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Nitric Oxide-Releasing NO–Curcumin Hybrid Inhibits Colon Cancer Cell Proliferation and Induces Cell Death In Vitro

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Abstract: Cancer is a leading cause of death worldwide, and most of the currently available drugs for cancer treatment have limited potential. Natural products and their relatives continue to represent a very high percentage of the drugs used for cancer treatment. Curcumin is one of several natural drugs that has recently attracted much attention due to its putative cancer-preventive and anticancer properties. As well, Nitric Oxide (NO) holds a great potential for NO-based treatments for a wide variety of diseases. Here, for the first time, we tested the anti-cancer activities of an NO–Curcumin hybrid, hypothesizing that by joining the effects of curcumin and NO in one compound, the hybrid compound would be more potent than curcumin alone in treating colon cancer. To compare the anti-cancer activities of curcumin and NO–curcumin, we treated different colon cancer cell lines with either curcumin or NO–curcumin and tested their effects on cell proliferation and death. Our results show that NO–curcumin is more effective in reducing cell proliferation and increasing cell death when compared to curcumin. In addition, NO–curcumin has a lower IC50 compared to curcumin. Altogether, our results demonstrate for the first time that an NO–curcumin hybrid has more potent anti-cancer activity compared to curcumin alone, making it a potential future treatment for cancer and perhaps other diseases.

Keywords: colon cancer; curcumin; hybrid; nitric oxide; NO

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1. Introduction

Colorectal cancer is one of the most commonly diagnosed cancers [1]. It is the third leading cause of cancer death in the USA, and its incidence is rapidly increasing in developing countries as well [1].

Although widely available, anti-cancer drugs are not very efficient, have many side effects, and are very expensive. Many of the chemotherapy drugs used for cancer treatment are natural products or their synthetic structural relatives [2]. It is estimated that about 83% of the anti-cancer drugs recorded worldwide are either natural products or their derivatives [3]. Curcumin is a natural product that is derived from *Curcumin longa*. It is well documented that curcumin is a potent anti-inflammatory and anti-cancer product. It has been found that curcumin induces its anti-cancer effects by modulating different signaling pathways. These pathways include cell proliferation, cell death, cell survival, and different kinase pathways, as reviewed in [4]. On the molecular level, curcumin has been shown to suppress cancer initiation, growth, and progression by regulating various transcription

factors, growth factors, inflammatory cytokines, protein kinases, and other enzymes [5,6]. However, due to its hydrophobic structure curcumin is poorly soluble in water, which leads to low bioavailability [7]. To overcome this obstacle, different curcumin derivatives have been synthesized and tested in different cancer models both in vitro and in vivo. Several of these derivatives have been shown to enhance different curcumin activities, including its anti-tumor activities [8].

In colon cancer cells, curcumin has been shown to inhibit nuclear factor kappaB (NFκB) activity and to reduce cyclooxygenase-2 expression and β-catenin transactivation, which are changes that play a role in curcumin-mediated apoptosis [9–12]. Furthermore, different studies have demonstrated that curcumin exerts its anti-cancer effect by increasing reactive oxygen species (ROS) generation [13–17].

Following its discovery as a crucial signaling molecule, great hopes have been generated for new NO-based treatments for a wide variety of diseases, including cancer. In cancer, NO appears to play dual antagonistic effects. While low NO levels are believed to promote cancer initiation and progression, high and sustained NO levels have tumor-suppressing and cytotoxic effects [18]. Several studies have highlighted the anti-neoplastic role of NO. NO was shown to induce apoptosis in cancer cells via different mechanisms, including induction of p53 pro-apoptotic function [19–21], inducing degradation of anti-apoptotic mediators by the proteasome [22], increasing mitochondorial release of cytochrome C [23], and induction of Smac release [24]. Based on NO anti-tumor signaling, various NO donors have been tested for their anti-neoplastic activity. Indeed, several different classes of NO donors have been tested in cancer therapy, as reviewed in [25]. A few of these NO donors are present on the market, such as, for example, sodium nitroprusside and organic nitrates. In addition, a large number of animal and clinical studies have been conducted demonstrating their advantageous features [26]. A new and novel approach to designing NO-releasing compounds is the synthesis of hybrid NO donor drugs. Examples of such NO hybrids include NO-releasing non-steroidal anti-inflammatory drugs (or nitroaspirins) and NO–indomethacin [27]. In addition, a recent approach to discovering novel anti-tumor agents is the generation of NO-donor/natural product hybrids. For example, furoxan/oridonin hybrids and novel furoxan-based nitric oxide (NO)-releasing derivatives of glycyrrhetinic acid (GA) have shown anti-neoplastic effects [28,29].

In this article, we tested for the first time the anti-cancer activity of an NO–curcumin hybrid. Our results clearly show that NO–curcumin is more potent than curcumin alone in reducing cell proliferation and inducing cell death. Moreover, our hybrid compound has a lower IC50 than curcumin, which makes it a future potential anti-cancer drug either alone or in combination with other compounds.

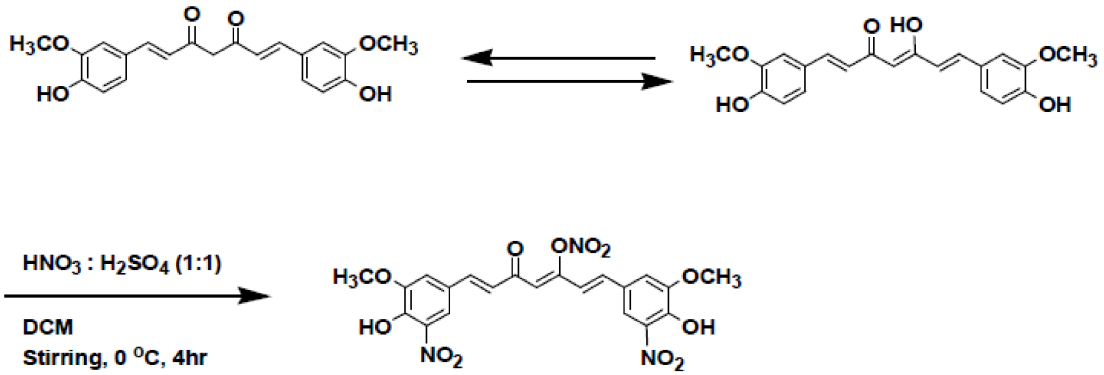
2. Materials and Methods

2.1. Curcumin Extraction

Twenty grams of ground turmeric (*Curcuma longa* L.) rhizome (≥80%, Merck KGaA, Darmstadt, Germany) was dissolved in 50 mL of dichloromethane (≥99.8%, Sigma Aldrich, Rehovot, Israel). Then, the mixture was stirred with a magnetic stirrer and heated at reflux for 1 h. Afterwards, the mixture was suction-filtered and the filtrate was concentrated in a hot water bath maintained at 50 °C. Then, the reddish yellow oily residue was triturated with 20 mL of hexane (≥95%, Sigma Aldrich, Israel).

2.2. Synthesis, Purification, and NMR Analysis of Nitro-Oxy Curcumin

The synthesis of nitro-oxy curcumin was carried out as illustrated in Scheme 1. Curcumin (1 eq. mo) was dissolved in dichloromethane (DCM) and cooled to 0 °C.



Scheme 1. Nitrate esterification/ nitration of curcumin.

Then, a mixture of concentrated HNO_3 (≥99.8%, Sigma Aldrich, Israel) and concentrated H_2SO_4 (≥99.99%, Sigma Aldrich, Israel) (10 eq. mol) was added dropwise to the solution over 30 min under stirring. This reaction mixture was then stirred at 0 °C for 2 h in the dark. The mixture solution was saturated by NaCl (≥99.0%, Sigma Aldrich, Israel) and then extracted with CH_2Cl_2. The extract was dried over Na_2SO_4(≥99.0%, Sigma Aldrich, Israel), filtered, and the solvent was distilled off under reduced pressure. The reddish yellow oily residue was triturated with 10 mL of hexane and the resulting solid was collected by suction filtration. Curcumin and nitro-oxy curcumin were purified using flash column chromatography on Merck silica gel 60 (particle size 230–400 mesh), with the eluent being a combination of CH_2CL_2 and CH_3OH (≥99.8%, Sigma Aldrich, Israel) in various ratios. To observe the purity of the compounds, analytical TLC was performed on silica gel 60F254-percolated plates (Merck KGaA, Darmstadt, Germany) and visualized using UV light or I_2 vaper.

NMR purity data were captured on a Varian VXR-300 (300 MHZ) spectrometer with a 5-mm switchable probe and processed using VNMR software. The internal standard was tetramethyl silane Me4Si (0.00 ppm) (Sigma Aldrich, Israel), and the solvents were chloroform-d (≥99.8%, Sigma Aldrich, Israel) and dimethyl sulfoxide-d6 (≥99.8%, Sigma Aldrich, Israel). The abbreviations for the splitting patterns are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, unresolved multiple due to instrument field strength; and dd, doublet of the doublet.

2.3. Assessment of Nitric Oxide Release

Nitrite (NO) was estimated according to a Greiss reagent kit (Promega Corporation Madison, WI, USA). In brief, concentration was calculated using a standard curve of sodium nitrite of serial dilution. Reaction volume was 150 µL in total and contained 50 µL of sample, 50 µL of 1% sulphanilamide (≥98.0%, Sigma Aldrich, Israel) in 5% phosphoric acid (≥85.0%, Sigma Aldrich, Israel), and 50 µL of 0.1% napthaylamine diamine dihydrochloric acid (≥85.0%, Sigma Aldrich, Israel) in water. Finally, NO was measured at 550 nm using an ELISA reader.

2.4. Cell Culture

HT29, HCT116, RKO, and $CaCO_2$ colon cancer cell lines were grown in DMEM supplemented with 10% FBS (Gibco), glutamine, and penicillin/streptomycin (Biological industries, Beit Haemek, Israel). THP-1 cells (10^5/well) were grown in RPMI-1640 supplemented with 10% FBS (Gibco), glutamine, and penicillin/streptomycin (Biological industries, Israel).

2.5. Propidium Idodide Staining

To first test whether our treatments would have any effect on cell viability, 0.5 million of the different cells were cultured in 60 mm plates in triplicates. One day afterwards, cells

were treated with different concentrations of either vehicle, curcumin, or NO–curcumin. At 24 h and 48 h post treatment, PI was added directly to the cell culture media at a final concentration of 1 μg/mL and incubated for 10 min in a CO_2 incubator. Later, the cells were observed under inverted fluorescent microscope (Olympus CKX 41) and imaged.

2.6. Cell Count

To determine the effect of our compounds on cell growth and cell death, 0.5 million of the different cells were cultured in 60 mm plates in triplicates. One day afterwards, cells were treated with different concentrations of either vehicle, curcumin, or NO–curcumin. At 24 h, 48 h, and 72 h post treatment, both adherent and floating cells were collected and counted using a trypan blue exclusion assay to detect the percentage of dead cells.

2.7. XTT Assay and IC50 Determination

To test the effect on cell proliferation, 1500 cells of different cancer cell lines were seeded in triplicates in 96-well plates. 24 h later, cells were treated with different concentrations of NO–curcumin or curcumin. In the following days, the difference in cell proliferation rates was tested using a commercially available XTT kit (Biological industries, Israel) according to the manufacturer's instructions. In order to determine the IC50, XTT results were blotted and the dose needed to kill 50% of the cells was determined using Prism software.

2.8. RNA Extraction, cDNA Synthesis, and Real-Time PCR

Total RNA was prepared using TRI reagent (Sigma Aldrich, Jerusalem, Israel), as described by the manufacturer. One microgram of RNA was used for cDNA synthesis using a First-Strand cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) in a 20 μL reaction mix that contained 4 μL of 5X iScript reaction mix, 1 μL iScript reverse transcriptase, and up to 20 μL nuclease-free water. The reaction protocol was as follows: 5 min at 25 °C, 20 min at 46 °C, 1 min at 95 °C, and hold at 4 °C. Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA). The PCR mix contained 20 ng cDNA, 10 μL of X2 reaction mix, 0.7 μL of 10 μM primer mix, and nuclease-free water up to 25 μL. The PCR cycling program was as follows: 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All measurements were performed in triplicate and standardized to the levels of UBC. The primer sequence of the tested target genes is described in Table 1.

Table 1. Primer sequence of target genes.

Target Gene	Forward Primer	Reverse Primer
P21	5′-cgtcaaatcctccccttcct-3′	5′-atgggttctgacggacatcc-3′
Bax	5′-ggttgtcgccctttctact-3′	5′-aagtccaatgtccagcccat-3′
COX2	5′-tcccttccttcgaaatgcaa-3′	5′-aggttagagaaggcttcccag-3′
CYCLIN E1	5′-ggaagaggaaggcaaacgtg-3′	5′-tgcattattgtcccaaggctg-3′
EGFR	5′-tcaataactgtgaggtggtcc-3′	5′-gacataaccagccacctcct-3′
BCL2	5′-gccctgtggatgactgagta-3′	5′-gaaatcaaacagaggccgca-3′
CYCLIN D1	5′-tacaccgacaactccatccg-3′	5′-ttcaatgaaatcgtgcgggg-3′
EGR1	5′-agcagcaccttcaaccctc-3′	5′-ccagcaccttctcgttgttc-3′

3. Results

3.1. Synthesis and Chemical Characterization of Nitro-Oxy Curcumin

NO–Curcumin was prepared in three steps. In the first step, a solid mixture of the three curcuminoids was obtained from finely powdered turmeric (yield = 15%). In the second step, the curcuminoid mixture was purified by column chromatography (silica gel) to afford pure (1E,6E)-1,7-bis(4-Hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione

(curcumin), with a yield of 80%; m.p. 183–185 °C (lit. 184–185 °C); FTIR (KBr) 3500, 1735, 1680, 1605, 1504, 1427, 1375, 1100, 800 cm−1; 1 H-NMR (DMSO) δ 3.85 (s, 6H), 6.1 (s, 1H), 6.65 (d, J = 15.7 Hz, 2H), 6.95 (d, J = 8.5 Hz, 2H), 7.15 (d, J = 8.5 Hz, 2H), 7.38 (s, 2H), 7.58 (d, J = 15.7 Hz, 2H). In the third step, pure curcumin was nitrated to generate nitro-oxy curcumin, as illustrated in Scheme 1 of Section 2 with a yield of 85% and modification percent of 70% (Griess Method); FTIR (KBr) 1735, 1640, 1525, 1280, 1040, 1020, 840 cm^{-1}. 1 H- NMR (DMSO) δ 3.85 (s, 6H), 6.1 (s, 1H), 6.65 (d, J = 15.7 Hz, 2H), 7.15 (s, 2H), 7.38 (s, 2H), 7.58 (d, J = 15.7 Hz, 2H). In order to assess the release of NO from NO–Curcumin, we used a Greiss reagent kit. Our results revealed that NO concentration was about seventeen-fold higher in extracts isolated from cells treated with NO–Curcumin as compared to extracts isolated from control untreated cells.

3.2. NO–Curcumin Changes Cell Morphology and Cell Density

Curcumin is known to induce cancer cell death and apoptosis [10,13]. To test whether nitrification enhances this anti-cancer effect of curcumin, we treated HCT116 and RKO colon cancer cells with the same concentration of either curcumin or NO–curcumin for different time points. As shown in Figure 1A,B, NO–curcumin lowered cell density in a more drastic manner compared to curcumin. We noticed changes in cell morphology as well; in order to assess whether these changes were cell-death related, we stained treated cells with propidium iodide (PI). As shown in Figure 1C,D, more cells were stained with PI upon NO–curcumin treatment compared to cells treated with curcumin alone, meaning that more dead cells were observed following NO–curcumin treatment. Interestingly, NO–curcumin had a stronger effect on more proliferative and more aggressive cell lines (HCT116 and HT29) compared to less aggressive cells, e.g., $CaCO_2$ and RKO cells (data not shown). Of note, NO–Curcumin did not show a cytotoxic effect on THP-1 normal cells (data not shown). Overall, these results indicate that NO–curcumin has a stronger cytotoxic effect on colon cancer cells compared to curcumin alone.

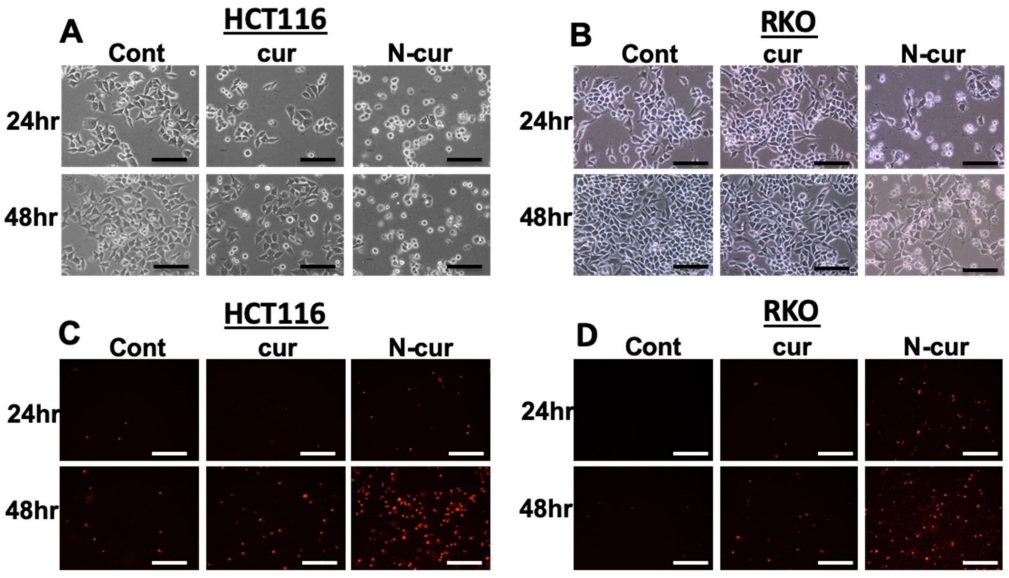


Figure 1. Effect of NO–curcumin on colon cancer cell morphology and cell death. (**A**,**B**) Representative phase contrasting microscopic micrographs showing the effect of curcumin (cur) and nitro-curcumin (N-cur) on HCT116 (**A**) and RKO (**B**) cell morphology and cell density after treating the cells with 40 uM of both compounds for the indicated time points. Untreated cells were used as controls. (**C**,**D**) Representative fluorescent microscopic micrographs of propidium idodide staining showing the effect of curcumin (cur) and nitro-curcumin (N-cur) on cell death after treating the cells with 40 μM of both compounds for the indicated time points. Untreated cells were used as controls; size bar = 100 μm.

3.3. NO–Curcumin Has a More Potent Effect on Cell Proliferation and Cell Death Compared to Curcumin Alone

After showing qualitatively that NO–curcumin reduces cell density, which may indicate a cytotoxic effect on colon cancer cell lines, we quantitatively tested the effect of NO–curcumin on cell death and cell proliferation using Trypan blue exclusion and XTT assays. While curcumin reduced cell proliferation by about 16% and 10% in HCT116 and RKO respectively, NO–curcumin reduced cell proliferative index by about 80% and 60% in HCT and RKO cells, respectively (Figure 2A,B). Of note, these numbers were obtained in relation to the relative proliferation index of control untreated cells after 48 h. In the cell death assay, 40 μM curcumin increased cell death to 5% and 2% in HCT116 and RKO cells, respectively, after 24 h, and to 8% and 4% by 48 h. On the other hand, the same concentration of NO–curcumin increased cell death in HCT116 cells to 30% and 50% after 24 and 48 h, respectively, and to about 35% after 24 and 48 h in RKO cells. (Figure 2C,D). Of note, and as discussed above, NO–curcumin has a stronger effect on more aggressive colon cancer cells. These results prove that NO–curcumin has a stronger effect on cell proliferation and cell death compared to curcumin alone.

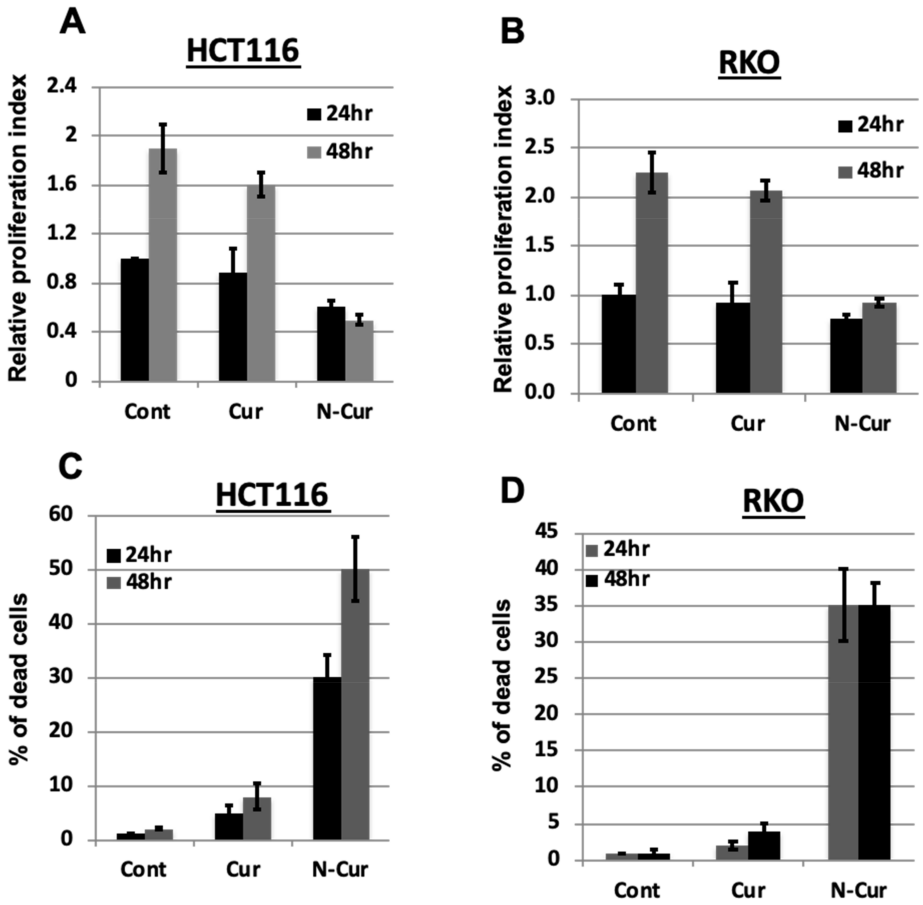


Figure 2. Effect of NO–curcumin on colon cancer cell proliferation and cell death. (**A**,**B**) Representative statistical analysis of the effect of curcumin (cur) and nitro-curcumin (N-cur) on HCT116 (**A**) and RKO (**B**) cell proliferation as measured by XTT assay after treating the cells with 40 μM of both compounds for the indicated time points; untreated cells were used as control and day zero, the day of treatment, was used as a reference point. (**C**,**D**) Representative statistical analysis of the effect of curcumin (cur) and nitro-curcumin (N-cur) on HCT (**A**) and RKO (**B**) cell death as measured by trypan blue exclusion assay after treating the cells with 40 μM of both compounds for the indicated time points; untreated cells were used as control.

3.4. NO–Curcumin Has a Lower IC50 Compared to Curcumin Alone

IC50 is usually measured in order to compare drug activities. To determine which compound was more potent, we titrated the effects both curcumin and NO–curcumin on cell proliferation using an XTT test over a wide range of concentrations. As shown in Figure 3A,B, NO–curcumin was able to show activity even at low concentrations (5 and 10 μM), while curcumin showed significant effects only at higher concentrations (40 μM and above). For example, while 20 μM of curcumin had no effect on cell proliferation over 48 h, the same concentration of NO–curcumin was found to be more potent than 40 μM of curcumin. After showing that 40 μM of curcumin was not enough to result in more than about a 50% effect on cell proliferation, in order to compare the IC50 of the compounds we used a wider range of concentrations up to 100 μM. As can be seen in Figure 3C,D, NO–curcumin has a lower IC50 (about 40 μM) than curcumin (bout 80 μM). Of note, we could not achieve 50% inhibition of cell proliferation with curcumin treatment in any of the tested cell lines, for example RKO and $CaCO_2$, in the range of compound concentrations we used (data not shown). The only cell line we succeeded with was HT29. These results indicate that NO–curcumin is more potent than curcumin, which may indicate a safer therapeutic window.

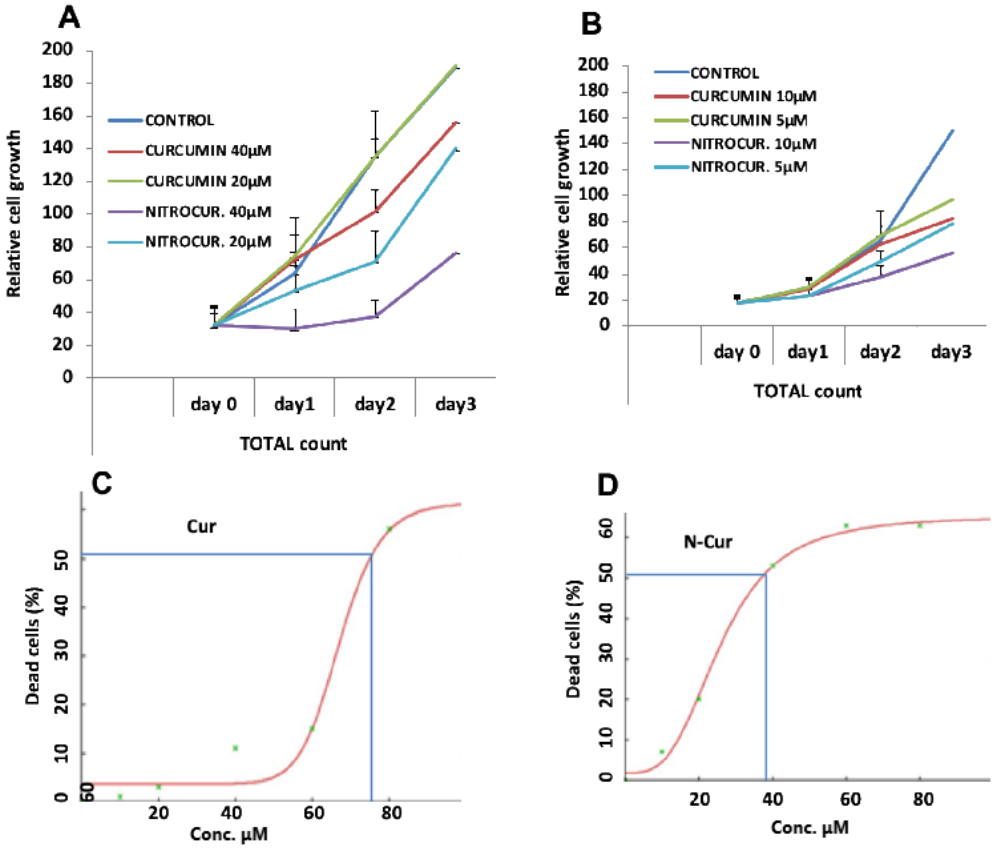


Figure 3. Dose-dependent effect of NO–curcumin on colon cancer cells and IC50. (**A**,**B**) Representative statistical analysis of the effect of curcumin (cur) and nitro-curcumin (N-cur) on HT29 cell proliferation using an XTT assay after treating the cells with the indicated concentrations. (**C**,**D**) XTT analysis of cell proliferation after treating HT29 cells with the indicated doses of curcumin (**C**) and nitro-curcumin (**D**) for the indicated time points. The X-axis intersection point with the 50% value on the Y-axis represents the IC50 of each compound as determined by GraphPad Prism statistics software (San Diego, CA, USA).

3.5. NO–Curcumin Reduces the Expression of Pro-Proliferative Genes and Increases the Expression of Pro-Apoptotic Genes

On the molecular level, it has been shown that curcumin induces its cytotoxic effect in part by modulating the expression of pro-proliferative and pro-apoptotic genes [30]. To elucidate whether NO–curcumin does as well, we measured the mRNA expression levels of different pro-proliferative and pro-apoptotic genes using real-time PCR. Our results show that NO–curcumin reduces the expression of all pro-proliferative genes tested in a similar manner to curcumin alone (Figure 4A). However, while NO–curcumin was able to lower the expression of the pro-apoptotic genes Bax and p21 in HCT116 cells after 12 h (Figure 4B) or even less time (data not shown), in our study curcumin required more time to change the expression of these genes (data not shown). Overall, these results show that NO–curcumin induces its effect in part by modulating the expression of pro-proliferative and pro-apoptotic genes, and that NO–curcumin has a faster effect on pro-apoptotic genes compared to curcumin.

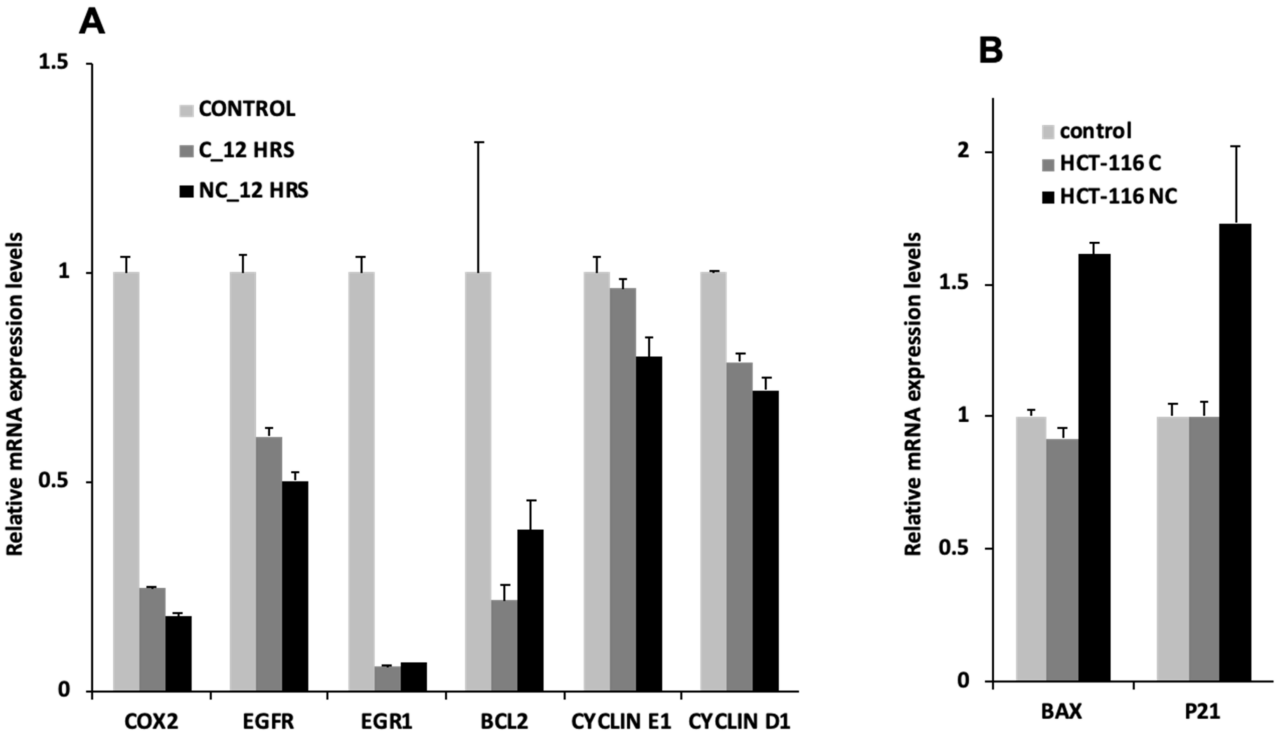


Figure 4. Comparison of the effect of curcumin and NO–curcumin on cell survival genes. (**A**) Statistical analysis showing the effect of curcumin and nitrocurcumin on the relative mRNA expression level of different pro-survival genes and (**B**) proapoptotic genes; the results represent data obtained from three different biological experiments performed in triplicate.

4. Discussion

Many anti-cancer drugs suffer from resistance and lack of selectivity. These facts make it a pressing need to either improve the selectivity and activity of currently used drugs or to develop new anti-cancer treatments with higher activity and selectivity. In this work, we successfully generated an NO–curcumin hybrid that has a more potent effect on colon cancer cell proliferation and apoptosis than curcumin alone. The most successful anti-cancer drugs on the market are those that target the unique hallmarks that distinguish cancer cell from normal cells. One such cancer cell hallmark is metabolism. Cancer cell metabolism is characterized by reduced oxidative activity and increased glycolytic activity [31]. This abnormal cell metabolism results in enhanced generation of reactive oxygen species (ROS). One of the cytotoxic effects of curcumin has been shown to be induced by increased generation of reactive oxygen species [32]. Interestingly, Syng-ai

et al. showed that normal cells were not affected by curcumin treatment and that depletion of glutathione, an important intracellular radical scavenger, further sensitized cells to apoptosis [32]. In addition, NO and the reactive nitrogen species (RNS) that result from it were shown to induce genotoxic effects through different mechanisms, including direct chemical modification of DNA and inhibition of DNA repair, which are events that lead to DNA mutagenesis and damage (reviewed [33]). In our present work, it seems that combining the ROS-induced genotoxic effects of curcumin and NO is the mechanism behind potentiating the anti-cancer activity of curcumin, an intriguing hypothesis which requires further investigation. In fact, our hypothesis is supported by published data showing that, while moderate levels of ROS in cancer cells represent an advantage that supports tumorigenesis and cancer progression [34,35], excessive levels of ROS stress can be toxic to cancer cells; indeed, cancer cells under increased oxidative stress were shown to be more sensitive to damage by further ROS insults induced by exogenous agents [36,37]. Therefore, manipulating oxidative stress using reagents that can change ROS levels, (such as NO–curcumin in the present case) represents a way to selectively kill cancer cells without causing significant toxicity to normal cells [37,38].

In fact, we are not the first to utilize NO donors for the treatment of cancer. For example, NO–aspirin (NCX-4016) showed a 1000-fold greater effect on colon cancer cells compared to the parent compound in vitro [39]. In an in vivo $APC^{min/+}$ mouse model of early intestinal neoplasia, NO–aspirin reduced polyp formation by 59% compared to the control treatment [40].

In the present study, we do not believe that the increased anti-cancer activity of the hybrid compound is solely related to the synergistic effect of NO on curcumin's anti-cancer effect; rather the addition of NO to curcumin overcomes the problem of curcumin's poor solubility and bioavailability by increasing its solubility and bioavailability, an issue that we now intend to investigate in an in vivo model.

In addition to drug effectiveness and targeting, drug safety is an important issue in drug development. In the present study, we showed that NO–curcumin is more potent and has a lower IC50 compared to curcumin alone. This means that lower concentrations of NO–curcumin are needed in order to achieve the target effect, thus rendering it effective and safe over a wider range of concentrations.

While curcumin has been shown to induce its anti-cancer activity by regulating many different pathways and gene expression that finally lead to cell death, different studies have highlighted the antineoplastic role of NO. It has been shown that NO can induce apoptosis through different mechanisms, including induction of the proapoptotic protein p53 function [19–21], inducing the degradation of anti-apoptotic mediators by the proteasome [22], increasing mitochondorial release of cytochrome c [23], and induction of Smac release [24]. Here, we have shown that NO–curcumin regulates the expression of different genes related to cell proliferation and apoptosis; downregulating the expression of genes that promote tumor growth and increasing the expression of pro-apoptotic genes. Of note, NO–curcumin had a more potent effect on these genes compared to curcumin alone. In addition, NO–curcumin showed a faster effect on these genes.

5. Conclusions

While our study has shown promising results, the exact mechanism of action of NO–curcumin needs to be fully elucidated; this is currently under investigation. Discovering this mechanism would help to predict which types or subtypes of a specific cancer will respond to NO–curcumin and which will not. In addition, this should open the door for using NO–curcumin with other anti-cancer drugs that share the same target in order to potentiate their action. Importantly, the combination between NO and curcumin can be supposed to be selective against cancer cells, as it is predicted to target the oxidative stress unique to cancer cells. Because NO-curcumin is designed to target a common cancer cell hallmark shared between almost all cancer types, our hybrid is predicted to be beneficial in the treatment and prevention of different types of cancer. Finally, in this work we succeeded

in making, using, and combining together the biological activities of NO and curcumin, and showed for the first time that this hybrid has a level of potent anti-tumor activity which makes it a promising potential future anti-cancer therapy.

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