Cytotoxic Profiling of Plant Secondary Metabolites on P53 Variant Human Colon Carcinoma Cell Lines

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Abstract

Chemoprevention strategies employ the use of compounds to inhibit the initiation, promotion, and progression phases of carcinogenesis. The successful chemopreventative candidate must therefore (1) selectively inhibit growth of transformed cells and (2) be administered on a frequent basis to confer maximal protection. Phytochemicals are a subclass of bioactive plant secondary metabolites that exhibit antioxidative, anticarcinogenic, and anti-inflammatory properties contributing to proper cell function. To assess the effectiveness of these compounds warrants an understanding of their cytotoxic mode of action. In this study, p53 variant human colon carcinoma cell lines were chronically exposed to varying concentrations of the phytochemicals—curcumin, andrographolide, and d-limonene—to determine the role of p53-induced cytotoxicity, with p53-mutant and p53-deficient cell lines representing precancerous lesions. Cytotoxicity was assessed using clonogenic assays and macroscopic colony counts were used to quantify cell survival. The results demonstrate that each phytochemical exhibits selective cytotoxicity toward nonfunctional p53 cell lines, suggesting a p53-mediated role in inhibition of cell clonogenicity and potential chemopreventative properties. Although each compound displays this described effect, only the D-limonene demonstrates considerable chemoprotection, suggesting it might have practical implications in vivo.

Introduction

Chemoprevention is an anticancer strategy that involves the use of compounds to inhibit the initiation of carcinogenesis and delay its promotion and progression. Mutations in the genome of cells are caused by the introduction of various carcinogens into the cellular environment (e.g. radiation) that are associated with increases in oxidative stress and chronic inflammatory responses that exacerbate the effect.[1,2] Therefore, the successful chemopreventative candidate must have potent antioxidative, immunomodulatory, and tumoricidal properties that counteract the carcinogen at the source as well as modify the development of accompanying systemic effects. As carcinogenic processes constitute a long latency period, the candidate must be administered on a frequent basis to confer maximal chemoprotection. Unlike chemotherapeutic compounds, the candidate must exert low cytotoxicity toward nonmalignant cells while exerting high specificity and maximal lethality toward transformed cells. Specifically, these compounds aim to target deregulation of the cell cycle and target only cells that delineate from proper cell function.[3,4] Cellular deregulation might include alteration in tumor suppressor function via p53 transcription pathway and RAS oncogene activation. These criteria warrant a cytotoxic examination of these compounds to ensure demonstrable high tissue tolerance and efficacy.

Despite synthetic analogues in the fields of oncology and chemotherapy, chemopreventative strategies usually rely on the use of dietary natural products in exerting their effects on cellular targets.[5,6] Plant secondary metabolites represent a class

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of natural compounds that have recognized antioxidative, anti-inflammatory, and growth inhibitory anticarcinogenic properties in vivo.[7-9] Phytochemicals refer specifically to those plant-derived compounds that exhibit bioactive antioxidative capabilities. Subclasses of these phytochemicals include polyphenolic, terpene, and diterpene compounds.[10] Although there are thousands of such phytochemical compounds, not each is compatible with consumption given their toxicity profiles in vivo. Therefore, only a few have been given notable attention with regard to their observed tumoricidal effects.[11]

Aims

Cytotoxic profiles of phytochemicals on isogenic cell lines might provide important information on the mechanism of these substances to determine the targets of growth inhibition. Specific compounds might interfere in certain pathways that are conducive to carcinogenic processes and therefore act to inhibit these processes. Additionally, these mechanisms might provide us with information on modifications that enhance the specific activity of the substances. This study investigates the interaction of the phytochemicals—curcumin, andrographolide, and D-limonene—with p53 variant human colon carcinoma cell lines—HCT116 p53 wild-type (wt), HT29, and HCT116 p53 null—where HT29 and HCT116 p53 null cell lines represent precancerous lesions given their nonfunctional p53 status. The dose–response of these interactions provides a basic mechanistic effect in terms of phytochemical p53-mediated cytotoxicity and chemoprevention, whereby our criterion of chemoprevention is selective cytotoxic effects toward nonfunctional p53 cell lines.

Materials and Methods

Subculture

HCT116 p53 wt, HT29, and HCT116 p53 null cell lines were cultured in T75 flasks (Falcon) containing RPMI 1640 medium (Gibco), Fetal Bovine Serum (Gibco), 1000 mM Penicillin–streptomycin solution (Gibco), and 2 mM L-glutamine (Gibco). Cells were maintained in an incubator at 37°C with 95% air and 5% CO_2. Subculture was routinely performed when cells were 80% to 100% confluent using a solution of 0.25% Trypsin (Gibco), Dulbecco's Phosphate-Buffered Saline (Gibco), and 1 mM EDTA (Versene; Gibco) at 37°C.

Compounds

Curcumin, andrographolide, and D-limonene are lipophilic compounds and were thus dissolved in the organic solvent, ethanol, as an intermediate prior to application into clonogenic flasks to increase solubility and therefore cell bioavailability. Stock containers of curcumin (>99.9% purity), andrographolide (>99.9% purity), and D-limonene (>99.9% purity) were purchased from MilliporeSigma. Working concentrations of curcumin were made in a ratio of 1 mg/mL 100% ethanol,

andrographolide in 1 mg/mL 100% ethanol, and D-limonene was miscible in a 10:1 ratio of 100% ethanol.

Clonogenic Assay

Clonogenic technique by Puck and Marcus was used for cell survival analysis. Briefly, compounds were generated as per the dose optimization section and administered into T25 flasks (Falcon). Cells were detached from stock T75 flasks (Falcon) and resuspended in medium to generate a single-cell suspension. Sample aliquot of the cell suspension was counted with the Z2 Cell Counter (Beckman Coulter) to generate values of viable cells. Following administration of compounds into flasks containing varying concentrations of each compound, cells were plated into each T25 flask (Falcon). Cell cultures were incubated for their respective clonogenic period, approximately 9 days for HCT116 p53 wt and HCT116 p53 null containing flasks and 11 days for HT29 flasks. Cells were stained following their clonogenic period of incubation with 25% carbol fuchsin in water where macroscopic colonies equal to and over 50 cells satisfy the criteria of reproductive cell survival. Total of 3 independent experiments were conducted with 3 replicates per experiment (n = 3).

Dose Function Analysis

Exposure of cells to growth inhibitory compounds comprises of 2 characteristic values in a dose–response curve, the nonminimal inhibitory concentration (NIC) and the minimal inhibitory concentration (MIC). The NIC value refers to the minimal dose at which the compound begins to inhibit cell growth, whereas the MIC refers to the minimal dose that exerts maximal growth inhibition.

In this study, cytotoxicity curves and corresponding NIC and MIC values were constructed using data from the clonogenic assay cell survival criteria. The inhibitory concentration for each compound was determined using the statistical software analysis package GraphPad Prism 7. The NIC and MIC software interpolated values were determined utilizing a Gompertz function dose–response curve based on the Lambert and Pearson method of determining antibiotic susceptibility in bacterial strains. The function assigns NIC and MIC values corresponding to intersections of the slope of the inflection point with the upper and lower asymptote, respectively. The 3-parameter Gompertz function is a type of sigmoidal logistic growth and decay function that describes cell survival by the equation $f(x) = a \times \exp[-b \times \exp.(-c \times x)]$. Values of $a < 0$, $b > 0$, and $c > 0$ are representative of decreases in cell survival and therefore the characteristic cytotoxicity curve that describes the situations of inhibitory growth applicable to administration of antitumorigenic compounds to cancerous cells.

Statistical Analysis

The One-way analysis of variance (ANOVA) between groups with follow-up Tukey multiple comparisons test was conducted

using the statistical software package GraphPad Prism 7 to examine the effects of phytochemical exposure on varying p53 function between each cell. The NIC and MIC values define a measured and computed value that characterizes each curve in terms of the biological effect end point. The input of each test therefore consists of the NIC and MIC values of each curve along with their associated standard deviation (SD) and degrees of freedom to assess differences between groups. The Tukey multiple comparisons test reports multiplicity adjusted P values that pool the SD of each group and therefore report conservative levels of significance. The 95% confidence interval and multiplicity adjusted P value <.05 were taken to be significant.

Results

Figures 1 to 3 represent the dose cytotoxicity profiles of each phytochemical between p53 variant human colon carcinoma cell lines and graphically describe the derivation of Gompertz

NIC and MIC best-fit values. The tabulated results in Table 1 put into perspective the relative cytotoxicity of each compound and p53-mediated effects between cell lines. From Table 1, the curcumin had the lowest NIC and MIC values in each of the cell lines, followed by andrographolide and D-limonene. The calculated NIC and MIC values of each of the phytochemicals were lower for the HT29 and HCT116 p53 null cell lines than the HCT116 p53 wt, with the exception of andrographolide where the MIC values were higher for the HT29 and HCT116 p53 null cell lines. Table 2 characterizes the steepness of the response via the NIC/MIC ratio value. The HCT116 p53 wt cell line exhibited steep survival on exposure to each of the compounds, whereas the broad profile of the curves of HT29 and HCT116 p53 null cell lines suggests a less pronounced and effective response per dose of the compounds. The differential effects between the function and nonfunctional p53 cell lines suggest that there might be a p53-mediated mechanism of cell cytotoxicity.
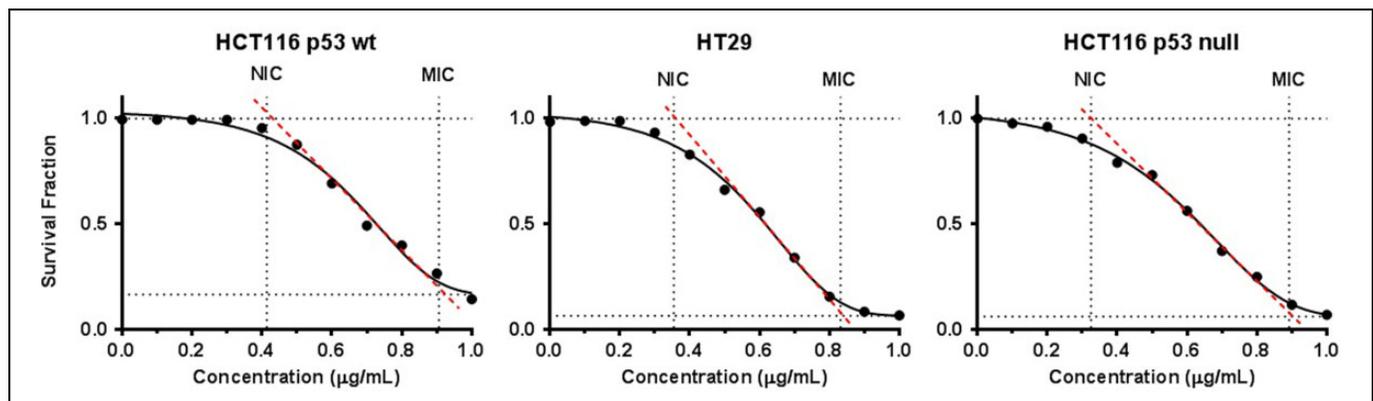
Figure 1. Curcumin cytotoxicity data on 3 p53 variant human colon carcinoma cell lines are representative of triplicate experiments. Values shown in each graph are expressed as the mean survival fraction of 3 independent experiments (n = 3). The solid line corresponds to the best-fit Gompertz function of the data. The dashed line represents the slope of the inflection point of the Gompertz function. Intersections of the inflection point with the upper and lower asymptote of the Gompertz function represent the NIC and MIC, respectively. Concentration data are expressed in micrograms per milliliter. MIC indicates minimal inhibitory concentration; NIC, nonminimal inhibitory concentration.
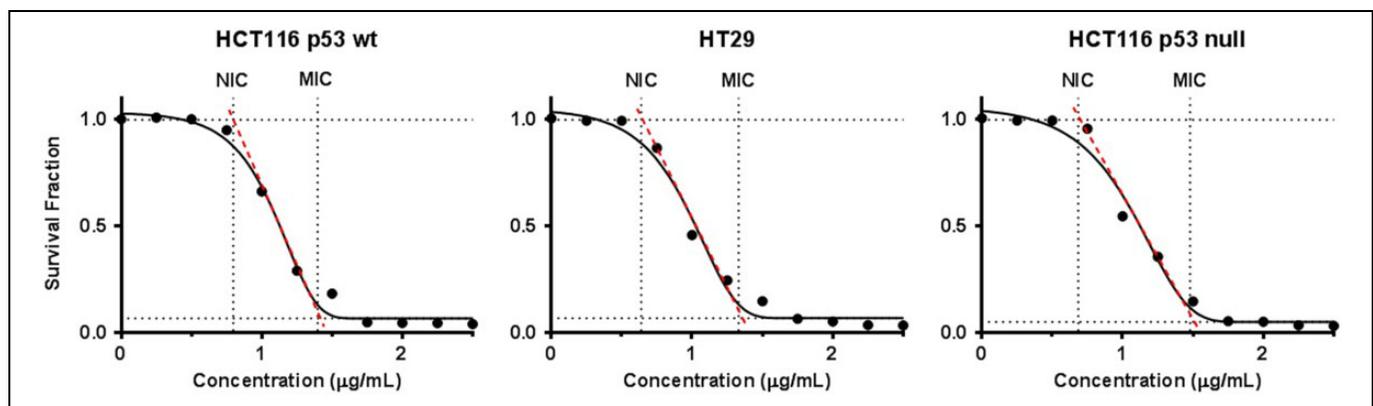
Figure 2. Andrographolide cytotoxicity data on 3 p53 variant human colon carcinoma cell lines are representative of triplicate experiments. Values shown in each graph are expressed as the mean survival fraction of 3 independent experiments (n = 3). The solid line corresponds to the best-fit Gompertz function of the data. The dashed line represents the slope of the inflection point of the Gompertz function. Intersections of the inflection point with the upper and lower asymptote of the Gompertz function represent the NIC and MIC, respectively. Concentration data are expressed in micrograms per milliliter. MIC indicates minimal inhibitory concentration; NIC, nonminimal inhibitory concentration.
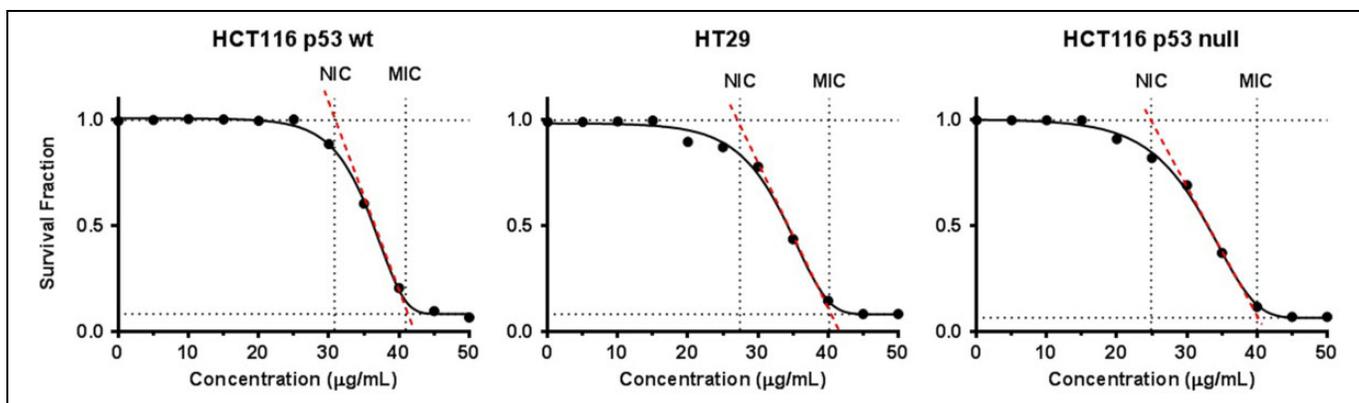
Figure 3. D-limonene cytotoxicity data on 3 p53 variant human colon carcinoma cell lines are representative of triplicate experiments. Values shown in each graph are expressed as the mean survival fraction of 3 independent experiments (n = 3). The solid line corresponds to the best-fit Gompertz function of the data. The dashed line represents the slope of the inflection point of the Gompertz function. Intersections of the inflection point with the upper and lower asymptote of the Gompertz function represent the NIC and MIC, respectively. Concentration data are expressed in micrograms per milliliter. MIC indicates minimal inhibitory concentration; NIC, nonminimal inhibitory concentration.

Table 1.[a].

Compound	HCT116 p53 wt		HT29		HCT116 p53 null	
	NIC	MIC	NIC	MIC	NIC	MIC
Curcumin	0.4144	0.9066	0.3548	0.8319	0.3254	0.8926
Andrographolide	0.7986	1.398	0.6394	1.331	0.6849	1.48
D-Limonene	30.83	40.97	27.44	40.17	24.9	40

Abbreviations: MIC, minimal inhibitory concentration; NIC, nonminimal inhibitory concentration; wt, wild type.
[a]Values are expressed in μg/mL.

Table 2.

Compound	HCT116 p53 wt	HT29	HCT116 p53 null
	NIC/MIC	NIC/MIC	NIC/MIC
Curcumin	0.457092	0.426494	0.364553
Andrographolide	0.571245	0.480391	0.462770
D-Limonene	0.752502	0.683097	0.622500

Abbreviations: MIC, minimal inhibitory concentration; NIC, nonminimal inhibitory concentration; wt, wild type.

Evaluation of the statistical significance in Table 3 of the best-fit values in the curcumin treatments showed there was significance in the comparison between the NIC and MIC values of the HCT116 p53 wt versus HT29 and HCT116 p53 wt versus HCT116 p53 null cell lines and the MIC values of HT29 versus HCT116 p53 null cell lines. Cytotoxicity comparisons in the andrographolide treatments demonstrate the significance between the NIC values of HCT116 p53 wt versus HT29 and the MIC values of HCT116 p53 wt versus HCT116 p53 null and HT29 versus HCT116 p53 null cell lines. The D-limonene demonstrated high significance between both the NIC and MIC values of HCT116 p53 wt versus HT29, HCT116 p53 wt versus HCT116 p53 null and the NIC value of HT29 versus HCT116

p53 null cell lines. Table 3 therefore suggests that there is a role for p53-mediated effects in cell survival at low-dose phytochemical exposure, otherwise we might anticipate that there are no differential effects between each of the cell lines, especially the HCT116 p53 wt and HCT116 p53 null variant cell lines. The observations of chemopreventative effects as per the experimental criteria are mostly in line with the D-limonene as it demonstrates high selective cytotoxicity toward the p53 mutant HT29 and p53-deficient HCT116 p53 null cell lines.

Discussion

The results demonstrate that there is likely a p53-mediated role in the induction of growth inhibition and cytotoxicity in response to curcumin, andrographolide, and D-limonene including their respective metabolites on the HCT116 p53 wt, HT29, and HCT116 p53 null colon carcinoma cell lines. Functional p53 is known to operate via binary response mechanisms, that is, at low doses to DNA damaging agents, the cell undergoes G1 cell cycle arrest allowing for the cell to undergo critical repair mechanisms prior to progression to S phase of the cell cycle.[12] At high doses, the cell accumulates p53 and causes apoptosis in response to sufficient damage to the cells.[12] Chemopreventative strategies targeting cancerous cells require activation of p53 and restoration of its ability to induce apoptosis, a strategy which is not an option in p53-deficient cell lines. Otherwise, it requires a bypass of this mechanism and uses an alternative p53-independent pathway to perform this function. The differential selection between the HCT116 p53 wt, HT29, and HCT116 p53 null cell lines is representative of the latter case. These observations are in line with the finding that the HCT116 p53 wt cells generally exhibit higher NIC and MIC values than HT29 and HCT116 p53 null cells that lack functional p53 transcription factors. At low doses, the compounds might initiate cell pathways that require p53-mediated cell cycle arrest to bypass the cell mechanism, which might be representative of slight xenohormetic basis for

Table 3. [a,b]

Compound	HCT116 p53 wt vs HT29		HCT116 p53 wt vs HCT116 p53 null		HT29 vs HCT116 p53 null	
	NIC	MIC	NIC	MIC	NIC	MIC
Curcumin	$P = .0035$[c]	$P = .0002$[d]	$P < .0001$[e]	$P = .6279$ NS	$P = .1835$ NS	$P = .0017$[c]
Andrographolide	$P = .0092$[c]	$P = .1063$ NS	$P = .0697$ NS	$P = .0410$[f]	$P = .6200$ NS	$P = .0003$[d]
D-Limonene	$P < .0001$[e]	$P = .0218$[f]	$P < .0001$[e]	$P = .0054$[c]	$P < .0001$[e]	$P = .8117$ NS

Abbreviations: MIC, minimal inhibitory concentration; NIC, nonminimal inhibitory concentration; ns, nonsignificant; wt, wild type.
[a]P value chart displays the results of p53-mediated phytochemical interactions and summarizes the 1-way analysis of variance (ANOVA) with subsequent Tukey multiple comparisons test. P values are multiplicity adjusted P values with 95% confidence interval.
[b]Level of significance.
[c]$P = .001$ to .01.
[d]$P = .0001$ to .001.
[e]$P < .0001$.
[f]$P = .01$ to .05.

these compounds. It is noteworthy that the phytochemicals serve as insecticides in plants.[13] Alternatively, the compound might enhance the activity of p53 and allow it to bypass this mechanism via an alternate pathway. Therefore, these compounds must operate in a p53-dependent way that selectively targets cells that do not have functional p53 and that might require functional p53 operation to inhibit other pathways. At the NIC dose of each compound, the HCT116 p53 wt exhibits a steep decline in cell lines. Therefore, at high doses, the compound might activate the p53-dependent apoptotic mechanism that explains the steep portion of the graph representative of the NIC/MIC ratio. Therefore, only those cells that lack functional p53 undergo premature cytotoxic effects.

In terms of oral dosing implications, the NIC value is the relevant value as the MIC value of nonfunctional p53 cell lines HT29 and HCT116 p53 null coincides to a significant extent with that of the HCT116 p53 wt cell lines and is therefore likely to cause toxic effects in otherwise nonmalignant cells. The successful chemopreventive candidate therefore has to have practical dose discrepancies between the NIC values of wt and mutant p53 cell lines to demonstrate in vivo effects. In this respect, the D-limonene satisfies this criterion more so than curcumin and andrographolide as the dose discrepancy between the NIC values of the HCT116 p53 wt and HT29 and HCT116 p53 wt and HCT116 p53 null cell lines are 3.39 μg/mL and 5.93 μg/mL, respectively. Although the cytotoxicity curves of the curcumin and andrographolide also demonstrate significant difference in NIC values between cell lines, the discrepancy between these values are very low; therefore, selective dosing might not be clinically practical. From the literature, D-limonene is known to bind to the RAS and downplay the cell hyperproliferation, which is an additional attribute to its cell regulatory mechanism.[14] Although this study does not address the role of RAS, it uses both the HCT116 p53 wt and HCT116 p53 null cell lines, each of which contain the RAS mutations; therefore, attributed effects must be independent of the mechanism of RAS inhibition.[15]

Conclusion

The use of phytochemicals in chemoprevention might have an important role in targeting various precancerous cell lines through enhancing cell regulatory mechanisms. Their appeal involves their noninvasive and practical route of administration via oral consumption. The compounds require frequent intake to confer maximal protective effects; in this regard, cytotoxic profiling of chronic long-term exposures to these compounds and their metabolites is important as it serves to provide guidance in this field. In this study, chronic exposures to curcumin, andrographolide, and D-limonene on p53 variant cell lines demonstrated that each of the compounds had some level of chemopreventative effect. The most pronounced effect though was observed in the D-limonene as demonstrated by the NIC dose discrepancies required to inhibit the growth of nonfunctional p53 cell lines. Therefore, further investigation might be warranted in the molecular pathways in which D-limonene inhibits these effects.

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