Administering the Optimum Dose of L-Arginine in Regional Tumor Therapy

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Abstract The purpose of this study is optimizing the L-arginine (L-Arg) doses on the basis of chemical structure in regional accessible tumor therapy to settle down a new protocol for the treatment of cancer. ^3H -thymidine-based cell proliferation assay was performed in vitro on tumor cell lines of fibrosarcoma (FS), lymphosarcoma-ascitic and on normal cell line of NIH 3T3 after treatment with different concentrations of L-Arg in phosphate buffered saline (PBS). The cultures were harvested after 22 h and the incorporated radioactivity was counted to identify their histologic grades as described in earlier studies. In vivo therapy of murine tumors was conducted where FS cells injected subcutaneously at ventro-lateral position of mice. Various drug delivery schedules were injected into the centre of tumor base, once a day for 4 days. Tumor diameter and survivals were monitored where the day of sacrifice was considered for monitoring the survival period. By identifying the histologic grades of the treated cultures in vitro and in vivo by different concentrations of L-Arg, the corresponding energy of such concentrations were determined. An efficient model with a good fit ($R^2 = 0.98$) was established to describe the energy yield by L-Arg dose. The equivalence between the tumor histologic grade and energy of the L-Arg dose delivered in saline (PBS) environment is the optimum condition for regional tumor

therapy achieves higher survival rate. The selective cytotoxicity to tumor cells with minimal damage to normal cells by L-Arg due to its chemical structure suggests to be considered the most promising drug for regional therapy of the accessible tumors like breast cancers of early stage with no distant metastasis.

Keywords Emad formula · Histologic grade · Stimulating apoptosis · Cell membrane damage · ^3H -thymidine-based cell proliferation assay · ^{51}Cr Chromium release assay

Introduction

The existing cancer therapies can act in two pathways, either by induction of apoptosis or by necrosis. The use of arginine (Arg) as antitumor agents is one of the effective approaches for the treatment of human cancer in both pathways.

Arg is a dibasic, cationic, semi-essential amino acid with numerous roles in the cellular metabolism. As an essential nutrient, it plays an important role in cell division, the healing of wounds, removing ammonia from the body, immune function such as stimulating T- and natural killer cell activity and influencing pro-inflammatory cytokine level, and the release of hormones [1–3]. Many studies have been shown that Arg has dual effects as toxic or protective effects against toxicity in the cells [4–6]. Arg serves also as an intermediary in the urea cycle and as a precursor for protein, polyamine, creatine and nitric oxide (NO) biosynthesis which is involved in the physiological control of different tissue at lower levels. Nevertheless, Arg can act as a toxic mediator in cells causing a genotoxicity at higher levels. The ability of Arg to inhibit the tumor growth based

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on the metabolism of Arg or based on its chemical structure has been verified and confirmed. Arg when incubated in medium for 24–72 h had been shown to kill gastric cancer cells through apoptosis based on the metabolism of Arg [7, 8]. Yurtcu et al. [4] have reported also that based on the metabolism Arg could induce DNA damage with rising of NO in lymphocytes. Through a non metabolic process, Arg in phosphate buffered saline (PBS) induce also cell membrane damage (CMD) to the tumor cells. The electrostatic attraction between the negatively charged components of cancer cells and the positively charged anticancer peptides (ACPs) contained in Arg is believed to play a role in the strong binding and selective disruption of cancer cell membrane. A direct damage of cancer cell mitochondria by the ACPs has also been suggested to explain cytotoxic selective activity ACPs as cancer cell plasma and mitochondrial membrane potential are typically higher than those of normal cells [9]. These findings suggest Arg as an anticancer molecule based on either of the metabolism of Arg or based on its chemical structure. Current approach investigates the anticancer effect of Arg based on its chemical structure in regional tumour therapy. Recently, Moawad [10, 11] improved models of clinical and pathology based staging of the cancer at the cellular level in which the rate of cell proliferation has been expressed by cell growth energy (CGE) which is the energy of all cellular kinetics of all the aberrant genetic variations that drive normal cell to cancer. The relation between CGE and cell doubling time (t_D) has been derived and presented by Moawad and is known by Emad formula as follows:

$$CGE = \ln \left[\ln \frac{\ln 2}{t_D} \right]^2 \text{Emad}, \quad (1)$$

$$\text{Emad} = 23234.59 \text{ MeV}, \quad (2)$$

where tumor t_D is the hypoxic tumor cell t_D [10–15].

Thus CGE should be assessed as a one of the essential factors for types of cancer staging, since it expresses the rate of cell growth and through which the anatomic extent which the disease has spread and the histologic classification can be identified. Hopefully, through Emad technology of advanced cellular mechanics [10–15] besides to what have been introduced about the molecular Arg physical background will prompt further exploration into the mechanism(s) of action of Arg and thus introduce new optimizing applications for this anticancer molecule, whose full therapeutic potential is yet to be realized.

Methods and Materials

Present study introduces a mathematical model that describes the capacity of L-Arg in stimulating the early

apoptotic process in normal cells and the ability of L-Arg in PBS to induce tumor cell membrane damage to be predictable and controllable. Our model is based on staging and grading the histopathologic findings in samples of tumor cell lines, and those transplanted in mice. The histologic grade (H_G) of those samples without L-Arg ($H_{G,\text{Control}}$) or with L-Arg doses can be identified from ^3H -thymidine-based cell proliferation assay by the same way described for staging of the cancer shown by Moawad on condition of cell number constancy for all ^3H -TDR proliferation assay samples according to the following proposed equation:

$$E_{\text{L-ArgDose}} = E_{\text{SA}} = H_{G,\text{control}} - H_{G,\text{Sample}}, \quad (3)$$

where $H_{G,\text{Sample}}$ is the histologic grade of the sample after treatment by the studied drug, while $E_{\text{L-ArgDose}}$ is the energy of the added L-Arg dose consumed in stimulating apoptosis (SA) in the treated sample [10, 11]. Whereas $H_{G,\text{control}}$ is the histologic grade of the detected sample in the absence of L-Arg which is equal to

$$H_{G,\text{control}} = U \% \times E_{^3\text{H-TDR}} \text{MeV}, \quad (4)$$

where $U \%$ is the unlabeled fraction of the detected cells by ^3H -TDR and $E_{^3\text{H-TDR}}$ is the energy of the used tritiated thymidine. The increase in labeled index (Li) by ^3H -TDR in case of cell number constancy expresses the decrease in $H_{G,\text{Sample}}$ than $H_{G,\text{control}}$ as a result of SA due to treatment by the studied drug. And then $E_{\text{L-ArgDose}}$ can be measured through ^3H -TDR-Based cell proliferation assay in which $E_{\text{L-ArgDose}}$ is equivalent to the increase in Li by ^3H -TDR induced by that L-Arg dose from that of the control sample.

$$E_{\text{L-ArgDose}} = \% \text{ increase in Li by } ^3\text{H-TDR} \times E_{^3\text{H-TDR}}. \quad (5)$$

While the decrease in Li of ^3H -TDR due to CMD and the decrease in number of living cells requires determining each of the number of killed cells (C_K) and energy consumed in stimulating apoptosis (E_{SA}) to identify $E_{\text{L-ArgDose}}$.

In such a case

$$E_{\text{L-ArgDose}} = E_{\text{CMD}} + E_{\text{SA}}. \quad (6)$$

C_K in a treated sample can be determined through ^{51}Cr release assay (CRA) which measures the target CMD and death which relates to the loss of plasma membrane integrity leading to the leakage of cellular components which might vary considerably between different detected samples according to their histologic grade and the added doses of L-Arg. Hence, the percent of specific release of ^{51}Cr by different L-Arg doses represents the percent of C_K with respect to the initial number of the control sample (C_0). Thus,

$$C_K = \% \text{ of specific release of } ^{51}\text{Cr} \times C_0. \quad (7)$$

Accordingly, the mechanism by which energy of the L-Arg dose ($E_{L-ArgDose}$) acts on those samples of cancer cell lines can be divided into two dependent actions:

- (1) Stimulating Apoptosis (SA) by decreasing cell cycle arrest (CCA) for all the treated cancer cells, where part of energy of L-Arg dose that consumed in SA is denoted by E_{SA} .

$$E_{SA} = (C_0 - C_K) \times \left(\frac{H_{GControl}}{C_0} - \frac{H_G}{(C_0 - C_K)} \right) \quad (8)$$

- (2) CMD that occurs when $(E_{SA}/Cell) > (H_{GControl}/Cell)$, where part of energy of L-Arg dose that consumed to induce CMD is denoted by E_{CMD} .

$$E_{CMD} = \frac{C_K}{C_0} \times H_{GControl} \quad (9)$$

Thus, an efficient estimation model can be established to describe the energy yield by L-Arg dose through each of 3H -TDR assay and CRA to check the presented staging model for the efficiency of L-Arg therapy.

In-Vitro Staging of Normal and Tumor Cell Lines Treated with L-Arg

As conducted and described by Shukla et al. [16]; normal cells of human, murine erythrocytes, murine splenic lymphocytes, NIH 3T3 cell lines and lymphosarcoma ascites (LSA) and fibrosarcoma (FS) are tumors of Swiss mouse origin and have been described earlier [17, 18]. Tumor cell lines FS and LSA were cultured in RPMI-1640 medium, while NIH 3T3 was cultured in DMEM medium. Both RPMI1640 and DMEM were supplemented with sodium bicarbonate (2 g/L), 2-mercaptoethanol (5×10^5 M), 10 % heat inactivated fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 µg/mL). L-Arg free RPMI-1640, PBS and DMEM medium was used to prepare various L-Arg solutions for studies. In case of L-Arg free RPMI-1640 and PBS known amount L-Arg powder was added to make desired concentration. DMEM containing 0.48 mM of L-Arg and RPMI 1640 containing 1.1 mM of L-Arg was used for 24 h culture. The cytotoxicity of FS, LSA and NIH 3T3 cell lines was assessed by 3H -thymidine-based cell proliferation assay. Briefly, tumor cells (FS and LSA) ($10^5/100$ µL) in 96 well plate were treated with different concentrations of L-Arg in PBS. After 1 h incubation at 37 °C, 100 µL of medium containing 20 % FCS was added to all the wells. After 24 h, the cells were pulsed with 3H -thymidine (1 µCi/well; specific activity: 6.5 Ci/mmol, Board of Radiation and Isotope Technology, Department of Atomic Energy, Mumbai, India). The cultures were harvested after 22 h on glass fiber filters using a Multimash-2000 harvester

(Dynatech Laboratories Inc., USA). The incorporated radioactivity was counted in a toluene-based scintillation fluid using LS Analyzer (TriCarb 2900 TR Packard, A Packard Bioscience Company). The effect of treatment with L-Arg in PBS environment up to 50 mM on normal cells of human and murine erythrocytes, murine splenic lymphocytes and NIH 3T3 cell lines were investigated by 3H -thymidine-based cell proliferation assay ($5 \mu Ci/10^6$ cells). Radioactivity in the tumor cell lines FS, LSA were expressed as Labeled index (Li) shown in Fig. 1 and Table 1, while Li of normal cell line NIH 3T3 groups are shown in Table 2. Controls indicate thymidine incorporation in cells treated in the absence of L-Arg. The involvement of NO, NOs (nitrite + nitrate) levels in the supernatant of LSA tumor cells ($1 \times 10^6/mL$) treated with 10 mM of Arg in PBS environment were assayed using the commercial nitric oxide assay kit (Calbiochem, Germany) as per the manufacturer's protocol.

^{51}Cr Chromium Release Assay (CRA)

As conducted and described by Shukla et al. [16]; CRA with tumor cell line LSA was performed. Briefly, LSA tumor cells ($18 \times 10^6/320$ µL) were labeled with 180 µL $Na_2^{51}CrO_4$ (180 µCi; specific activity of 4.3 mCi/mmol of ^{51}Cr in the form of $Na_2^{51}CrO_4$ in isotonic saline; Board of Radiation and Isotope Technology, Department of Atomic Energy, Mumbai, India) by incubating at 37 °C. for 1 h. The ^{51}Cr labeled cells were washed with excess of medium to remove free $Na_2^{51}CrO_4$ and finally suspended either in PBS or medium. Labeled cells ($10^5/100$ µL) in 96 well plate were treated with different concentration of L-Arg in PBS or medium for 1 h at 37 °C in hexareplicates. After incubation the plates were centrifuged and 50 µL supernatants were added to dioxane-based scintillation cocktail (10 g PPO, 250 mg POPOP, 100 g naphthalene dissolved in 1 l dioxane). The radioactivity was measured in LS Analyzer (TriCarb 2900 TR Packard, A Packard Bioscience Company) using an energy window of 0–2,000 keV. Radioactivity was expressed as counts per minute (cpm) which was used for statistical analysis. Appropriate low controls (LC), which indicates the spontaneous release in the supernatant in the absence of L-Arg in the respective group and high controls (HC), which indicate the radioactive release in the supernatant in 10 % sodium dodecyl sulphate (SDS) treated cells were included. Percent specific release of ^{51}Cr was shown in Table 3 and calculated by the following formula:

$$\begin{aligned} \% \text{ Specific } ^{51}Cr \text{ release} = & \\ & \frac{[(\text{Radioactivity in Arg-treated group} - LC) / (HC - LC)]}{\times 100} \end{aligned} \quad (10)$$

Fig. 1 Li of Arg-treated FS and LSA groups by ^3H -TDR versus L-Arg dose in mM

Table 1 L-Arg dose in mM and the corresponding Li of Arg-treated FS and LSA groups by ^3H -TDR

L-Arg dose in mM	Li of FS treated-groups (%)	Li of LSA treated-groups (%)
0 (Control)	66.67	50
10	76.67	25
50	44.67	11.25
150	38.67	7.0833
300	32	2.5

Table 2 L-Arg dose in mM and the corresponding Li of Arg-treated NIH-3T3 groups by ^3H -TDR

L-Arg dose in mM	Li of NIH-3T3 treated-groups (%)
0	83.33
10	85.33
20	87.5
40	85.33
50	87.5

Table 3 L-Arg doses in mM added to LSA samples and the corresponding % of the specific ^{51}Cr release

L-Arg dose in mM	% of the specific ^{51}Cr release (%)
0	0
10	15
50	19
150	21
300	36

In-Vivo Staging of Murine Tumors After L-Arg Therapy

As conducted and described by Shukla et al. [16]; single cell suspension of the FS cells were prepared as reported earlier and injected subcutaneously at ventro-lateral position of mice on day 0 [18]. FS therapy was initiated on day 7, post tumor transplantation when the average tumor diameter was 4.5–5.6 mm. The treatment was continued on

day 8, 9 and 10th day of tumor transplantation. Various drug delivery schedules as mentioned in results were injected intratumorally 0.02 ml/gram body wt. into the centre of tumor base, once a day. Injection was performed slowly over 30 s without moving the needle, to ensure uniform diffusion of the solution. For these injections, no leakage of solution around the needle or out of the tumor was observed. Tumor diameter and survivals were monitored. When tumor diameter reached 25 mm in FS bearing mice, animals were sacrificed. The day of sacrifice was considered for monitoring the survival period. The mice which were tumor free on day 30 were monitored for the presence of visible tumors. Tumor free mice surviving on day 120 of tumor transplantation were considered cured of tumor.

Results and Analysis

In-Vitro Staging of Tumor Cell Lines by ^3H -TDR-Based Cell Proliferation Assay and ^{51}Cr Release Assay

The total number of cells decreased compared to the initial total number of cells in all treated samples of tumor cell line LSA. Accordingly, Labeled index (Li) of LSA treated groups by ^3H -TDR was decreased in dose dependent manner due to C_K rate induced by L-Arg doses as shown in Fig. 1 and Table 1. While, the total number of cells was constant in the treated FS groups up to 10 mM and then decreased by increasing the added L-Arg dose. Accordingly, the Li by ^3H -TDR of FS treated groups up to 10 mM were increased and then decreased by the increase of L-Arg dose due to C_K as shown in Fig. 1 and Table 1. On the other hand, in CRA which was conducted for LSA cell line only, the percentages of the specific ^{51}Cr release for the detected samples which reflects the loss of plasma membrane integrity due to L-Arg doses compared to that of the highest control of LSA cell line (without L-Arg) are shown in Table 3.

Accordingly, the histologic grades of the control and treated groups of the tumour cell lines can be identified as follows:

The released energy of 1 μCi of tritiated thymidine (half-life time = 12.32 years, decay energy = 0.01859 MeV [19]) during the incubation of 22 h is equivalent to:

$$E_{\text{H-TDR}} = 1 \times 10^{-6} \times 3.7 \times 10^{10} \times \frac{12.32 \times 3.16 \times 10^7}{\ln 2} \times \left(1 - e^{-\frac{\ln 2 \times 22 \times 60 \times 60}{12.32 \times 3.16 \times 10^7}}\right) \times 0.01859 = 5.44641941 \times 10^7 \text{ MeV}.$$

Thus, from Eq. 4 and Table 1 the histologic grades of the control samples are:

$$H_{\text{G,control.FS}} = (100 - 66.67) \% \times 5.44641941 \times 10^7 \\ = 1.81547314 \times 10^7 \text{ MeV},$$

$$H_{\text{G,control.LSA}} = (100 - 50) \% \times 5.44641941 \times 10^7 \\ = 2.72320971 \times 10^7 \text{ MeV}$$

From Table 1, the tumor cell line FS with 10 mM L-Arg is the unique sample of increased Li by 10 % induced by L-Arg dose. Accordingly the energy yield by 10 mM L-Arg along with the histologic grade of the FS sample can be identified as follows:

% increase in Li = 10 %,

$$E_{\text{H-TDR}} = 5.44641941 \times 10^7 \text{ MeV}.$$

And from Eq. 5, the energy yield by 10 mM of L-Arg was equivalent to:

$$E_{\text{L-Arg 10mM}} = 10 \% \times 5.44641941 \times 10^7 \\ = 5.44641941 \times 10^6 \text{ MeV}.$$

Then from Eq. 3, the histologic grade of the FS sample after the treatment with 10 mM of L-Arg was identified as follows:

$5.44641941 \times 10^6 = 1.81547314 \times 10^7 - H_{\text{G,FS + 10mM}}$
 $\Rightarrow H_{\text{G,FS+10mM}} = 1.27083120 \times 10^7 \text{ MeV}$, while energy yield by other L-Arg doses in the rest FS treated samples can't be determined in similar way through Eqs. 5 and 3 due to the induced killed cells (C_K) by those doses and the decrease in the number of cells of those samples as previously explained. While, the histologic grade of the

treated LSA sample by 10 mM of L-Arg is proposed according to Eq. 3 to be equivalent to

$$H_{\text{G,LSA+10mM}} = 2.72320971 \times 10^7 - 5.44641941 \times 10^6 \\ = 2.17856776 \times 10^7 \text{ MeV}.$$

Such staging value can be reached also from Table 3 and through Eqs. 6–9 as follows:

From Table 3 the percentage of the specific ^{51}Cr release of LSA sample by 10 mM of L-Arg was 15 %, then according to Eq. 7 number of C_K in this sample is equivalent to $C_K = 15 \% \times 10^5 = 15,000$ cells.

Thus from Eq. 9, the part of energy of 10 mM dose of L-Arg consumed in CMD was as follows:

$$E_{\text{CMD}} = (15,000/100,000) \times 2.72320971 \times 10^7 \\ = 4.08481456 \times 10^6 \text{ MeV}.$$

While from Eq. 6, the other part of energy of 10 mM dose of L-Arg consumed in SA was as follows:

$$5.44641941 \times 10^6 = 4.08481456 \times 10^6 + E_{\text{SA}} \Rightarrow \\ E_{\text{SA}} = 5.44641941 \times 10^6 - 4.08481456 \times 10^6 \\ = 1.36160485 \times 10^6 \text{ MeV}$$

Thus from Eq. 8, the histologic grade of the treated LSA sample by 10 mM of L-Arg ($H_{\text{G,LSA+10mM}}$) was as follows:

$$1.36160485 \times 10^6 = (10^5 - 15,000) \\ \times \left(\frac{2.72320971 \times 10^7}{10^5} - \frac{H_{\text{G,LSA+10mM}}}{10^5 - 15,000} \right)$$

Thus, $H_{\text{G,LSA+10mM}}$ was equivalent to $2.17856776 \times 10^7 \text{ MeV}$ which is 100 % identical to what have been previously identified to confirm the reconciliation between the model of staging samples of constant number of cells before and after the treatment through Eqs. 3–5, and that of staging samples of decreasing number of cells due to cell death and boost the confidence in the hypothesized Eqs. 6–9 to estimate E_{SA} , E_{CMD} , and $E_{\text{L-ArgDose}}$.

Since Li by $^3\text{H-TDR}$ of all other treated LSA samples were decreased in dose dependent manner due to the induced C_K , then the histologic grade of the other treated LSA samples ($H_{\text{G,LSA}}$) would be decreased proportionally to the decrease in their corresponding Li by $^3\text{H-TDR}$ according to Eq. 11.

Table 4 L-Arg doses in mM added to LSA samples and the corresponding, C_K , $H_{\text{G,LSA+10mM}}$, E_{SA} , E_{CMD} , and $E_{\text{L-ArgDose}}$ in MeV

L-Arg dose	C_K	$H_{\text{G,LSA}}$ in MeV	E_{SA} in MeV	E_{CMD} in MeV	$E_{\text{L-ArgDose}}$ in MeV
0 (Control)	0	2.7232097×10^7	0	0	0
10	15,000	2.17856777×10^7	1.36160485×10^6	4.08481456×10^6	5.44641941×10^6
50	19,000	9.80355494×10^6	1.22544437×10^7	5.17409844×10^6	1.74285421×10^7
150	21,000	6.17260866×10^6	1.53407480×10^7	5.71874038×10^6	2.10594884×10^7
300	36,000	2.17856776×10^6	1.52499743×10^7	9.80355494×10^6	2.50535293×10^7

$$H_{G,LSA} = \frac{Li_{LSA+LArg}}{Li_{LSA+10mM}} \times H_{G,LSA+10mM} \quad (11)$$

Thus, $H_{G,LSA}$ of the other treated LSA samples were identified according to Eq. 11 and tabulated in Table 4.

Accordingly in CRA, from the percentage of the specific ^{51}Cr release derived from Eq. 10 shown in Table 3 the number of C_K in other LSA treated samples can be derived from Eq. 7. Consequently from Eqs. 9 and 8, the part of L-Arg dose energy consumed in CMD (E_{CMD}) along with the corresponding rest energy of L-Arg dose that consumed in stimulating apoptosis (E_{SA}) were identified for other LSA treated samples and hence from Eq. 6, $E_{L-ArgDose}$ for the other L-Arg doses were identified and tabulated in Table 4.

In order to test the hypothesis of estimating the energy yield by L-Arg doses in treating tumor cell line LSA through Eqs. 6–9, the estimated values of $E_{L-ArgDose}$ shown in Table 4 should be equivalent to the fractions of $E_{^3H-TDR}$ that corresponds to the differences induced by those L-Arg doses in Li by ^3H-TDR of tumor cell line FS samples.

$$i.e. E_{L-ArgDose} = [Li_{FS+10mM} - Li_{FS+LArgdose}] \times E_{^3H-TDR} \quad (12)$$

Accordingly, from Table 1 and Eq. 12

$$E_{L-Arg 50mM} = [0.7667 - 0.4267] \times 5.44641941 \times 10^7 \\ = 1.74285421 \times 10^7 \text{ MeV,}$$

$E_{L-Arg 150mM} = [0.7667 - 0.3867] \times 5.44641941 \times 10^7 = 2.06963938 \times 10^7 \text{ MeV,}$ and $E_{L-Arg 300mM} = [0.7667 - 0.32] \times 5.44641941 \times 10^7 = 2.43273400 \times 10^7$ which are 100, 98, and 97 %, respectively identical to the estimated value for $E_{LArg 50mM}$, $E_{LArg 150mM}$, and $E_{LArg 300mM}$ in Table 4 to provide a clear-cut criterion for accepting the hypothesis of the provided staging model in case of decreasing number of cells than that of the initials and leaving no room for doubt to accept the estimated values for $E_{L-Argdose}$. The comparison between $H_{G,LSA}$, E_{SA} , E_{CMD} and $E_{L-Argdose}$ in MeV in treated LSA groups by L-Arg doses shown in Fig. 2 clarify a non-linear relationship between the L-Arg dose and its corresponding energy which

indicates that energy yield of single dose more than 100 mM is less than that of 2 fractions dose of 50 mM each.

Also constancy in E_{SA} induced by each of 150 and 300 mM shown in Fig. 2 doesn't mean that both doses have the same effect in SA where the living cells in both treated samples were different by 21 %. The histologic grade at the cellular level of each treated LSA sample ($E_{LSA Cell}$) can be calculated as follows:

$$E_{LSACell} = \frac{H_{G,LSA}}{(C_0 - C_K)} \text{ MeV} \quad (13)$$

which shows that $E_{LSA Cell}$ was decreased gradually from 272.32 MeV in the control sample in dose dependent manner to 256.3, 121.0, 78.13 and 34.0 MeV (87.5 %) in the LSA treated sample by 10, 50, 150 and 300 mM L-Arg dose, respectively. Those staging grades presented through our staging model are consistent with the experimental findings of the extensive tumor cell lines membrane damage leading to its death caused by L-Arg delivered in PBS (L-Arg-P) up to 50 mM, and that L-Arg at 150 mM and above irrespective of chirality and incubation vehicle became an effective antitumor molecule against all the cell lines tested.

Moreover, the perfect correlation between the quantity of L-Arg dose and its corresponding energy ($r = 0.99$) boost the confidence to establish the following efficient estimation model shown in Fig. 3 with a good fit ($R^2 = 0.98$) to describe the energy yield by L-Arg dose rate;

$$E_{L-ArgDose} = 5545,667.294 \times \ln(\text{Dose}_{L-Arg}) \\ - 6336,299.039 \text{ MeV,} \quad (14)$$

where Dose_{L-Arg} is the quantity of L-Arg dose in mM while $E_{L-ArgDose}$ is the corresponding energy yield of that dose in MeV.

In NOs assay, Arg at 10 mM delivered in PBS and not in medium demonstrated that there is no increased production of NO in Arg-P group compared to the control group. These results indicate that NO is not contributing to the anticancer effect of Arg through its chemical structure.

Fig. 2 $H_{G,LSA}$, E_{SA} , E_{CMD} and $E_{L-Argdose}$ in MeV in treated LSA groups by L-arginine

Fig. 3 Energy in MeV yield by L-arginine doses in mM

In-Vivo Staging of Murine Tumors After L-Arg Therapy

Tumor necrosis, shrinkage and dose-dependent tumor cure of solid FS were observed. L-Arg cured mice bearing solid tumor (FS) at concentration of 287 mM (1,000 mg/Kg body wt.) per injection administered intratumorally either in medium or PBS at the tumor site with 100 % survival rate. Murine skin at the tumor injection site healed with no scarring. No any systemic toxicity was observed in the Arg-treated mice in all the experimental groups. Thus, the histologic grade of the FS tumour ($H_{G,FS,Tumour} = H_{FS,Tumour}$) was equivalent to the energy yield by L-Arg dose of 287 mM delivered in the 7th, 8th, 9th, and 10th days post tumor transplantation, i.e. $H_{G,FS,Tumour} = 4 \times E_{L-Arg,Dose,287}$

From the estimation model shown in Eq. 14, the predicted value of the energy yield by L-Arg dose of 287 mM ($E_{L-Arg,Dose,287}$) is equal to 2.505×10^7 MeV.

Accordingly, the identified histologic grade of the treated FS tumour ($H_{G,FS,Tumour}$) by 4 doses of 287 mM of L-Arg experimentally was as follows:

$$H_{G,FS,Tumour} = 4 \times 2.505 \times 10^7 = 1.002 \times 10^8 \text{ MeV.}$$

In order to check the identified $H_{G,FS,Tumour}$ experimentally and the results of our estimation model for the energy yield by L-Arg, a clinical staging for the FS tumor was performed according to the provided data of FS tumor monitoring and the clinical model of cancer staging introduced by Moawad [10–13, 15]. Such clinical model is based on measuring tumor t_D which expresses the rate of growth by imaging techniques and estimating the percentage of the tumor hypoxic cells ($H\%$) whose value can be estimated by the following equation:

$$H = 1.711936952 [E_{Hypoxic,cell} (\text{MeV}) / (M\% \times C_0)] - 1.448074675, \quad (15)$$

where C_0 the total number of tumor cells and $M\%$ is the malignant tumor fraction which can be estimated also by the following equation:

$$M = 79.53 E_{Hypoxic,cell} - 313.94, \quad E_{Hypoxic,cell} \leq 5.2 \text{ Emad} \\ (R^2 = 0.98), \quad M = 100 \quad \text{for} \quad E_{Hypoxic,cell} \geq 5.2 \text{ Emad}, \quad (16)$$

while $E_{Hypoxic,cell}$ is the energy of the hypoxic cell can be estimated from Eqs. 1 and 2 after measuring the tumour t_D [10, 11]. Thus, providing that patient-specific histologic grade ($H_{G,Tumour}$) is the summation of energies of the hypoxic cells ($\sum E_{Hypoxic,cell}$) then:

$$H_{G,Tumour} = \sum E_{Hypoxic,cell} \\ = \%M \times \%H \times C_0 \times E_{Hypoxic,cell} \\ \times 23234.59 \text{ MeV.} \quad (17)$$

[10, 11].

Thus, from the provided data of the FS tumor monitoring; 100 μg (10^5 cells) of single cell suspension of FS cells were transplanted in mice and L-Arg tumor therapy was initiated on day 7, post tumor transplantation when the average tumor diameter (4.5–5.6 mm) was 5.05 mm.

Thus, tumor weight became 65.646342 mg of $t_D = 0.747977531$ d.

Then from Eq. 1, the energy of the hypoxic cell was identified as follows:

$$E_{Hypoxic,Cell} = \ln \left[\left(\ln \frac{\ln 2}{0.747977531 \times 24 \times 60 \times 60} \right)^2 \right] \\ = 4.88 \text{ Emad.}$$

While from Eqs. 16, 15 and 17 $\Rightarrow M\% = 73.8\%$, $H\% = 1.18\%$.

Thus, the identified histologic grade of the FS tumour clinically according to the clinical model introduced by Moawad is equivalent to $\Rightarrow H_{G,FS,Tumour} = 0.738 \times 0.0118 \times 4.88 \times 23234.59 \times 10^5 = 9.874 \times 10^7$ MeV which is 99 % identical to what has been identified experimentally for the histologic grade of the FS tumour through treatment by L-Arg. This matching adds a strong boost to the results of the L-Arg energy yield estimation model.

Effects of L-Arg on Normal Cells

The effect of treatment with L-Arg in PBS environment up to 50 mM on normal cells like human and murine erythrocytes, murine splenic lymphocytes and NIH 3T3 cell lines was investigated. The treatment of both murine and human erythrocytes with L-Arg-P up to 50 mM for 1 h was

non toxic suggesting that L-Arg stabilizes the erythrocyte membrane and prevents hypotonic induced hemolysis and significantly enhanced proliferation of splenocytes, when compared to the control as revealed in ^3H -thymidine-based cell proliferation assay. As shown in Table 2, the Li by ^3H -TDR ($5 \mu\text{Ci}/10^6$ cells) increased from 83.33 % in the control group of 10^6 normal cells to 85.33 % in 10 mM to 87.5 % in 20 mM and then decreased to $85\frac{5}{12}$ % in 40 mM to increase once again to 87.5 % in 50 mM L-Arg-P treated group. Thus, the histologic grade of the control group of normal cell ($H_{G.NIH3T3Control}$) was decreased to $H_{G.NIH 3T3+10mM}$ and to $H_{G.NIH 3T3+50mM}$ by the percentage of increase in Li by ^3H -TDR induced by those doses of L-Arg [10, 11]

$$\text{i.e. } (H_{G.NIH3T3Control} - H_{G.NIH3T3+10mM}) \\ = [U_{Control} \% - U_{NIH3T3+10mM} \%] \times E_{^3H-TDR}$$

$$\text{Thus } (H_{G.NIH3T3Control} - H_{G.NIH3T3+10mM}) = [(1 - 83.33 \%) - (1 - 85.33 \%)] \times 5 \times 5.44641941 \times 10^7 = 5.44641941 \times 10^6 \text{ MeV.}$$

$$\text{While } (H_{G.NIH3T3Control} - H_{G.NIH3T3+50mM}) = [U_{Control} \% - U_{NIH3T3+20mM} \% + U_{NIH3T3+40mM} \% - U_{NIH3T3+50mM} \%] \times E_{^3H-TDR} [10, 11] \Rightarrow [(1 - 83.33 \%) - (1 - 87.5 \%) + (1 - 85.33 \%) - (1 - 87.5 \%)] \times 5 \times 5.44641941 \times 10^7 = 1.72651495 \times 10^7 \text{ MeV.}$$

Those reductions in the histologic grade of the normal cells NIH 3T3 are 100 and 99 %, respectively identical to what have been measured for the energy yield by the 10 and 50 mM doses of L-Arg in the in vitro staging of tumor cell line LSA shown in Table 4 and confirmed in testing the in vitro staging of tumor cell line FS which confirms and provides a clear-cut criterion for accepting the hypothesis of the presented staging model in case of decreasing number of cells than that of the initials and leaving no room for doubt to administer the appropriate L-Arg dose by the provided estimation model as conducted with high efficacy in the introduced regional L-Arg therapy for murine tumors.

Discussion

The purpose of this study is to improve L-Arg regional therapy and develop future strategies to settle down a new protocol for optimizing the proper ranges of L-Arg doses. A pathologic methodology for staging normal, cancer cell lines and a clinical one for staging tumors were conducted as described in earlier studies to determine the energy yield of L-Arg doses [10, 11]. The efficient estimation model of current approach ($R^2 = 0.98$) to estimate energy yield of L-Arg dose enables to find out dose equivalency between L-Arg doses and different drugs used for therapeutic interventions. Since apoptosis is considered a normal self adaptation process belongs to normal cells of doubling time

ranged from that of cells at natural background radiation (NBR) to that of cells at maximum tolerated dose (MTD) settled by Moawad [13]. Accordingly, cells of doubling time longer than that range fail to the spontaneous apoptosis inducing CCA as commonly shared by the malignant cells. As shown from Table (3) rates of killed cells (C_K) in the treated tumor cell line LSA by L-Arg were increased in dose-dependent manner decreasing CCA and stimulating apoptosis. Thus, L-Arg acts via restoring the normal range of cell doubling time to undergo the apoptotic process. Such decrease in CCA occurs due to the electrostatic binding between the negatively charged components of cancer cells and the positively charged ACPs contained in the Arg. The CCA decreases gradually by the increase of the added L-Arg dose that induce a gradual CMD till cell killing through necrosis shown by the release of cytochrome c and the leakage of cellular components and consequently the increase in killed cells as observed in CRA. Such mechanism of the anticancer effect of the Arg is not metabolically driven but through its chemical structure which has been supported and confirmed through each of the presented efficient model to describe the energy yield by L-Arg dose and the NO assay. In the presented NO assay, LSA tumor cells treated with 10 mM of Arg in PBS environment does not produce NO in the supernatant, whereas all doses of Arg in PBS less than 50 mM damage the tumor cell membrane in the cell proliferation assay [16]. These findings confirm that the anticancer effect of the CMD observed was through a non metabolic process and that Arg pathway via the enzyme NOs does not seem to contribute to the anticancer effect of Arg through its chemical structure, i.e., was due to the strong binding of the electrostatic attraction between the negatively charged components of cancer cells and the positively charged ACPs contained in Arg. On the contrary, the mechanism of the anticancer effect of the Arg based on metabolism involves the production of NO besides to the use of NOS inhibitor. L-Arg is utilized by the body to make Arg derived NO. NO synthase (eNOS, or NOS I) is the enzyme responsible for this in the endothelium. NO dilates blood vessels that increase blood flow to tumors that may induce metastasis could be prevented using NOS inhibitor [20]. Arg derived NO production diminishes tumor blood flow but it is not clear that the enhanced blood flow due to Arg supplementation would enhance tumor growth. Thus targeting the anti-cancer effect of L-Arg on the basis of chemical structure whenever possible avoids the arguments against the anti-cancer effect on the basis of the metabolism that produces NO. The strong inverse correlation ($r = -0.98$) between the histologic grade of the treated tumour cell line LSA samples $H_{G.LSA}$ and the gradual loss of mitochondrial membrane potential without complete damage and cell killing represented by E_{SA} indicates that

through the electrostatic attraction between the negatively charged components of cancer cells and the positively charged ACPs in L-Arg, the process of CMD starts by the activation of mitochondria to lose membrane integrity gradually that down regulate the expression of oncogenes that could also play a role in the clearing of virus infected cells. Thus, by the increase of L-Arg dose E_{SA} increases and conversely H_G decreases gradually till vanishing at direct CMD. Consequently, the mechanism by which L-Arg acts on malignant cells especially when delivered in PBS is to activate several caspases, both the initiator caspases-8 and -9, the effector caspase-3, and caspases-1 and -2 that secondly activates the mitochondria as a self adaptation process to release the cytochrome c into the cytoplasm loosing its membrane potential exerting direct effects on tumor cells, including the down-regulation of oncogene expression and induction of tumor suppressor genes. Such adaptation process disrupts the mitochondrial function as a reflection of E_{SA} reducing cell doubling time (t_D) once again, and decreasing the frequency of oncogene to undergo apoptosis by the release of pro-apoptotic factors through the PT-pore [21]. Rate of apoptosis would be accelerated gradually by the increase of L-Arg dose in dose dependent manner, where by restoring the normal rates of the cell doubling time, rate of apoptosis becomes normal [21]. On the contrary, L-Arg has not shown neither increasing in rate of apoptosis nor DNA damage in normal cell lines like NIH 3T3 cell lines up to 20 mM, while treatment of human and murine erythrocytes up to 50 mM was non toxic. Moreover, L-Arg-P treatment significantly enhanced the induced proliferation of normal cell line NIH 3T3 when compared to the control without inducing CMD suggesting that L-Arg stabilizes the normal cell membrane for its low cytolytic activities to normal mammalian cells and erythrocytes. Thus, the cytotoxicity of L-Arg was selective to tumor cells, which is the most important parameter for the selection of an anticancer drug of low side effects. Thus, these findings suggest that the appropriate dose of L-Arg when delivered in saline (PBS) environment at the pharmacological concentration becomes an ideal anticancer molecule for targeting the accessible solid tumor with minimal damage to normal cells. Improving delivery skills can be performed by the use of a multi-hole needle to improve the distribution of the injectate drug over the regular end-hole needle and to ensure uniform dose absorption, avoiding leakage outside the cell of interest as well [12]. Injection skills using ultrasound or fluoroscopic guidance techniques have made precise needle insertions routinely feasible, which could be applied for intra-tumoral injection of these agents to ensure maximizing the tumor dose uniform distribution while monitoring its response to therapy in order to stop ineffective therapies and avoid complications caused by tumor progression [12]. This can

be detected through monitoring the tumor response through newer imaging techniques that combine SPECT with CT, or PET with CT, may improve quantitation of targeted radiodiagnostic agents so that non responding tumors can be identified early to modify the administered dose [12].

Conclusion

Targeting the anti-cancer effect of L-Arg on the basis of chemical structure whenever possible in cancer therapy avoids the confusions about targeting that based on drug metabolism during which NO is produced. The selective cytotoxicity to tumor cells with minimal damage to normal cells by L-Arg due to its chemical structure suggests to be considered the most promising drug for regional therapy of the accessible tumors like breast cancers of early stage with no distant metastasis. Regional L-Arg therapy shows that administering the optimum dose, skillful injection techniques in PBS environment and monitoring tumor response to therapy are three completely dependent objectives towards the treatment success.

Conflict of interest The author declares that there is no conflict of interest concerning this paper.

References

1. Tapiero H, Mathé G, Couvreur P, Tew KD. L-Arginine. *Biomed Pharmacother.* 2002;56(9):439–45 REVIEW. PMID 12481980.
2. Stechmiller JK, Childress B, Cowan L. Arginine supplementation and wound healing. *Nutr Clin Pract.* 2005;20(13):52–61 REVIEW. PMID 16207646.
3. Witte MB, Barbul A. Arginine physiology and its implication for wound healing. *Wound Repair Regen.* 2003;11(6):419–23 REVIEW. PMID 14617280.
4. Yurtcu E, Kasapoglu E, Sahin F. Protective effects of β -carotene and silymarin on human lymphocytes. *Turk J Biol.* 2012;36:47–52.
5. El-Missiry MA, Othman AI, Amer MA. L-Arginine ameliorates oxidative stress in alloxan-induced experimental diabetes mellitus. *J Appl Toxicol.* 2004;24(2):93–7.
6. Lin WT, Yang SC, Chen KT, Huang CC, Lee NY. Protective effects of L-arginine on pulmonary oxidative stress and antioxidant defenses during exhaustive exercise in rats. *Acta Pharmacol Sin.* 2005;26:992–9.
7. Nanthakumaran S, Brown I, Steven DH, Schofield AC. Inhibition of gastric cancer cell growth by arginine: molecular mechanisms of action. *Clin Nutr.* 2009;28:65–70.
8. Wolf C, Bruss M, Hanisch B, Gothert M, von Kugelgen I, Molderings GJ. Molecular basis for the antiproliferative effect of agmatine in tumor cells of colonic, hepatic, and neuronal origin. *Mol Pharmacol.* 2007;71:276–83.
9. Lemesko VV. Potential-dependent membrane permeabilization and mitochondrial aggregation caused by anticancer polyarginine-KLA peptides. *Arch Biochem Biophys.* 2010;493:213–20.
10. Moawad EY. Clinical and pathological staging of the cancer at the nanoscale. *Cancer Nano.* 2012;3:37–46.

11. Moawad EY. Reconciliation between the clinical and pathological staging of cancer. *Comp Clin Pathol*. 2012. doi:[10.1007/s00580-012-1603-6](https://doi.org/10.1007/s00580-012-1603-6).
12. Moawad E. Isolated system towards a successful radiotherapy treatment. *Nucl Med Mol Imaging*. 2010;44:123–36.
13. Moawad EY. Radiotherapy and risks of tumor regrowth or inducing second cancer. *Cancer Nano*. 2011;2:81–93.
14. Moawad EY. Optimizing bioethanol production through regulating yeast growth energy. *Syst Synth Biol*. 2012;6:61–8.
15. Moawad EY. Safe doses and cancer treatment evaluation. *Cancer Oncol Res*. 2013;1:6–11. doi:[10.13189/cor.2013.010102](https://doi.org/10.13189/cor.2013.010102).
16. Shukla J, Thakur VS, Poduval TB. Arginine: appropriate dose and delivery environment makes it an anticancer molecule. *Open Cancer J*. 2010;3:1–15.
17. Thakur VS, Shankar B, Chatterjee S, Premachandran S, Sainis KB. Role of tumor-derived transforming growth factor- β 1 (TGF- β 1) in site-dependent tumorigenicity of murine ascitic lymphosarcoma. *Cancer Immunol Immunother*. 2005;54:837–47.
18. Poduval TB, Seshadri M, Sundaram K. Lectin potentiation of BCG-contact-mediated antitumor action. *J Natl Cancer Inst (Bethesda)*. 1984;65:909–12.
19. Barbalace K. Periodic table of elements—H—hydrogen. 1995–2011. <http://environmentalchemistry.com/yogi/periodic/H-pg2.html>. Accessed online: 21 Dec 2011.
20. Lu W, Schroit AJ. Vascularization of melanoma by mobilization and remodeling of preexisting latent vessels to patency. *Cancer Res*. 2005;65:913–8.
21. Thyrell L, Erickson S, Zhivotovsky B, Pokrovskaja K, Sangfelt O, Castro J, Einhorn S, Grandér D. Mechanisms of interferon-alpha induced apoptosis in malignant cells. *Oncogene*. 2002; 21(8):1251–62.