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Novel Anticancer Curcumin Derivative with Conserved Functional Groups

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Abstract

Background: Curcumin, a polyphenol from turmeric, has potent anticancer properties in human cancer cell lines. Nevertheless, its multi-targeting clinical application in cancer and other diseases is hampered by its poor systemic bioavailability. A novel curcumin derivative "NCD" was developed through covalent modification of the curcumin "CUR" molecule on sites remote from its natural functional groups.

Methods: The NCD was tested for its anticancer effect against six human cancer cell lines in comparison to CUR and the standard anticancer drug Doxorubicin "DOX". *In vivo* structure-action relationship to the dose-dependent inducible plasma Glutathione S-transferase (GST) was studied. Plasma GST activity was estimated one and half and 24 hours after oral administration of each of CUR and the NCD.

Results: The NCD showed significantly lower Half-maximal inhibitory concentrations (IC50s) as compared to the DOX with each of HELA, the human cervical, MCF7, the human breast and HCT116, the human colonic cancer cell lines. Significant improvements in terms of lower IC50 as compared to CUR with all tested human cancer cell lines were also found. *In vivo*, the NCD showed significant elevations in inducible plasma GST in the 1.5 hr and the 24 hr duration studies as compared to CUR.

Conclusion: The IC50 anticancer effect of the NCD significantly was improved against six human cancer cell lines in comparison to CUR. The NCD retained an improved potency in terms of *in vivo* structure-action relationship to dose-dependent induction of plasma GST. With GST as biomarker for cancer, it should not be taken as parameter in studies and/or clinical trial involving both cancer and CUR as GST is dose-dependently inducible by CUR and some of its derivatives.

Introduction

While cancer initiation and development implicates often hundreds of genes and/or signaling cascades, most conventional chemotherapeutic agents used today were designed to hit a single intracellular target, meanwhile, exerting a number of frequent severe adverse side effects, together with lack of safety. Hence, efforts are directed to the development of multi-targeting therapies especially from natural phytochemicals which are safe and inexpensive.

Fulfillment of such potentials was found with curcumin (diferuloylmethane), a natural polyphenol from the rhizomes of *Curcuma longa*. Either native or modified, it exhibits the ability to affect multiple intracellular targets [1,2] potentially useful for both prevention and treatment of multifactorial diseases such as cancer, by inhibiting specific molecular signaling pathways involved in carcinogenesis [3-5]. The reported activity of curcumin against leukemia, lymphoma, gastrointestinal, genitourinary, breast, ovarian, head and neck squamous cell carcinoma, lung, melanoma, neurological cancers, and sarcomas reflects its ability to affect multiple targets [1-6]. *In vitro*, curcumin has been reported to be antiproliferative in a number of cells lines [4,5,7], with toxicity significantly higher in tumor cells compared to the normal cells [8]. Therefore, curcumin can be considered as an ideal lead compound for anticancer drug development with safety up to12 g/day [9-11], but its very poor bioavailability in both plasma and tissues [12,13] hampers the advancement of its clinical use.

To overcome such limitations, several approaches have been

tested; including the covalent modification of the curcumin molecule. For example, multiple myeloma remains an incurable malignancy despite the recent approval of new molecular targeted agents; however, a newly synthesized curcumin analogue showed 7 to 12 fold more potent growth suppression of myeloma cells [14]. Nevertheless, such covalent modifications need to be tested in terms of *in vivo* structure-action relationships. In this respect, several types of cellular and body fluids components were recognized as biomarkers for curcumin action, whose efficacy appears to be related to dose dependent induction of glutathione-S-transferase (GST), inhibition of prostaglandin-E2 production and suppression of oxidative DNA adduct M1G formation [15]. Glutathione S-transferases (GSTs), are a family of phase II detoxification enzymes that catalyze the conjugation of glutathione with a number of hydrophobic compounds. On the other hand, it is highly expressed in tumors and plasma of cancer patients; and is considered a useful prognostic factor to determine the clinical outcome of chemotherapy [16].

In the present study, a novel curcumin derivative "NCD" was developed through covalent modification of the curcumin molecule on sites remote from its natural functional groups. The first aim of the present work was to evaluate the anticancer effect of the "NCD" against some human cancer cell lines with the assumption of improvements to its natural potencies in terms of significantly lower micro molar IC50, in comparison with natural curcumin "CUR" and the standard anticancer drug Doxorubicin "DOX".

Secondly, to determine whether the "NCD" still retains the essential potencies of natural curcumin, as applied to living cells/organism. Accordingly, a comparative *in vivo* study of the plasma levels of the dose-dependent inducible GST, as a biomarker of curcumin action was conducted. Based on such planning, it should be possible to shed

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light on the firm conservation of the original biochemical, functions/ potencies of natural curcumin within the novel curcumin derivative.

Materials and Methods

A novel curcumin derivative "NCD" was developed through covalent modification of the curcumin molecule on sites remote from its natural functional groups. It was presented free of charge to the participating researchers as a personal non-profit scientific gift to help advancement of cooperation in national medical research. The novel derivative, (PCT/EG2008/000044, WO 2010/057503, Regional phase European Patent Application No. 08878223) was registered as international patent protected by the rights of "The Patent Co-operation Treaty" and is the personal property of its inventors, Rezq *et al.*, 2008 [17].

Test compounds: Standard Doxorubicin hydrochloride "DOX" (Adria-mycin, with mol. mass 579.98), natural curcumin "CUR" (with mean mol. mass 338.00) and the novel curcumin derivative "NCD", (with estimated mol. mass 636.00).

Human cell lines: Six different human cancer cell lines were used in this study together with the normal nonmalignant melanocytes cell line (HFB4). The human cancer cell lines included: the cervical cancer cell line (HELA), laryngeal carcinoma cell line (HEP2), breast cancer cell line (MCF7), hepatocellular cell line (HEPG2), colorectal cancer cell line (Caco2) and colonic cancer cell line (HCT116). All cell lines were purchased from the American Type Culture Collection (ATCC) Manassas, VA USA frozen in liquid nitrogen, then stored and maintained at the laboratories of the National Cancer Institute, Cairo, Egypt, by serial sub-culturing. Cell viability was determined by trypan blue exclusion method using the inverted microscope according to Stoddart, 2011[18].

Assay of anti cancer cytotoxic activity: The assay was carried out according to the SRB assay of Skehan *et al.*, 1990 [19]. The sensitivity of the human cancer cell lines to increasing serial concentrations equivalent to 0, 5, 12.5, 25 and 50 µg/ml of each of the standard Doxorubicin hydrochloride "DOX" (Adriamycin), natural curcumin "CUR" and the novel curcumin derivative "NCD" were compared.

Dose-dependent inducible plasma glutathione-S-transferase: One and half and 24 hours' duration studies were conducted to determine the dose response characteristics of curcumin CUR and its novel derivative NCD on inducible plasma GST. The study was car-ried on 170 male white albino rats, of an average weight 150-200 gm from an inbred colony (Curl: HEL 1), at the Kasr Al-Aini experimental animals unit, Faculty of Medicine, Cairo University. Rats were bred and maintained in air-conditioned pathogen-free conditions, with 12:12 hr daylight/darkness cycles and allowed free access to chow and water. Ethical protocols for animal treatment were followed with approval from the Institutional Animal Care Committee. The rats were divided into 17 groups, each of 10 rats: a control group, 4 duplicated groups that received pure curcumin suspended in 1 ml of water orally at doses equivalent to 37, 74,148 and 296 µM/kg

body weight respectively. In addition another 4 duplicated groups received the novel curcumin derivative dissolved in 1 ml of water orally on the same equimolar bases. The control group received an equal oral volume of water. One and half hour and 24 hours after oral administration of the respective doses of each compound, venous blood samples were withdrawn from the tail veins of the proper corresponding group, to estimate plasma GST activity according to the method of Habig *et al.*, 1974 [20].

Calculations and statistical analysis: Half-maximal inhibitory concentrations (IC50s) dose-response curves were constructed after conversion of the concentrations of the experimental test compounds with different molecular masses to micro molar equivalents for proper accurate comparisons. The (IC50s) were determined using the software package "OriginPro-8" of OriginLab Corporation USA. A nonlinear sigmoidal curve fit based on the "Boltzmann" model was applied using the equation: y = A2 + (A1-A2)/(1 + exp((x-x0)/dx)), where y = IC50. Standard and test compounds IC50 values in µmol/ml were calculated from the appropriate linear segment of the correlation line for each of the tested normal and human cancer cell lines. Each curve was constructed from 6X6 increasing serial concentrations of the test compound.

Arithmetic means of IC50 each representing six points with intra-assay S.D. of each tested compound were subjected to the Student t-test at p<0.05 to determine the significance of difference of mean IC50 values in µmol/ml using the software package "Statistica-8" of Statsoft Inc. USA. The percentage of difference between the IC50 of the three test compounds was calculated to compare their rela-tive potencies and/or improvements in terms of significantly lower micro molar IC50 if any.

The one and half hour and the extended, (24 hours) inducible plasma GST dose response curves for each of curcumin CUR and its novel derivative NCD were constructed using mean GST U/L ± S.D as percentage change relative to the normal control levels. The student t-test was applied at p<0.05 to test the significance of differences of mean plasma inducible GST levels allover the whole induction dose range of serial doubling equimolar concentrations of CUR and NCD. The non-linear distance weighted least square fit procedure was applied to construct correlation dose response lines of plasma inducible GST percentage of change to each of CUR and NCD.

Results

Following the mathematical and statistical analysis models applied in this study it was possible to reach solid inferences about the significance of difference of mean IC50 values in micro molar expres-sion and percentage of difference between the IC50 of each of the three tested compounds relative potencies and/or improvement.

The results of the comparative *in vivo* plasma levels of inducible glutathione-S-transferase, for the 1.5 and the 24 hours' duration studies are presented as mean percent change relative to the normal control levels together with the statistical analysis of the significance of differences in dose response characteristics of CUR and its novel derivative NCD on inducible plasma GST with respect to time and to each other.

Mean IC50 values: Table 1 and Figure 1, show the statistical analysis of the mean IC50 of the three tested compounds in µmol/ml ± intra-assay six point S.D. as tested against the one normal and six human cancer cell line cultures using the Student t-test at p-values <0.05, while Table 2 and Figure 2 show the percentage difference analysis of the same results.

It is clear from Table 1 and Figure 1 that both CUR and NCD have high human anticancer survival inhibitory potentials in the micro molar range against several human cancer cell line cultures. As for DOX, the most sensitive was the HFB4 human normal nonmalignant melanocyte cell line with a mean IC50 of 5.23±0.24 µmol/ml and the least sensitive was the HCT116 human colonic cancer cell line with a mean IC50 of 7.46±0.95 µmol/ml. Curcumin showed the same towards the HFB4 human normal nonmalignant melanocyte cell line with a mean IC50 of 7.64±0.08 µmol/ml and was least effective against the CACO2 human colorectal cancer cell line with a mean IC50 of 5.27±0.19 µmol/ml. However, as for NCD, it showed improved lower mean IC50 dose response as compared to CUR, being most effective against the HCT116 human colonic cancer cell line with a mean IC50 of 5.34±0.43 µmol/ml and least effective against the CACO2 human colorectal cancer cell line with a mean IC50 of 6.13±0.50 µmol/ml. An over all comparison showed that the micro molar IC50 of CUR is significantly higher than the standard DOX with all tested human cancer cell lines and that NCD has significantly lower mean IC50 dose response as compared to DOX in case of HELA, the human cervical cancer cell line, MCF7, the human breast cancer cell line and the HCT116 human colonic cancer cell line. However, NCD showed a statistically non-significant difference from DOX with both HEP2, the larynx human cancer cell line and HEPG2, the human hepatic cancer cell line, while it was less effective than DOX against the HFB4 human normal nonmalignant melanocyte cell line and CACO2 the human colorectal cancer cell line. Nevertheless, going with the research assumption, the NCD showed the "desirable" high improvement; with statistically significantly lower mean IC50 dose response as compared to CUR with all tested human cancer cell lines.

Mean IC50 percentage change: Table 2 and Figure 2 express the results in terms of percentage difference of IC50 of the three tested compounds against the one normal and six human cancer cell lines to compare the relative potencies and/or improvements in terms of significantly lower IC50 if any.

The micro molar IC50 of CUR is higher than the standard DOX with all tested human normal nonmalignant and cancer cell lines ranging from 108.7 to 245.0 percent for HELA, the human cervical cancer cell line and CACO2 the human colorectal cancer cell line respectively. The same applies for NCD against the standard DOX with statistically significantly lower micro molar IC50 ranging from 71.6 to 96.8 percent for HCT116 human colonic cancer cell line and HEP2, the larynx human cancer cell line respectively except with HFB4 normal nonmalignant melanocytes cell line and the human colorectal cancer CACO2 which showed higher percent values of 113.2 and 116.3 % respectively.

On the other hand, and going with the research assumption, the NCD showed high improvement with statistically significantly lower micro molar IC50 than CUR with all tested human normal nonmalignant and cancer cell lines ranging from 42.5 to 77.5 percent for HEP2, the larynx human cancer cell line and HFB4 normal nonmalignant melanocytes cell line respectively.

Inducible GST: Table 3 and Figure 3 show the results of the comparative *in vivo* plasma levels of inducible glutathione-S-transferase, for the 1.5 and the 24 hours' duration studies as mean percent change
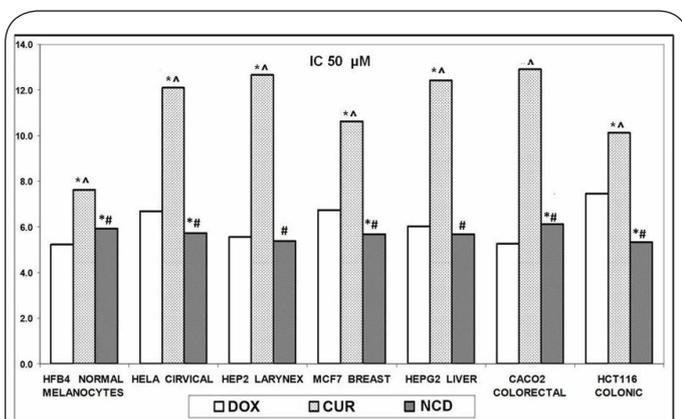

Figure 1. Relative micro molar IC50 of DOX, CUR and NCD against different human cancer cell lines
   * = Statistically significantly different from DOX.
   # = Statistically significantly different from CUR.
   ^ = Statistically significantly different from NCD.

Table 1. Statistical analysis of the difference in IC50 of CUR and NCD as compared to DOX against different human cancer cell lines.

CELL LINE TYPE	DOX IC50 µmol/ml MEAN ± S.D.	CUR IC50 µmol/ml MEAN ± S.D.	NCD IC50 µmol/ml MEAN ± S.D.
HFB4 NORMAL NONMALIGNANT MELANOCYTES	5.23 ± 0.24	7.64 ± 0.08 t-test vs. DOX p<0.001 ↑	5.92 ± 0.61 t-test vs. DOX p<0.05 ↑ vs. CUR p<0.001 ↓
HELA CERVICAL CANCER	6.69 ± 0.49	12.09 ± 1.16 t-test vs. DOX p<0.001 ↑	5.73 ± 0.54 t-test vs. DOX p<0.05 ↓ vs. CUR p<0.001 ↓
HEP2 LARYNEX CANCER	5.56 ± 0.43	12.67 ± 1.09 t-test vs. DOX p<0.001 ↑	5.38 ± 0.39 t-test vs. DOX = N.S. ↓ vs. CUR p<0.001 ↓
MCF7 BREAST CANCER	6.75 ± 0.87	10.63 ± 0.82 t-test vs. DOX p<0.001 ↑	5.67 ± 0.59 t-test vs. DOX p<0.05 ↓ vs. CUR p<0.001 ↓
HEPG2 HEPATIC CANCER	6.03 ± 0.58	12.43 ± 0.78 t-test vs. DOX p<0.001 ↑	5.68 ± 0.61 t-test vs. DOX = N.S. ↓ vs. CUR p<0.001 ↓
CACO2 COLORECTAL CANCER HCT116 COLONIC CANCER	5.27 ± 0.19	12.91 ± 0.47 t-test vs. DOX p<0.001 ↑	6.13 ± 0.50 t-test vs. DOX p<0.005 ↑ vs. CUR p<0.001 ↓
HCT116 COLONIC CANCER	7.46 ± 0.95	10.12 ± 0.72 t-test vs. DOX p<0.001 ↑	5.34 ± 0.43 t-test vs. DOX p<0.001 ↓ vs. CUR p<0.001 ↓

Table 2. Percentage difference in IC50 of CUR and NCD as compared to DOX against different human cancer cell lines.

CELL LINE TYPE	DOX IC50 μmol/ml MEAN ± S.D.	CUR IC50 μmol/ml MEAN ± S.D.	NCD IC50 μmol/ml MEAN ± S.D.
HFB4 NORMAL NONMA-LIGNANT MELANOCYTES	5.23 ± 0.24	7.64 ± 0.08 percent % of DOX = 146.1 ↑	5.92 ± 0.61 percent % of DOX = 113.2 ↑ % of CUR = 77.5 ↓
HELA CERVICAL CANCER	6.69 ± 0.49	12.09 ± 1.16 percent % of DOX = 108.7 ↑	5.73 ± 0.54 percent % of DOX = 85.7 ↓ % of CUR = 47.4 ↓
HEP2 LARYNEX CANCER	5.56 ± 0.43	12.67 ± 1.09 percent % of DOX = 227.9 ↑	5.38 ± 0.39 percent % of DOX = 96.8 ↓ % of CUR = 42.5 ↓
MCF7 BREAST CANCER	6.75 ± 0.87	10.63 ± 0.82 percent % of DOX = 157.5 ↑	5.67 ± 0.59 percent % of DOX = 86.0 ↓ % of CUR = 52.7 ↓
HEPG2 HEPATIC CANCER	6.03 ± 0.58	12.43 ± 0.78 percent % of DOX = 206.1 ↑	5.68 ± 0.61 percent % of DOX = 94.2 ↓ % of CUR = 45.7 ↓
CACO2 COLORECTAL CANCER	5.27 ± 0.19	12.91 ± 0.47 percent % of DOX = 245.0 ↑	6.13 ± 0.50 percent % of DOX = 116.3 ↑ % of CUR = 47.5 ↓
HCT116 COLONIC CANCER	7.46 ± 0.95	10.12 ± 0.72 percent % of DOX = 135.7 ↑	5.34 ± 0.43 percent % of DOX = 71.6 ↓ % of CUR = 52.8 ↓

Table 3. Plasma inducible GST (U/L), Mean ± S.D., percent change from control and statistical analysis of differences with respect to time and CUR vs. NCD.

CONTROL = 164.23 ± 8.56 U/L				
CUR / TIME	CUR 37 μM/kg	CUR 74 μM/kg	CUR 148 μM/kg	CUR 296 μM/kg
1½ hr	162.71 ± 8.12 Percent -0.59 ↓	161.26 ± 8.25 Percent -1.61 ↓	207.27 ± 13.50 Percent 26.63 ↑	213.49 ± 11.16 Percent 30.41 ↑
24 hr	164.87 ± 5.46 Percent 0.59 ↑	162.10 ± 6.34 Percent -1.04 ↓	172.53 ± 10.16 Percent 5.28 ↑	181.97 ± 9.98 Percent 11.05 ↑
t-test vs. 1½ hr	N.S. ↑	N.S. ↓	$p < 0.001$ ↓	$p < 0.001$ ↓
NCD / TIME	NCD 37 μM/kg	NCD 74 μM/kg	NCD 148 μM/kg	NCD 296 μM/kg
1½ hr	155.24 ± 9.61 Percent -5.36 ↓	158.14 ± 10.63 Percent -3.41 ↓	210.61 ± 9.99 Percent 28.56 ↑	249.22 ± 19.96 Percent 51.70 ↑
t-test vs. CUR	$p < 0.05$ ↓	N.S. ↓	N.S. ↑	$p < 0.001$ ↑
24 hr	153.96 ± 6.50 Percent -6.08 ↓	160.88 ± 13.47 Percent -1.64 ↓	177.33 ± 10.73 Percent 8.11 ↑	205.73 ± 20.59 Percent 25.36 ↑
t-test vs. 1½ hr	N.S. ↓	N.S. ↓	$p < 0.001$ ↓	$p < 0.001$ ↓
t-test vs. CUR	$p < 0.001$ ↓	N.S. ↓	N.S. ↑	$p < 0.005$ ↑

N.S. = Statistically Non-Significant difference.

Table 4. Non-linear distance weighted least square fit of dose response curves of mean plasma inducible GST percentage of change to each of CUR and NCD.

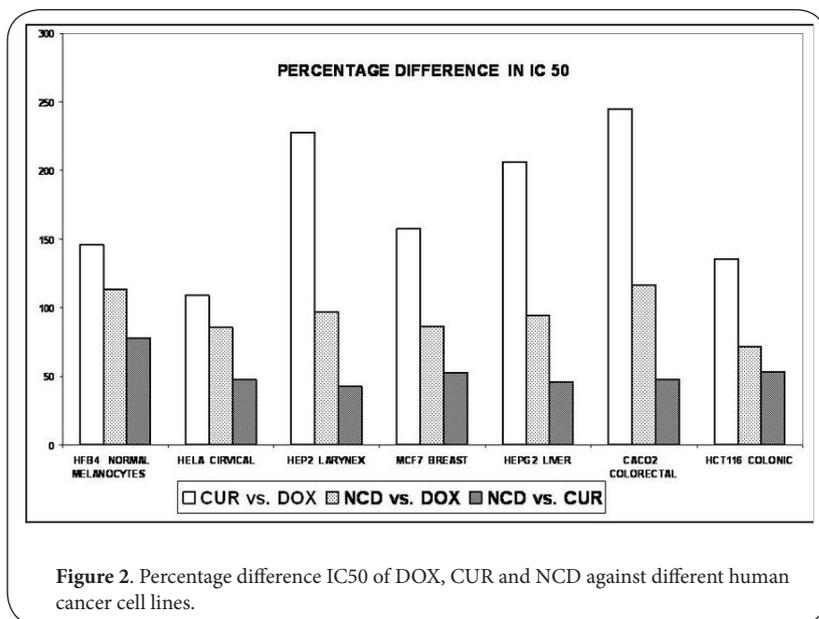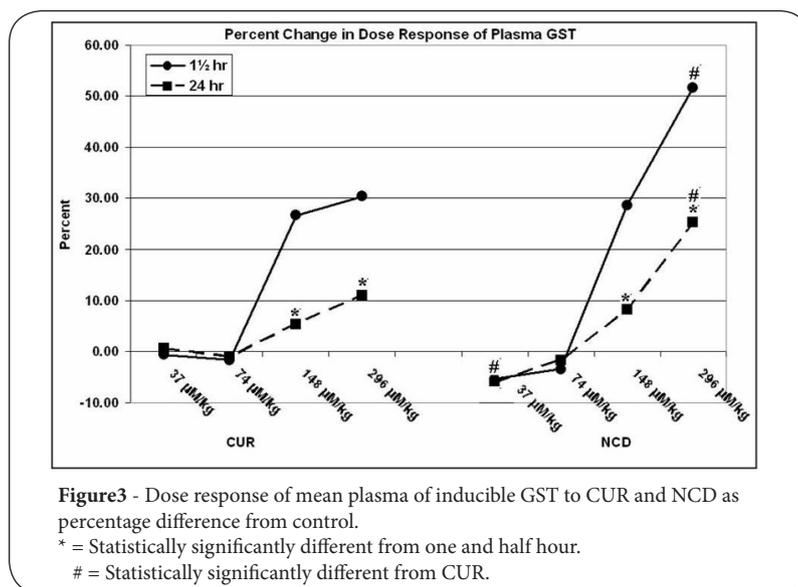| STUDY	Distance weighted L. S. F.	Coefficient	Significance
CUR 1.5 hr	y = -4.60 + 0.13 * x	r = 0.75	p < 0.001
CUR 24 hr	y = -2.35 + 0.05 * x	r = 0.55	p < 0.001
NCD 1.5 hr	y = -14.42 + 0.23 * x	r = 0.92	p < 0.001
NCD 24 hr	y = -10.44 + 0.12 * x	r = 0.81	p < 0.001

Figure 2. Percentage difference IC50 of DOX, CUR and NCD against different human cancer cell lines.

Figure3 - Dose response of mean plasma of inducible GST to CUR and NCD as percentage difference from control.
* = Statistically significantly different from one and half hour.
\# = Statistically significantly different from CUR.

relative to the normal control levels together with statistical significance of differences in dose response characteristics of CUR and NCD on inducible plasma GST with respect to time and to each other.

The results point out to an almost identical curvilinear dose response curves of mean plasma inducible GST to the serial doubling concentrations of CUR and the NCD. A slight gradual decrease ranging from -0.059 to -1.04 % was found with the lower CUR doses of 37, 74 µM/Kg body weight mg/kg body weight for the 1.5hr and the 24 hour's duration studies, with one exceptional increase of 0.59 % with the starting dose of CUR after 24 hours.

On the other hand, the extended 24 hour study points out to statistically non-significant differences lower CUR doses of 37, 74 µM/Kg body weight, with highly statistically lower values for the higher doses, (p<0.001).

Almost the same applies for the NCD, but with a higher range of decrease in the values of inducible plasma GST of -1.64 to -6.08 %, pointing to higher potency, with p-values of p<0.05 to p<0.001 in favor of the NCD. Nevertheless, increasing the oral dose of both CUR and NCD to 148 and 296 µM/Kg body weight has changed the whole picture completely to elevations in the range of 5.28 to 30.14 % for CUR and 8.11 to 51.70 % for the NCD for the 1.5 and the 24 hours' durations studies, ending in statistically highly significant percent elevations in inducible plasma GST with p-value of p<0.005 to p<0.001 in favor of the NCD over CUR. However, the extended 24 hour study points out to statistically non-significant differences lower NCD doses of 37, 74 µM/kg body weight, with highly statistically lower values for the higher doses, (p<0.001). The inducible response of curcumin biomarker GST is more sharp and last longer in case of the NCD in comparison to CUR.

GST Dose response curves: Table 4 shows the nonlinear distance weighted least square fit of dose response lines of mean plasma inducible GST percentage of change from control to each of CUR and NCD. The dose dependent relationship is clear from the table.

There are statistically highly significant correlations of the equimolar increasing doses of each of CUR and NCD with the percentage change of inducible plasma GST. However, the correlation coefficient (r) is higher for the NCD than CUR for the 1.5 hr and the 24 hr studies. Curcumin showed (r) values of 0.75 and 0.55, while the NCD showed 0.92 and 0.81 for the respective studies.

From another point of view, the slopes of the correlation lines are more important for evaluating the dose-response relationship in the form of "impact", as the slope (b) represents the change in y, (the percentage change in inducible plasma GST) per unit change of x, (the micro molar concentration of each of the NCD and CUR). In this respect; Table 4 shows clearly higher slopes for the NCD as compared to CUR of 0.23 and 0.12 to 0.13 and 0.05 percent increase in inducible plasma GST for both the 1.5 and the 24 hr studies, pointing again to more sharp and extended effect in case of the NCD in comparison to CUR.

Discussion

While cancer initiation and development implicates often hundreds of genes and/or signaling cascades, most conventional chemotherapeutic agents were designed to hit a single intracellular target and lead to severe adverse side effects, together with lack of safety. Efforts are directed to the development of multi-targeting therapies from safe and inexpensive, natural phytochemicals, despite difficulties to secure intellectual property rights to plant-based products.

In this respect, curcumin (diferuloylmethane), a natural hydrophobic polyphenol from the rhizomes of *Curcuma longa*, either native or formulated has shown significant promises against cancer [1] with ability to affect multiple intracellular targets [2] and inhibit specific molecular signaling pathways involved in carcinogenesis [3-5]. While reported to be antiproliferative in a number of cells lines [4,5,7], it has low systemic bioavailability due to poor absorption, rapid metabolism and systemic elimination [12,13]. With its toxicity significantly higher in tumor cells than in normal cells [8], curcumin

could be considered as an ideal lead compound for anticancer drug development, with high dose safety up to 12 g/day [9-11].

To overcome these limitations, several covalent modifications of the curcumin molecule were tried. For example, multiple myeloma remains hardly curable malignancy, but with newly synthesized curcumin analogs, a 7-12 fold more potent growth suppressors of myeloma cells could be achieved [14].

The present work demonstrated that the anticancer effect of the "NCD" is retained and significantly improved in terms of lower micro molar IC50 against one normal and six human cancer cell lines in comparison to natural curumin. The results agree with countless number of published studies; only few will be referred to here.

Adams *et al*., 2004 [21], synthesized a series of curcumin analogs with higher degrees of anticancer potency than the chemotherapeutic drug, cisplatin. Following, Ohori *et al*., 2006 [22] pointed to a modified curcumin derivative with growth-suppressive activity 30 times that of natural curcumin. Expression of cancer-related genes usually affected by curcumin indicated that the derivative induced the down-regulation of β-catenin, Ki-ras, cyclin D1, c-Myc, and ErbB-2 at one eighth the concentration of curcumin. Much recently, Lin *et al*., 2010 [23] designed a novel molecule from curcumin to inhibit constitutive STAT3 signaling and demonstrated that it is an effective inhibitor of STAT3 phophorylation, DNA binding activity, downstream target gene expression and induces apoptosis in human multiple myeloma, glioblastoma, colorectal and liver cancers.

The present data demonstrate the conserved and improved *in vivo* structure-action relationships with the dose dependent induction of plasma GST. Park *et al*., 2010 [24] reported that curcumin induces GST expression by signaling through the nuclear erythroid-derived 2-related factor 2 (NRF-2) and NF-κB *via* an anti-oxidant response element. However, increased expression of GST isoenzymes is linked to the development of resistance to alkylating cytostatic drugs and their deficiency reportedly increased predisposition to various forms of cancer. Hence, GST status may be a useful prognostic factor to determine the clinical outcome of chemotherapy [16]. The present results agree with the published observation that GST enzyme activity has been shown to be up or down-regulated in rat tissues after oral consumption of curcumin, depending on the dose and route of administration [16]. In three patients on 36 mg of curcumin daily, lymphocytic activity of GST was decreased with time to reach 41% of control on day 29 of treatment. This decline was not observed at the higher dose levels [16]. The effects of curcumin on GST expression are complex and may involve competitive enzyme inhibition as well as indirect enzyme induction [25]. Genetic polymorphisms in GST and its altered expression and activity are associated with oxidative DNA damage with subsequent risk of cancer susceptibility [26]. The inducible response of curcumin action biomarker GST is sharper and lasted longer in case of the NCD in comparison to CUR.

The nonlinear distance weighted least square fit of the dose response lines of mean plasma inducible GST percentage of change from control to each of CUR and NCD showed statistically highly significant correlations of the equimolar increasing doses of each of CUR and NCD with the percentage change of inducible plasma

GST. However, the correlation coefficient (r) is higher for the NCD than CUR for the 1.5 hr and the 24 hr studies.

From another point of view, the slopes of the correlation lines are more important for evaluating the dose-response relationship in the form of "impact", as the slope (b) represents the change in y, (the percentage change in inducible plasma GST) per unit change of (x), of micromolar concentration of each of the NCD and CUR.

In this respect higher slopes for the NCD as compared to CUR for both the 1.5 hr and the 24 hr studies were observed, pointing again to more sharp and extended effect in case of the NCD in comparison to CUR. However, with GST considered a biomarker for both CUR and cancer at the same time, it should not be used for either of them in studies or clinical trial of cancer treatment with CUR or its derivatives. Most recent studies have shown that the structure-action relationships of the symmetric curcumin molecule [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] are strictly related to its phenolic hydroxyl and methoxy groups and the conjugated enones of the heptadiene moiety. As concerns the anticancer and GST induction potencies, Labbozzetta *et al*., 2009 [27] elaborated the diketone system of curcumin into different analogues; which showed remarkable increases in the antitumor potency both in the parental MCF-7 breast cancer cell line and in the amultidrug-resistant MCF-7R variant. They implied that the diketone fragment of curcumin is not indispensable for the growth inhibition and pro-apoptotic activities. The ability of curcumin analogues to induce phase II detoxification enzymes e.g. GST may be linked to the presence of both the phenolic hydroxyl groups and the diketone functionality of curcumin [28] and the conjugated enones act as Michael acceptors for curcumin's anti-cancer activity [29].

In general, oxyaryl substituent with an adjacent, unsaturated C=C–C=O unit seems to offer antitumor and cancer cell cytotoxicity [30]. Meanwhile, its anti-inflammatory and anticancer activity, depends on low hydrogenation and a high level of methoxylation [31,32]. Other results indicates that the substitution of a hydroxyl group for a methoxy group at the meta positions of the phenyl rings in curcumin significantly enhanced the anti-inflammatory activity, and the removal of phenyl ring at the 7th position of the heptadiene back bone and addition of hydroxyl group significantly increased the anti-proliferative activity of curcumin [33].

At this point, it is important to confirm that the core difference in curcumin derivatization was the site of modification and the type of the substituted chemical group, which was in all cases made through reactions with the naturally occurring functional chemical groups of the curcumin molecule. Here comes the difference, as the present NCD was made through covalent modification to remote sites, apart from such groups.

Assumptions of both conserving and improving the natural potencies of curcumin as anti cancer and *in vivo* GST dose dependent inducer was verified true. With GST as biomarker for both CUR and cancer, it should not be taken as a parameter in studies and/or clinical trial involving both cancer and CUR or its derivatives as GST is dose-dependently inducible by CUR and some of its derivatives. The present study opens the door wide for further *in vivo* structure-

action relationship studies about the tremendous activities/potencies of the curcumin molecule in its novel covalently modified, conserved functional group form.

Conflict of interests

The authors declare that they have no competing interests.

Authors' contributions

Mohamed T.Abdel Aziz, Mohamed F.El-Asmar, Ameen M.Rezq participated in the design of the study and revised it critically; Hanan H.Fouad, Hanan H.Ahmed, Amira A.Hassouna, Fatma M.Taha carried out the performance of the animal experiments and the laboratory estimation of GST ; Hafez F.Hafez carried out the cell line study; Ameen M. Rezq performed the mathematical and statistical analysis and interpretation of data and drafted the manuscript. All authors read and approved the final manuscript.

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