in vivo *24*: 387-392 (2010)

Diindolylmethane (DIM) Spontaneously Forms from Indole-3-carbinol (I3C) During Cell Culture Experiments

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Abstract. *Indole-3-carbinol (I3C) when given orally is converted to diindolylmethane (DIM) and other oligomers catalyzed by stomach acid. This suggests that DIM is the predominant active agent and that I3C is a precursor, 'pro-drug' in vivo. However, in cell culture studies carried out in neutral solutions, I3C has been considered fully active. Materials and Methods: The stability of I3C in cell culture media was studied. Results: In the 8 different cell culture media tested, greater than 50% dimerization of I3C into DIM occurred in 24 hours. At 48 hour, greater than 60% conversion was found. When neutral synthetic cerebrospinal fluid (CSF) or peritoneal fluid (PF) was studied, a large peak, tentitively identified as I3C's linear trimer (LTR) conversion product by mass spectra, and two smaller peaks, were seen. When CSF or PF was diluted 1:1 with media, the formation of these additional peaks was diminished. Conclusion: Because of the greater biologic potency of DIM when studied in parallel with I3C in vitro, this extent of dimerization shows that DIM rather than I3C is the active agent in cell culture studies.*

Numerous studies have established the therapeutic potency of indole-3-carbinol (I3C) in *in vivo* studies in hormone-related diseases including breast and prostate cancer (1, 2). In *in vivo* studies, I3C is usually given orally in the diet or administered once daily by gavage to the stomach. In studies where diindolylmethane (DIM) or I3C were given by intraperitoneal (IP) administration to study liver enzyme activity (3, 4) or stimulation of immune function (5), DIM was considerably more potent than I3C. One study by Garikapati *et al*. (6), giving I3C IP or intravenously, found a modest effect in limiting the growth of transplanted prostate cancer. The question of whether the observed response to I3C was actually due to DIM and other I3C conversion products formed spontaneously in tissue fluids has not been addressed by prior investigators.

Previous studies have established that I3C is a labile compound *in vitro*, which readily rearranges to DIM, linear trimer (LTR), cyclic trimer (CTR), indoloc carbazole and other oligomers under acidic conditions (7). Thus, orally administered I3C is almost completely converted to DIM and the other compounds noted above in the acidic environment present in the stomach. Following oral administration of I3C, the blood level of I3C is transient and only found in animal plasma for 30 minutes (8) and not at all in human plasma (9-10). For this reason, it is generally considered that the conversion products in the stomach, primarily DIM, are the active agents and that I3C is an unstable precursor, functioning as a "pro-drug". Although I3C is less active than DIM when given IP, under cell culture conditions, at neutral pH, an active response to I3C has been observed in a large number of studies (2, 11, 12). This study tested the possibility that significant conversion of I3C to DIM also occurs under physiologically neutral cell culture conditions or in the presence of intraperitoneal fluid. The dimerization reaction showing the conversion is illustrated in Figure 1. Based on this reaction, a 2 molar concentration of I3C will be converted to a 1 molar concentration of DIM following a complete condensation reaction.

Materials and Methods

I3C and DMSO were obtained from Sigma-Aldrich (St. Louis, Mo, USA). All other reagents were obtained from VWR (West Chester, PA, USA). The 8 cell culture media (MEM, DMEM, Delbecco's, Iscove, RPMI, M199, Mccoy's, and Leibowitz L-15) were obtained from Gibco (Carlsbad, CA, USA). All transfers of reagents into the culture tubes were carried out in a laminar flow hood.

Synthetic extracellular peritoneal fluid (EX) was prepared by the method of Montenegro *et al*. (13). Synthetic cerebrospinal fluid (CSF) was prepared by the method on McKay *et al*. (14). See Table I for compositional details of these two media.

Cell culture media studies. Pure I3C (100 mg) was dissolved in 2 ml of DMSO and 30 μl of this solution was added to 4 ml of the

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Key Words: Indole-3-carbinol, I3C, diindolylmethane, DIM, culture media, linear trimer.

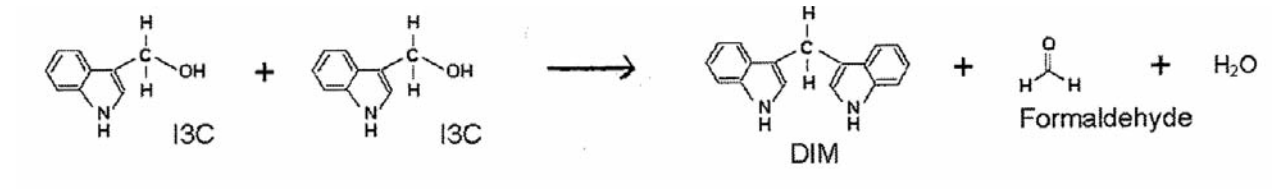

Figure 1. *Condensation of I3C into DIM and formaldehyde.*

8 different media in sterile tubes. The tubes were then tightly capped and placed in a cell culture incubator for 24, 48, or 96 hrs at 37˚C. Each of the tubes was then extracted twice with 2 ml of chloroform. The combined extracts were evaporated to dryness in a rotating vacuum evaporator. The residue was taken up in 30 μl of ethyl acetate and an aliquot of each was promptly spotted on 20 × 20 cm reverse-phase thin layer chromatography (TLC) plates (Merck Darmstadt, Germany) at 2 cm intervals and developed in 70/30 hexane-ethyl acetate. I3C and DIM reference standards were also spotted on the same plates. Under these TLC conditions I3C was completely stable with negligible conversion to DIM. The plates were developed with a spray of a methanolic solution of phosphomolybdic acid (4 gm per 10 ml of methanol). Figure 2 illustrates a sample run. The plates were then assayed using the ImageJ program developed at the National Institutes of Health (15) providing a quantitative intensity value for the each developed spot corresponding to I3C, DIM or unknown TLC isolate. The ratio of the signal intensity values for I3C and DIM were calculated. Additional runs were carried out with 1:10 serum-media mixtures and other TLC runs were carried out containing media + serum + 10^{-6} MCF-10A cells.

Synthetic intraperitoneal fluid and cerebrospinal fluid studies. A volume of 15 μl of I3C stock solution (100 mg of I3C in 2 ml of DMSO) was added to 4 ml of sterile CSF or EX (Table I) and incubated at 37˚C for 24 or 48 hours. The incubates were processed as described above. A sample run is illustrated in Figure 3.

Production of a reference curve for presence of I3C relative to DIM. The signal intensity assays were calibrated by spotting known quantities of I3C and DIM on similar TLC plates, which were then developed and quantitated as described above (Tables II and III). Both compounds that were part of a pair were run on the same plate. A sample run is illustrated in Figure 4.

Results

The aim of the calibration studies was to determine the ratio of intensities found for different known proportions of I3C and DIM. When 10 μg of DIM and 5 μg of I3C (1.19 μM ratio) were compared the signal intensity ratio was 1.23. To achieve the same intensity ratio starting with I3C, at least 60% of the I3C initially added in the various incubates would have to be converted to DIM. With 60% conversion, approximately 2/3 of the I3C will be converted to DIM. Since I3C condenses into DIM at a ratio of 2:1, the intensity ratio of roughly equal molar amounts of DIM [MW 246] (10

Table I. *Composition of synthetic media.*

Extracellular fluid (13) Mg/100 ml		Cerebrospinal fluid (14) Mg/100 ml	
NaCl	841 mg	NaCl	725 mg
KCl	20 mg	KCl	20 mg
$MgCl_2$	22 mg	$MgCl_2$	19 mg
Ascorbic acid	3 mg	$NaHCO_3$	210 mg
100 ml of 2 mM phosphate buffer adjusted to pH 7.1		NaH_2PO_4 pH adjusted to 7.1	120 mg
		Dextrose	450 mg

Table II. *Comparison of the intensity ratios for known molar concentration of samples of pure I3C and DIM.*

TLC of standards mass	DIM:I3C		
	5 μg to 10 μg molar ratio	5 μg to 5 μg molar ratio	10 μg to 5 μg molar ratio
	0.29	0.60	1.19
Ratio of image intensity	0.78	1.14	1.23

Table III. *Correspondence of DIM/I3C image intensity ratio to molar DIM:I3C ratio.*

DIM:I3C image intensity ratio	Molar ratio of DIM:I3C
0.5	0.97
1.0	1.22
1.50	1.35
2.0	1.44
2.5	1.51
3.0	1.57

Intermediate values were computed from a logarithmic equation (Y=0.3205(ln{x} +1.2183).

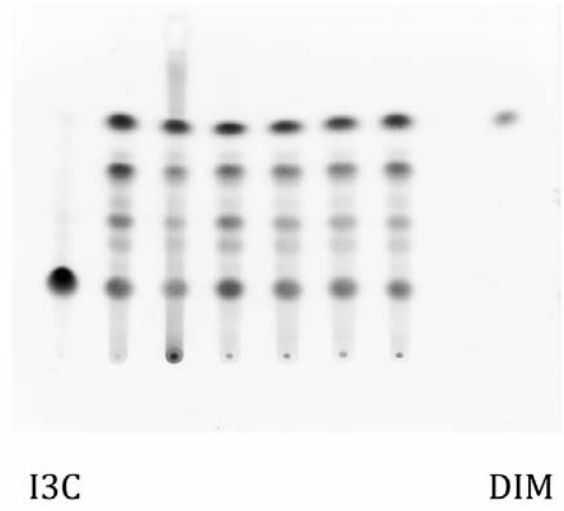

Figure 2. *Dimerization of I3C to DIM in different cell culture media.*

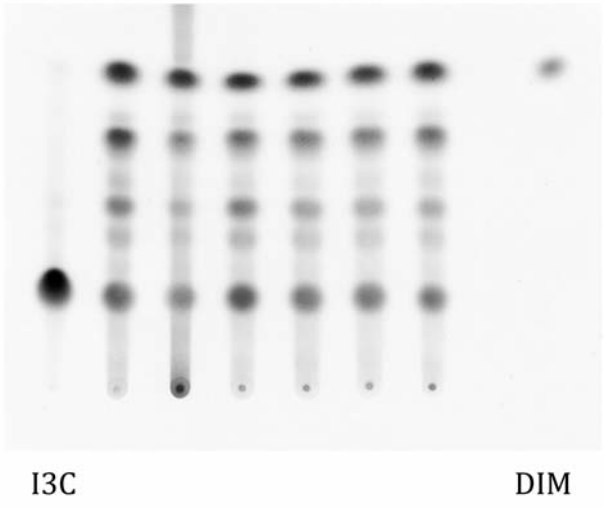

Figure 3. *Oligomerization of I3C to DIM and LTR in synthetic CSF and EX.*

Table IV. *Summary of TLC results expressed as ratios of DIM to I3C intensities in cell culture media.*

Time (hours)	24	36	48	72	96
Avg. DIM/I3C Ratio	1.28	1.50	2.44	2.58	2.49
Media alone	61%	62%	64%	65%	64%
Avg. DIM/I3C Ratio	1.05		1.12*		
Media+serum	60%		60%		
Avg. DIM/I3C Ratio	0.99***		1.06***		
Media +serum +cell	54%		60%		

*Media + 25% serum; ***media +25% serum+ cells; % represents % converson of I3C to DIM.

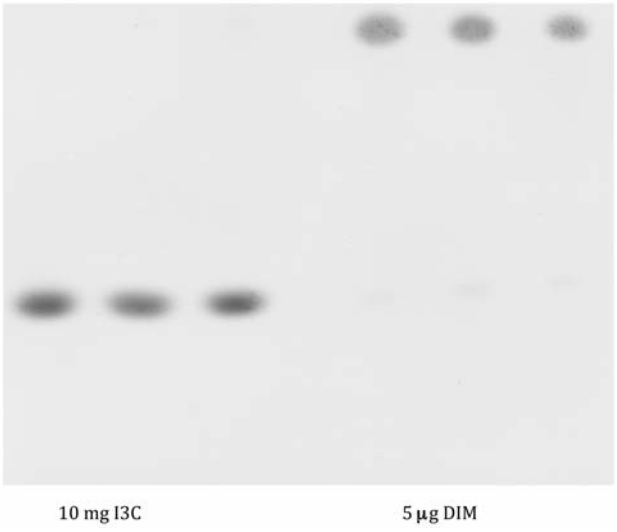

Figure 4. *Calibration of 10 µg of I3C and 5 µg DIM.*

mg) to I3C [MW147] (5 mg) is 1.23 (Table II). It should be noted that 1 mg of formaldehyde is generated per 100 mg of I3C converted to DIM. However, since the body normally generates and metabolizes 50 g of formaldehyde a day as part of one carbon metabolic cycle, the addition of 1 or 2 mg of additional formaldehyde would be inconsequential *in vivo* (16). In calculating how many mg of I3C must be converted to DIM to yield a 10 mg to 5 mg ratio, a correction is required for the fact that some mass is lost in converting I3C to DIM by multiplying 10 mg by 294 (2×147)/246 {mw of DIM} to correct for the larger mass of I3C required to yield 10 mg of DIM.

Significant and approximately similar conversion of I3C to DIM in cell culture media was observed in all cases. Based on intensity ratios greater than 1 after 24 hours of incubation, at least 50% of the I3C initially added was converted to DIM. The results are shown in Table IV. At the earliest time evaluated (24 hours) the extent of conversion of I3C to DIM was above 50% in all incubation conditions based on an image intensity ratio greater than 1.1 (Table II). The quantitative measurements indicate that an intensity ratio of 1.23 for DIM/I3C represents at least a 60% conversion of I3C to DIM (Tables III and IV). At this extent of conversion, I3C and DIM are approximately equimolar in the solution. Considering the greater potency of DIM over I3C, at this extent of conversion there is sufficient DIM present to account for the anti-proliferative and pro-apoptotic activity observed in most published I3C cell culture studies (11, 12).

Table V. *Metabolism of I3C in synthetic media +/–serum or cell culture media.*

Time	CSF	CSF*	CSF**	CSF***	CSF****	EX	EX*	EX**	EX***	EX****
24 hrs										
DIM/I3C	1.12	0.68	1.12	0.78	0.77	1.32	1.03	1.26	0.94	1.001.00
LTR/I3C	1.06	0.70	0.74	0.53	0.53	0.82	0.47	1.06	0.71	0.41
48 hrs										
DIM/I3C	1.12	0.68				1.74	1.18			
LTR/I3C	0.79	0.70				2.21	0.90			

+ 20% serum, **1:1 with Dulbecco's; ***1:1 with Icove's, ****1:1 with M199.

While the addition of serum or cells slightly reduces the conversion, there remains sufficient DIM generated to account for the anti-proliferative, pro-apoptotic activity observed in most reported cell culture experiments (17, 18).

The intensity ratio of 1.25 seen at 48 hours with I3C added to media plus serum and cells was also comparable to the ratio observed for approximately equimolar DIM and I3C (Table III). In order to explore the conversion of I3C to DIM in intraperitoneal fluid, synthetic EX and CSF were prepared. Incubation of I3C in these fluids in the absence of any protein addition was carried out. The conversion pattern (Table IV) was completely different. In addition to TLC peaks corresponding to I3C and DIM, a third major peak in between the I3C and DIM was seen. The formation of this compound was greater in (EX) than in CSF. When a larger scale experiment was carried-out, it was possible to tentatively identify this third peak as the LTR based on the GC-MS fragmentation pattern. Earlier work by DeKruif *et al*. (7) also reported the formation of LTR in aqueous systems. Dilution of EX and CSF with serum (1:1) decreased the formation of LTR. In addition 1:1 dilution of PF or CSF with any of three different tissue culture media (Dulbecco's, Iscove's, and M199) resulted in substantial suppression of the formation of LTR. This suggests that in the 2006 study by Garakapati *et al*. (18), where I3C was injected IP, the tumor growth inhibition observed was due primarily to DIM.

Discussion

Assuming at least a 50% conversion of I3C to DIM and approximately equimolar concentrations of I3C and DIM provides a basis for interpreting the observed activity of I3C in previous cell culture studies (2, 11, 12). In three out of four recent studies testing both I3C and DIM the minimum inhibitory concentration (MIC) directly measured for DIM was 25 μM, which would result from 66% conversion of 100 μM of I3C into DIM and would be reflected by signal intensity ratios near 1.2 (Tables V and VI). Thus, conversion of I3C to DIM *in vitro* provides a sufficient source of DIM to account for the observed growth inhibition in earlier published cell culture studies starting with pure I3C (17-20).

Table VI. *Comparison of reported minimal inhibitory concentrations for I3C and DIM to estimated presence of DIM from I3C in recent studies.*

Culture model	Cell type	Drug	IC_{50} (μM)	MIC (μM)	Predicted DIM from I3C at MIC (μM)	Reference
Breast cancer	MCF-7	I3C		100	25	(17)
		DIM		25		
Prostate cancer	DU-145	I3C	200	200	25	(18)
		DIM	50	25		
Prostate cancer	DU-145	I3C	160	100	25	(19)
		DIM	25	25		
Cervical cancer	C33A	I3C	200	100	25	(20)
		DIM	60	50		

IC_{50}: Inhibitory concentration resulting in 50% reduction of control cell numbers; MIC: minimum inhibitory concentrations for I3C or DIM resulting in a clear reduction in cell number.

Sufficient DIM is formed from I3C in cell culture to account for most of the previously reported *in vitro* activity of I3C (17, 18). It is likely that the same will be true for peritoneal fluid, though the rates may vary depending on pH and protein content. Based on the high rate of spontaneous conversion of I3C to DIM and LTR by 24 hours, cell culture results attributing activity at 48 hours to I3C need to be reconsidered in view of the substantial levels of DIM present due to conversion. Based on the rates of conversion deomonstrated here, many anti-proliferative and pro-apoptotic results reported for I3C are likely due to the effects of DIM, which is more potent, or alternatively, due to the combined effects of DIM and LTR.

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Received March 25, 2010
Revised May 30, 2010
Accepted June 4, 2010