began, and written informed consent was obtained from all participating patients. The participated BC patients (n = 1140) were asked to swallow 14 mg of aspirin with water only after they have eaten a meal containing bread, fat and proteins (meat, fish and cheese) once in 24h as described in details before [14].

Preparation of DCN-2

DCN-2 was prepared from the blood of the subjects suffering from ACS by using SDS- Poly acryl amide gel electrophoresis as described before in detail [7].
Assay of DCN-2

The plasma DCN-2 was determined by Enzyme Linked Immunosorbent Assay (ELISA) using antibody raised against electrophoretically purified DCN-2 as the antigen in New Zealand white rabbit that has been described in detail before [7].

**In vitro translation of DCN-2 synthesis in leukocytes**

Typically, the leukocytes suspension in Tyrode’s buffer pH 7.4, was incubated with different amounts of DCN-2 for different times at 37°C, the nucleic acids containing mRNA for DCN-2 were extracted by Trizol method [15], the nucleic acid extract was treated with the mixture of 1 nM of all 20 different amino acids and 1.0 µM ATP and the mRNA was translated by using plant leaf ribosomal particles [15]. The synthesized proteins that also contained DCN-2 was determined by ELISA as described above.

These experiments were carried out to determine the newly synthesized DCN-2 due to the presence of environmental stresses which might also release preformed DCN-2 from the leukocytes.

**Determination of NO**

NO was determined by methemoglobin method as described before [16,17]. The amounts of NO formed was verified by independent chemiluminescence method [18].

**Determination of blood glucose level**

The blood glucose level was determined by a glucometer (Behringer).

**Determination of blood pressure levels**

The systolic and diastolic blood pressures were measured by using mercury sphygmomanometer.

**Chemicals**

Aspirin was obtained from Medica Zydis Healthcare., N0- methyl-l-arginine acetate ester (NAME), Histopaque, Trizol, Goat anti rabbit IgG-HRP was obtained from Sigma Aldrich Co. St. Louis, MO, USA. ELISA maxisorb plates were from Nunc, Denmark.

**Statistical analysis**

Results shown are mean ± S.D of at least 6 different experiments by using blood samples from 6 different donors. The significance of the results was analyzed by Student’s t test. Significance, p < 0.05, was considered significant. The significance of the prevention of death due to ACS or AMI in the BC patients who received, and who did not receive aspirin in the study was analyzed by the Z test. Where appropriate, the coefficient of correlation (r) of the results was also determined by use of the Pearson test. Correlation Coefficient, Pearson score “r”, is such that -1 ≤ r ≤ +1 is acceptable. The (+) and (-) signs are used for positive linear correlations and negative linear correlations, respectively. The statistical analyses were performed by Graphpad Prism software.

**Results**

**Plasma levels of DCN-2 in BC subjects and in age matched normal female volunteers**

As described above several environmentally induced stresses are known to induce BC, and as some of the stresses were found to induce systemic synthesis of DCN-2, a protein with “double edged” atherosclerotic risk properties, being a diabetogenic and a hypertensive agent with simultaneously being a potent platelet aggregating agent through TXA2 synthesis [6]. The plasma DCN-2 level in BC subjects was compared to that in the plasma of the age matched normal volunteers. It was found that while the plasma level of the stress induced protein in the female volunteers was 13.50 ± 0.64 nM, the plasma level of DCN-2 in the BC patients was found to be increased to 36.75 ± 0.85 nM (n = 1140, p<0.0001). These results demonstrated that the plasma DCN-2 level in the BC patients was significantly higher than that in the age matched female volunteers (Table 2).

**The appearance of DCN-2 antibody in the circulation of the fBC patients**

As described above (Table 2), the plasma level of DCN-2 in the BC patient was found to be significantly higher than that in the normal counterpart; the possibility of the presence of DCN-2 antibody in the circulation of the BC patients was also compared to that in the normal female volunteers. It was found that while the plasma level of DCN-2 antibody (determined as a protein by ELISA using electrophoretically purified DCN-2 as the antigen) in BC patients was 1.38 ± 0.122 mg/1000 mg plasma protein (n=1140), the plasma DCN-2 antibody in the normal female volunteers was 0.75 ± 0.05 mg/1000 mg plasma protein (p<0.001) determined by the same procedure (Figure 2). These results demonstrated that the plasma DCN-2 antibody was significantly higher in the BC patients than that in the age matched normal female volunteers.

**Table 2. Quantification of the plasma DCN-2 antibody in plasma in breast cancer subjects and in age matched normal female volunteers.**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Plasma DCN-2 (nM)</th>
<th>Unpaired t test (Two-tailed)</th>
</tr>
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<tbody>
<tr>
<td>Breast cancer (n = 1140)</td>
<td>36.75± 1.7078</td>
<td>p&lt;0.0001, R squared = 0.9850</td>
</tr>
<tr>
<td>Normal female(n = 1140)</td>
<td>15.50 ± 1.2909</td>
<td>R squared = 0.837</td>
</tr>
</tbody>
</table>

Blood samples was obtained from newly diagnosed breast cancer patient who had to undergo therapeutic intervention of the condition including chemotherapy, radiation, surgery and the plasma sample was separated, the amount of plasma DCN-2 was quantified by ELISA by using electrophoretically purified DCN-2 as described in methods and materials. Results shown are the mean ± SD of 1140 patients compared to age matched normal female volunteers.

**Figure 2. Quantification of DCN-2 antibody in plasma in breast cancer subjects and in age matched normal female volunteers**

Citrated blood was collected from breast cancer volunteers and normal female volunteers and the plasma DCN-2 was determined by ELISA using antibody raised against electrophoretically purified DCN-2 as the antigen as described in Materials and Methods. The quantity of plasma DCN-2 level was determined as mg protein/1000 mg plasma protein. The values shown are represented as mean ± SD of twenty eight different breast cancer subjects and equal number of age matched normal female volunteers. (p<0.0168), Symbol [■] represents breast cancer volunteers and symbol [□] represents normal volunteers.
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supported the presence of DCN-2 in BC patients, a novel protein in the circulation produced due to the development of the malignancy which might have led to immunological stimulation for the antibody production in the system.

Effect of oral administration of aspirin on the plasma DCN-2 level and the reduction of the death rate due to ACS or AMI in BC subjects

We have also reported before that oral administration of only 14 mg of aspirin/70 kg body weight was capable of stimulating synthesis of maspin, an anti-BC protein in BC through NO synthesis [14]. When similar quantity of aspirin was orally administered to the BC patients who consented to receive aspirin for its possible beneficial effect on the malignant condition through the systemic increase of maspin [14], it was serendipitously found that the oral administration of 14 mg/70 kg body weight aspirin every 24h reduced the plasma DCN-2 concentration from mean 36.75 ± 0.85 nM (ranging from 32.8 nM to 34.8 nM) to 15.75 nM (ranging from 16.7 nM to 14.1 nM) within 30 days (from day-1 to day-30) (Figure 3) (n = 1140, age between 25-55 years) as described in the Materials and Methods. Those patients who wanted to continue to receive aspirin orally were asked to continue for 2 years. Interestingly, the actual number of deaths due to ACS or AMI in these patients was only 119. As described in table 3, it was found that death rate in normal population due to ACS or AMI was 50%. However, the rate of death due to ACS or AMI BC patients who had received 14 mg of aspirin/70 Kg body weight for 2years was 10.43% that contrasted the death rate (50%) who didn’t receive aspirin. Z-test analysis between the groups were performed where the calculated Z value of the death rate (50%) who didn’t receive aspirin. Z-test analysis between the Materials and Methods. Those patients who wanted to continue to receive aspirin orally were asked to continue for 2 years. Interestingly, the actual number of deaths due to ACS or AMI in these patients was only 119. As described in table 3, it was found that death rate in normal population due to ACS or AMI was 50%. However, the rate of death due to ACS or AMI BC patients who had received 14 mg of aspirin/70 Kg body weight for 2years was 10.43% that contrasted the death rate (50%) who didn’t receive aspirin. Z-test analysis between the groups were performed where the calculated Z value of the death rate due to ACS or AMI between the normal population and the BC patients who received aspirin was 17.36 which was greater than the critical two-tailed z value 2.17. Also, the z-value of the death rates due to ACS or AMI between the BC patients not receiving aspirin and the BC patients using aspirin was 27.34 which were again greater than the critical two-tailed z-value 2.33. The P-value for the two-tailed test in all the cases was found to be 0 which was less than the alpha value of 0.5 (i.e. P<0.5). Thus Z-test analysis of the death rate due to ACS or AMI in normal, BC patients and the BC patients who received aspirin demonstrated that the use of aspirin could be useful for the prevention of death rate due to ACS or AMI in BC patients.

The effect of oral administration of aspirin on the systemic blood pressure and blood glucose levels

We have reported before that systemic increase of NO level by the oral administration of aspirin was capable of normalizing hypertension to normotensive level [6]. None of the 1140 BC patients who received oral administration of aspirin didn’t report to develop hypertension in that the systolic and diastolic blood pressure were found to be in the ranges of 120-130 mm Hg and 90-95 mm of Hg respectively.

The plasma glucose level was in the range of 95-100 mg/dl suggesting the maintenance of euglycemia in the patients.

Discussion

The occurrence of ACS or AMI in BC patients has been reported to be the major cause of death in these victims [1]. As described in the results, the use of 14 mg aspirin/70 kg body weight reduced the death rate which was due to the development of ACS or AMI in BC that was supported by "Z" test analysis of the death rate in BC patients (Figure 3 and Table 3). It could be argued that the effect of aspirin due to the inhibition of cyclooxygenase on the reduction of death rate was an extension of the well known effect of aspirin on the reduction of death rate due to ACS or AMI in general [19-21]. Our results demonstrated that the reduction of death rate due to the malignant condition was related to the reduction of the plasma DCN-2 level which was the consequence of aspirin induced NO production due to the actual inhibition of DCN-2 synthesis, determined by in vitro translation of DCN-2 m-RNA and not due to the inhibition of prostaglandin synthesis [7]. And, as such the effect of aspirin on the reduction of ACS or AMI in general through the inhibition of cyclooxygenase [10,22], could be an erroneous presumption, particularly because, lack of prostaglandin synthesis had no effect on DCN-2 synthesis.

As described above, the synthesis of DCN-2 was determined by in vitro translation of mRNA of DCN-2 (Figure 2) which could be inhibited by inhibiting the aspirin induced NOS by using l-NAME, an inhibitor of NOS [23]. In other words, the effect of aspirin on the reduction of death rate in BC was not a consequence of the inhibition of platelet aggregation, but due to the inhibition of systemic synthesis of DCN-2 which we have reported first time ever as an anti-atherosclerotic agent.

Table 3. Death rate due to ACS or AMI in breast cancer patients with or without the use of aspirin.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Death Rate due to ACS or AMI</th>
<th>Significance by Z test Analysis</th>
</tr>
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<tbody>
<tr>
<td>Normal Subjects</td>
<td>34%</td>
<td>Z value between * and ** = 17.36 (P&lt;0.5)</td>
</tr>
<tr>
<td>Breast Cancer patients who received Aspirin</td>
<td>10.43%</td>
<td>Z value between * and ** = 35.31 (P&lt;0.05)</td>
</tr>
<tr>
<td>Breast Cancer patients who didn’t received aspirin</td>
<td>50%</td>
<td>Z value between * and ** = 27.34 (P&lt;0.05)</td>
</tr>
</tbody>
</table>

Death rates due to ACS or AMI in breast cancer patients with or without the use of aspirin for 2 years and in normal counterparts dying of ACS or AMI was compared by using Z test, a special case of null hypothesis.

![Figure 3. Effect of daily ingestion of aspirin of 14 mg/70 kg body weight in breast cancer subjects.](image-url)

Breast cancer subjects (n=1140, age between 45-60years) were asked to ingest aspirin of 14 mg/70 kg body weight every 24hr after full meal as described in materials and methods. The DCN-2 level in the plasma was quantified using DCN-2 ELISA as described. Each filled circle symbol (●) represent the plasma DCN-2 in each patient at day 0 and each filled triangle symbol (▲) represent the plasma DCN-2 at day 30 after daily ingestion of aspirin. As many of the subjects had similar amounts of DCN-2, many of the points shown in the figure are overlapped both at day 0 and day 30.
through its ability to be an anti-diabetic and an anti-hypertensive agent [7,24], which was also a potent novel platelet aggregating agent through the reduction of platelet NO level prior to the synthesis of TXA2 [6,7].

It should be mentioned here that the increase of the plasma DCN-2 level was not a unique event in the case of BC subjects only (Table 2). This atherosclerotic protein has also been reported to appear in the circulation of subjects affected by ACS or AMI, diabetes mellitus and hypertension before [6,7,24].

It has been reported before, that the continuous use of aspirin in cardiac patients where aspirin is used as an inhibitor of cyclooxygenase [10,23], usually led to the development of tachyphylaxis [25]. However, the use of aspirin to reduce the synthesis of systemic DCN-2 through the stimulation of NO synthesis did not lead to similar tachyphylaxis as we have reported before [7]. Furthermore, the synthesis of NO by NO2 led to the synthesis of NO due to the stimulation of the NO by NO itself, a rare phenomenon known as “feedback activation” of the enzyme by the product itself (i.e. NO), and as such a small quantity of aspirin used in breast cancer subjects might actually amplify the systemic NO level through the “feedback activation” and may thus “bypass” tachyphylaxis.

Conclusion

The appearance of DCN-2, an environmentally induced stress protein induced by alcohol consumption and smoking is considered to be a major risk factor in the BC and also reported to be a causative agent for ACS or AMI due to the increased aggregation of platelets. As aspirin is reported to inhibit DCN-2 synthesis in vivo independent of the aspirin induced inhibition of cyclooxygenase, the effect of chronic administration of 14 mg aspirin/70 kg body weight for 2 years that reduced the plasma DCN-2 level to normal condition was found to decrease the incidences of death rate due to ACS or AMI in BC patients by 10.43%.

Acknowledgement

Dr. Girish GV was supported by a Senior Research Associate Fellowship from CSIR, Government of India (IA-27413).

Contributions

D.B., G.G. and A.K.S. conceived and designed the experiments. D.B., G.G. and S.N. performed the experiments. D.B., G.G., S.N. and A.K.S. analyzed the data. R.B. and A.K.S. contributed reagents/materials/analysis tools. A.K.S., D.B. and G.G. wrote the manuscript. All authors reviewed the manuscript.

References


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