Research Article

In vitro Antioxidant, Inhibition of Oxidative DNA Damage and Antiproliferative Activities of Ethanolic Green Tea (*Camellia sinensis*) Extract

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ABSTRACT

Green tea is a famous beverage produced from the dry leaves of *Camellia sinensis*. It suggested that it has an important beneficial effect on human health. The present work aims to assess the total phenolic and flavonoids content and *in vitro* antioxidant, inhibition of oxidative DNA damage and antiproliferative activities of ethanolic green tea extract, as preliminary phase of our laboratory studies *in vivo*. Different methods were used; DPPH radical-scavenging, inhibition of lipid peroxidation, $OH^{\cdot}$-scavenging activity and DNA damage inhibition assays. In addition to antiproliferative activity which was evaluated using xCELLigence RTCA instrument. The extract presented high levels of phenolic compounds (700µg±1 µg of gallic acid equivalent/mg extract), flavonoids (33.74±0.05 µg of quercetin equivalent/mg extract), and 18 phenols were identified using HPLC-TOF/MS analysis. In DPPH free radical-scavenging assay, the extract showed remarkable activity; IC_{50} value 10.35±0.14 µg/ml and the highest percentage of the inhibition was 96% similar to vitamin C in the same concentration 25 µg/ml. On the other hand, it exhibited the inhibition of lipid peroxidation with IC_{50} value 333.29±17.90 µg/ml. The $OH^{\cdot}$-scavenging assay indicated that the ethanolic green tea extract had a significant effect on $OH^{\cdot}$ radical; IC_{50} value 12.83±0.63 µg/ml compared to ascorbic acid which was 10±0.72 µg/ml. The extract also exhibited a completed protection of plasmid DNA against oxidative damage and an interesting antiproliferative activity against PC3 cells. The results of this study confirmed that this ethanolic green tea extract is a potent source of beneficial antioxidant and anticancer.

Keywords: Green tea, Phenolic and flavonoid compounds, Antioxidant activity, Oxidative DNA damage inhibition, Antiproliferative activity.

INTRODUCTION

For the past decades, oxidative stress has been increasingly recognized as a contributing factor in the genesis of chronic diseases as cancers and cardiovascular diseases[1]. Plant polyphenols are natural compounds and most of their pharmacological properties are considered to be due to their antioxidant activity. They are able to scavenge endogenously generated oxygen radicals formed by various xenobiotics; radiation etc[2] and they may reduce the risk of development of several diseases caused by oxidative stress[3]. Green tea which is the most popular consumed beverage after water, obtained from the dried leaves of *Camellia sinensis* plant is one of the natural source of polyphenols[4]. The data in the literature points to the possible role of green tea as a chemopreventive agent against different types of cancers and it suggests that much of its antiproliferative effects are mediated by its polyphenols constituents[5-7].

It has also demonstrated other beneficial effects in studies of diabetes, Alzheimer's disease and obesity[8-10]. In the present study, we report the total phenolic and flavonoids content of a store Chinese green tea, usually used as beverage in Algeria, and we evaluated its antioxidant, inhibition of oxidative DNA damage and its antiproliferative activities *in vitro* to confirm their effect and as preliminary phase of our laboratory studies *in vivo*.

MATERIALS AND METHODS

Green tea extraction

Store Chinese green tea leaves (2000 g) were macerated with EtOH/H_2O (7:3 v/v) for 48 h three successive times. After filtration, the combined filtrates were concentrated in vacuum (up to 35°C).

Determination of total phenolic and flavonoid content

The total phenolic content of ethanolic green tea extract was determined using Folin-Ciocalteu reagent according to the method of Singleton.[11] 100 µl of Folin-Ciocalteu and 1580 µL of distilled water were added successively to 20 µl of each green tea extract prepared in methanol (1 mg/ml). Three min later, 300 µl of sodium carbonate (20 %) was added. The test tubes were shaken for 2 h at room temperature. The absorbance was evaluated at 765 nm. The standard curve was prepared using gallic acid solutions (0 to 500 mg/ml) prepared in MeOH/H_2O (1:9 v/v). The concentration of total phenolic compounds was determined as µg of gallic acid equivalent (GAE) per mg of extract using the gallic acid curve equation: Absorbance = 0.001 x gallic acid (µg).

Although, the total flavonoid content of ethanolic green tea extract was determined according to the method of Wang[12]. 0.5 ml of 2% $AlCl_3$ was mixed with 0.5 ml of sample and incubated for 1h at room temperature. The

absorbance was measured at 420 nm. The concentration of flavonoid was calculated from standard quercetin graph equation: Absorbance = 0.034 x quercetin (µg) + 0.015 and determined as µg of quercetin equivalent (QE) per mg of extract.

HPLC-TOF/MS analysis

For HPLC-TOF/MS analysis, stock solutions of the 23 phenolics (2,5 ppm) and dried crude extract (200 ppm) were prepared in methanol at room temperature. Samples were filtered passing through a PTFE (0,45 µm) filter by an injector to remove particulates.

Agilent Technologies 1260 Infinity HPLC System coupled with 6210 Time of Flight (TOF) LC/MS detector and ZORBAX SB-C18 (4,6x100 mm, 3.5 µm) column. Mobile phases A and B were ultra-pure water solution with 0.1% formic acid and acetonitrile, respectively. Flow rate was 0.6 ml/ min and column temperature was 35°C. Injection volume was 10 µl. Solvent program was as follow: 0–1 min 10% B; 1–20 min 50% B; 20–23 min 80% B; 23–25 min 10% B; 25–30 min 10% B. Retention times and *m/z* values of standard compounds were used on the determination step. Ionization mode of HPLC-TOF/MS instrument was ES (-) with gas temperature of 325 °C, gas flow of 10.0 l/min, and nebulizer of 40 (psi). Phenolic content of ethanolic green tea extract were determined making comparison retention times and *m/z* values of standard phenolic compounds.

Evaluation of the antioxidant activities

DPPH radical-scavenging activity assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging capacity of the ethanolic green tea extract was evaluated using the method described by Braca.[13] 3 ml of methanol DPPH solution (0.004%) were mixed with increasing concentrations (1, 2.5, 5, 10, 20, 25 µg/ml) of extract (dissolved in methanol). After 30 min incubation at room temperature, the absorbance was measured at 517 nm. Tests were carried out in triplicate and ascorbic acid was used as positive control.

The percentage of DPPH scavenging activity (I %) was calculated using the following equation (1): $I\% = (A_0 - A_1/A_0) \times 100$. Where A_0 is the absorbance of DPPH solution alone and A_1 is the absorbance of DPPH solution + extract or vitamin C. The half inhibition concentration (IC_{50}) of the green tea extract was calculated from the graph plotted of inhibition percentage against extract concentration.

Inhibition of lipid peroxidation

The capacity of ethanolic green tea extract to inhibit lipid peroxidation was evaluated according to the modified protocol of Cao and Ikeda using egg *vitellose*[14]. 50 µl of $FeSO_4$ (0.07 M) was mixed with 10% egg *vitellose* homogenate to induce the lipid peroxidation then incubated with increasing concentration of green tea extract or vitamin C at 37°C. After 30 min incubation, 1ml trichloroacetic acid 20% (TCA) and 1.5 ml thiobarbituric acid 1% (TBA) were added. The samples were mixed then incubated for 15 min at 95°C. After centrifugation (4000 rpm for 20 min), the resulting thiobarbituric reacting substance (TBARS) was measured in the supernatant at 532 nm. The lipid peroxidation inhibition was calculated as percentage (I %) according to equation (1). Where A_0 is the absorbance of the control (without extract or vitamin C) and A_1 is the absorbance of sample + extract or vitamin C.

OH^--scavenging activity assay

The OH^--scavenging capacity of green tea extracts was evaluated according to the literature procedure of Wang with few modifications[12]. 0.5 ml $FeSO_4$ (8 mM) was added to 0.8 ml H_2O_2 (6 mM) to generate the hydroxyl radicals by Fenton reaction followed by 0.5 ml distilled water, the various concentrations of ethanolic green tea extract or vitamin C (positive control) and 0.2 ml sodium salicylate (6 mM). The samples were mixed then incubated at 37 °C for 1h. The absorbance was measured at 562 nm. The scavenging was calculated using the following equation: $I\% = [1- (A_1 - A_2)/ A_0] \times 100$. Where A_0, is the absorbance of the control (without extract or vitamin C), A_1 is the absorbance of the extract or vitamin C addition and A_2 is the absorbance without sodium salicylate.

DNA damage inhibition efficiency

The potential of ethanolic green tea extract to inhibit DNA damage was tested by photolyzing 46966 plasmid DNA (extracted from *Escherichia coli*) via UV radiation in the presence of H_2O_2 and performing agarose gel electrophoresis with the irradiated DNA[15]. Into two polyethylenes microcentrifuge tubes, 1 µl aliquots of 46966 plasmid (200 µg/ml) were added followed by 50 µg of ethanolic green tea extract in one of the two tubes without the other which the irradiated control (C_R). Then 4 µl of 3% H_2O_2 was added into two tubes, and then they were placed directly on the surface of a UV transilluminator (300 nm) during 10 mn at room temperature. In another tube, 1 µl aliquot of 46966 plasmid DNA was placed and served as the non-irradiated control (C_0). All the samples were run on 1% agarose gel and then photographed using a Lourmat gel imaging system (Vilber).

Evaluation of the anticancer activity using xCELLigence system

Cell culture and preparation of Cell Suspension

PC3 (prostate cancer) cells were grown in Dulbecco's modified eagle's medium (DMEM, Sigma), supplemented with 10% (v/v) fetal bovine serum (Sigma, Germany) and 2% PenStrep solution (Sigma, Germany) at 37°C in a 5% CO_2 humidified atmosphere.

PC3 cells in the culture flask were detached from bottom of flask by 10 ml Trypsin-EDTA solution. After detachment, 10 ml of medium was added into the flask and mixed thoroughly. This suspension was transferred to

Falcon tubes and put in centrifuge. After this, supernatant was transferred onto the cells at the bottom of Falcon tube; 4 ml medium was added and mixed. Cell concentration of this cell suspension was measured by CEDEX HiRes Cell Counter which uses Trypan Blue.

Preparation of extract solution

Green tea extract was dissolved in DMSO to a final concentration of 20 mg/ml. 25 µl of this solution was mixed with 475 µl of medium.

Preparation of E-Plate 96 plate and treatment

50 µl of medium was added into each wells of E-Plate 96 and the plate was left in sterile cabinet for 15 min., then in incubator for another 15 min. After this time, a background measurement was performed. Next 100 µL of the cell suspension ($2,5x10^4$ cells/100 µl) was added into wells – except last three; these were left without cells as only medium – and the plate left in the hood for 30 min. Then the plate was inserted to xCELLigence station in the incubator and a measurement was performed for 80 min. After this, 50, 20 and 10 µl of green tea extract solution was added into the wells respectively for 250, 100 and 50 µg/ml concentrations and the final volume were completed to 200 ml with medium. No extract solution was added into control and medium wells. Then the plate was inserted to xCELLigence station in the incubator and measurement for 48 h was started.

STATISTICAL ANALYSIS

Data are expressed as mean ± SD and statistical interferences were based on student's test for mean values comparing green tea extract to standard using Graph Pad Prism version 6.

RESULTS AND DISCUSSION

The total phenolic and flavonoid content

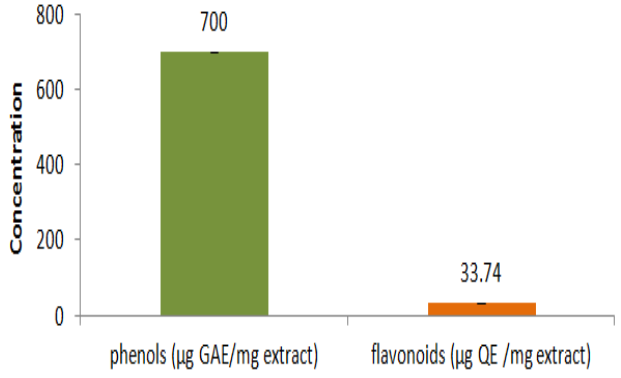

Figure 1: Total phenolic and flavonoid content of ethanolic green tea extract. Values are mean ± SD (n=3).

Phenols and flavonoids are very important plant constituents. They are generally involved in the defense against ultraviolet radiation or aggression by pathogens[2]. Ethanolic green tea extract presented high levels of total phenolic and flavonoid compounds 700±1 µg of gallic acid equivalent/mg extract and 33.74±0.05 µg of quercetin equivalent/mg extract respectively (Figure1).

HPLC-TOF/MS analysis

HPLC-TOF/MS method has the potential to separate and determine phenolics distributed in plants. In this study phenolics and flavonoids content of ethanolic green tea extract were separated and detected based on comparison with 23 standard phenolic compounds. Among 23 standard phenolic compounds used, 18 were found in this green tea extract (Figure 2). Compared to the quantity of the total phenolic detected, this green tea showed a high quantity of gallic acid, rutin, catechin, quercetin, gentisic acid, vanilic acid, salicylic acid, low quantity of caffeic acid in addition to other phenolics with less quantity[16] (Table 1).

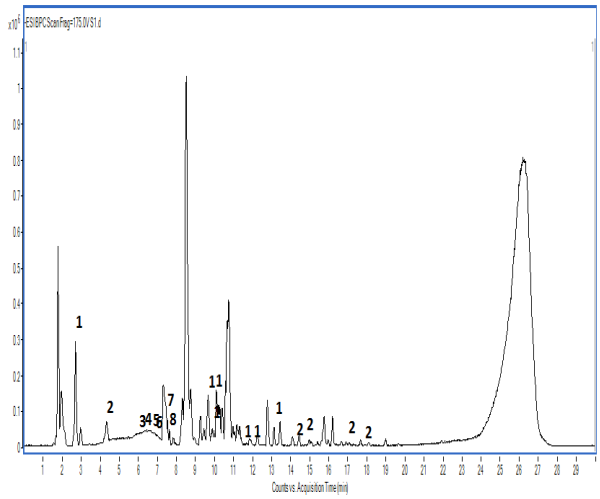

Figure 2: Chromatogram of ethanolic green tea extract. The chromatographic conditions are described under Materials and Methods. The numbers 1 to 23 are phenols and flavonoids detected in ethanolic green tea extract by HPLC-TOF/MS (Table 1).

Evaluation of the antioxidant activity

In the present study, the antioxidant activity of ethanolic green tea extract was evaluated using different assays. The free radical scavenging activity of this extract was evaluated through its ability to quench DPPH (2,2-diphenyl-1-picrylhydrazyl) radical. Figure 3 illustrated a significant dose dependent decrease in DPPH radical due to the scavenging ability of the extract. Compared to ascorbic acid (IC_{50}=5±0.1 µg/ml), ethanolic green tea extract showed a high activity (IC_{50}=10.35±0.14 µg/ml) and the highest percentage of the inhibition was 96% similar to vitamin C in the same concentration (25 µg/ml) (Figure 3).

The inhibition of the lipid peroxidation in egg *vitellose* homogenate induced by $FeSO_4$ system was also evaluated and the extract exhibited a remarkable effect (Figure 4) but it considered low (IC_{50}=333.29±17.90 µg/ml) compared to ascorbic acid (IC_{50}=20±1.06 µg/ml).

Table 1: The different phenols and flavonoids found in the green tea and their levels (expressed as mg per kg of dry leaves of green tea)

	Phenols	Retention time	mg phenolic/ kg plant
1	gallic acid	2.69	7473.79
2	gentisic acid	4.33	686.86
3	catechin	6.24	980.40
4	chlorogenic acid	6.34	123.98
5	4-hydroxybenzoic acid	6.8	8.16
6	protocatechuic acid	6.83	21.43
7	caffeic acid	7.61	34.10
8	vanilic acid	7.68	360.18
9	4-hydroxybenzaldehyde	9.08	0
10	rutin	9.67	2462.73
11	p-coumaric acid	10.01	72.09
12	ellagic acid	10.08	125.16
13	chicoric acid	10.25	0
14	ferulic acid	-	ND
15	hesperidin	-	ND
16	apigenin-7-glucoside	11.57	6.96
17	rosmarinic acid	12.11	4.73
18	protocatechuic acid ethyl ester	13.23	0
19	salicylic acid	13.37	233.45
20	resveratrol	14.37	1.03
21	quercetin	14.99	954.92
22	naringenin	17.04	9.51
23	kaempferol	17.94	26.18

ND: Not detected

The hydroxyl radical can be formed by the Fenton reaction in the presence of reduced transition metals (such as Fe^{2+}) and H_2O_2, which is known to be the most reactive of all the reduced forms of dioxygen and it is thought to initiate cell damage *in vivo*[17]. It can reduce disulfide bonds in proteins, specifically fibrinogen, resulting in their unfolding and scrambled refolding into abnormal configurations. The consequences of this reaction were observed in many diseases as cancer atherosclerosis and neurological disorders[18].

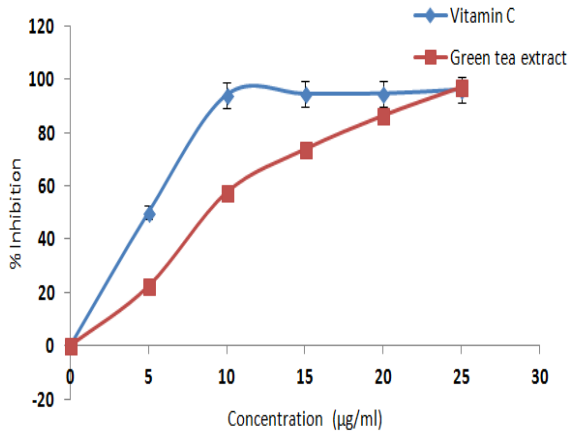

Figure 3: DPPH scavenging activity of green tea extract and vitamin C. Values are mean ± SD (n=3).

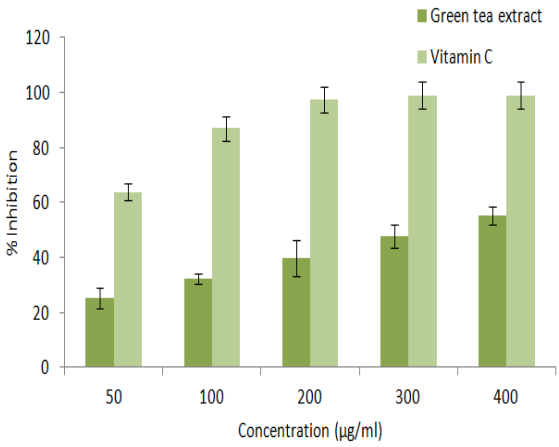

Figure 4: The effect of ethanolic green tea extract and vitamin C on inhibition of $FeSO_4$ induced lipid peroxidation of egg *vitellose*. Values are mean ± SD (n=3).

The OH^--scavenging assay used in this study indicate that this green tea extract had a significant high effect against this potent radical (IC_{50}=12.83±0.63 µg/ml) compared to ascorbic acid (IC_{50}=10±0.72µg/ml) and its effect was dose dependent (Figure 5).

The results of this study confirm that this green tea is a potent antioxidant. This potent antioxidant activity might result from its high contents of polyphenols and flavonoids especially catechins with 5,7-dihydroxyl groups on the A ring, di- or trihydroxyl groups on the B ring and D

ring in the case of catechingallate, which allow tea to react with the reactive oxygen species (superoxide radical, singlet oxygen, hydroxyl/peroxyl radical, peroxynitrite) and gave it a maximal of antioxidant activity [19], gallic acid[20], quercetin[21], rutin[22] and the others (Table 1).

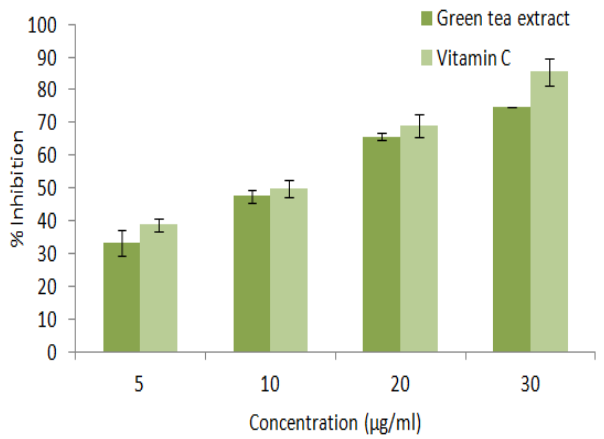

Figure 5: OH-scavenging activity of ethanolic green tea extract and vitamin C. Values are mean ± SD (n=3).

DNA damage inhibition efficiency

Many studies suggested that oxidative DNA damage may be an important factor risk for a variety of diseases including cancer in different organs[23,24], diabetes[25], neurodegenerative diseases[26] and cardiovascular diseases[27].

A high level of 8-hydroxy-2´-deoxyguanosine (8-OHdG) radical was revealed in patients samples compared to healthy controls[23,27]. This radical used as biomarker of oxidative DNA damage produced by the bound of hydroxyl radical to C8 position of the guanine ring. The results of the current study showed that ethanolic green tea extract exhibited a completed protection of plasmid DNA against oxidative damage caused by UV-photolysed H_2O_2 at a dose of 50 µg. UV-photolysis of H_2O_2 in ethanolic green tea extract sample showed a single band in agarose gel electrophoresis as in C_0 (untreated non irradiated DNA). This band represented the native form of supercoiled circular DNA. However C_R (untreated UV-irradiated DNA) showed two bands in agarose gel electrophoresis which indicated that the plasmid DNA was damaged by free radicals ($OH^{\cdot}$) generated by UV-photolysis of H_2O_2 (Figure 6).

This result agreed with the results of many other studies suggested that green tea can protect the DNA from the oxidative damage[28-30].

Evaluation of the anticancer activity

The anticancer activity of ethanolic green tea extract against PC3 cells was evaluated using xCELLigence RTCA instrument which allows as following the effect of the extract during all the 51 hours. The extract showed an interesting effect against PC3 cells especially during the first 24 h. The best effects observed compared to control and medium were at 25 h where 80% of cells died with 250 µg/ml, 60% with 100 µg/ml and 40% with 50 µg/ml. However after this time the cells started to proliferate again, and this may be due to because of the low ratio of bioactive molecules in the extract (Figure 7). These results confirmed many other studies which stated that green tea is natural potent anticancer agent against a variety of human malignances including prostate, lung, colon, stomach, kidney, pancreas and mammary glands[31]. This beneficial effect has been attributed to the presence of high amounts of polyphenols, which are potent antioxidants especially catechins[32].

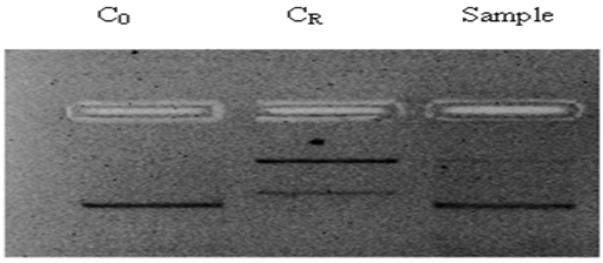

Figure 6: Effect of ethanolic green tea extract on the protection of 46966 plasmid DNA against oxidative damage caused by UV-photolysed H_2O_2. C_0=untreated non irradiated DNA, C_R=untreated UV- irradiated DNA and Sample= DNA UV-irradiated treated with ethanolic green tea extract.

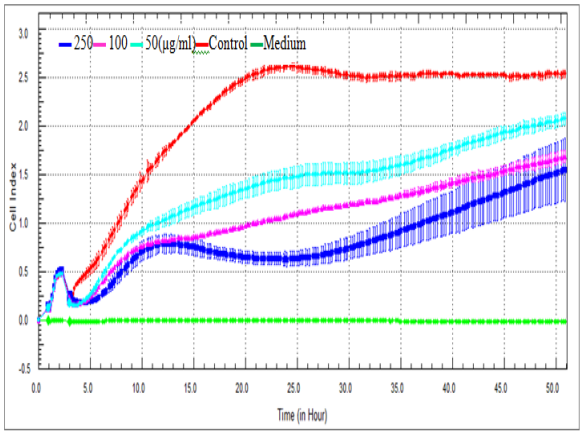

Figure 7: Anticancer activities of ethanolic green tea extract against PC3 ($2,5X10^4$ cell/well) cell line. Each substance was tested twice in triplicates using xCELLigence RTCA instrument. Several extract concentrations were applied to the cells represented by different color (50, 100, 250 µg/ml).

CONCLUSION

The polyphenols are very important natural compounds. They offer great hope for the prevention of chronic human diseases. Their role in human health is still a fertile area of research. This current study confirmed that the green tea which usually consumed in our country is an essential natural source of polyphenols and flavonoids and a potent antioxidant and anticancer agent and these encouraging results may open the way for many studies *in vivo* in our laboratory.

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