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# Indospicine cytotoxicity and transport in human cell lines

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ABSTRACT

Indospicine, a non-proteinogenic analogue of arginine, occurs only in *Indigofera* plant species and accumulates in the tissues of animals grazing on *Indigofera*. Canine deaths have resulted from the consumption of indospicine-contaminated meat but only limited information is available regarding indospicine toxicity in humans. In this study three human cell lines, Caco-2 (colorectal adenocarcinoma), HT29-MTX-E12 (colorectal adenocarcinoma) and HepG2 (hepatocellular carcinoma), were used to investigate the cytotoxicity of indospicine and its metabolite 2-aminopimelic acid in comparison to arginine. Indospicine and 2-aminopimelic acid were more cytotoxic than arginine, displaying the highest toxicity in HepG2 liver cells. Intestinal transport *in vitro* also revealed a 2-fold higher transport rate of indospicine compared to arginine. The sensitivity of HepG2 cells to indospicine is consistent with observed canine hepatotoxicity, and considering the higher *in vitro* transport of indospicine across an intestinal barrier, it is possible that similar ill effects could be seen in humans consuming contaminated meat.

## 1. Introduction

Indospicine (L-6-amidino-2-aminohexanoic acid), a naturally occurring non-protein amino acid, is an analogue of arginine (Fig. 1) with hepatotoxic properties. Indospicine is present in several species of the *Indigofera* plant genus that grow in grazing regions of Australia. The similarity in chemical structure to the proteinogenic amino acid arginine may disrupt arginine-related protein synthesis mechanisms. It has been demonstrated that indospicine inhibits, but is not hydrolysed by, arginase (Madsen & Hegarty, 1970) and acts as a competitive inhibitor for binding to arginyl-tRNA synthetase. This action prevents the incorporation of arginine, and other amino acids, in protein synthesis with secondary impacts on DNA synthesis (Christie, De Munk, Madsen, & Hegarty, 1971; Madsen, Christie, & Hegarty, 1970). Indospicine can also interfere with various other arginine-dependent liver enzymes acting as an antagonist and impeding arginine metabolism (Christie, Wilson, & Hegarty, 1975; Christie et al., 1971; Madsen & Hegarty, 1970; Praswati, 1989).

Indospicine produces a wide variety of toxic effects ranging from teratogenic and embryolethal effects (Pearn & Hegarty, 1970) to liver disease (Hegarty & Pound, 1970). Mild to severe indospicine-induced hepatotoxicosis has been described in ruminants and non-ruminants following dietary indospicine exposure with toxin sensitivities dependent upon the animal species (Fletcher, Al Jassim, & Cawdell-Smith, 2015; Hegarty & Pound, 1970; Nordfeldt et al., 1952; Suliman, Wasfi, Tartour, & Adam, 1983). Due to its structural similarity to arginine, indospicine toxicity is not only restricted to liver disease. It is also a teratogenic agent with very slow elimination rates from mammalian tissues and no clear evidence of any mammalian enzyme that can degrade the amidino group of indospicine (Hegarty, 1986).

High intake of *Indigofera* plant material in grazing cattle and sheep, as well as feeding trials in rats, mice and rabbits has confirmed liver damage in these species. Canines in particular are most vulnerable to indospicine hepatotoxicity, with reported secondary poisonings in dogs that consume indospicine-contaminated meat from horses and camels in Alice Springs in 1984 (Hegarty, Kelly, McEwan, William, & Cameron, 1988) and Perth in 2010 (FitzGerald, Fletcher, Paul, Mansfield, & O'Hara, 2011). Hence, an important characteristic of indospicine is that its toxicity is not only limited to the animals that first ingest it, but it also has the potential to impact on secondary consumers further down the food chain, including humans. However, little is known about toxicity in humans following consumption of indospicine or whether its metabolites may also produce hepatotoxicity or have teratogenic effects.

A recent study demonstrated that indospicine is not degraded under domestic cooking conditions or during human gastrointestinal digestion

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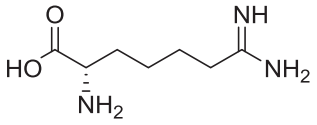
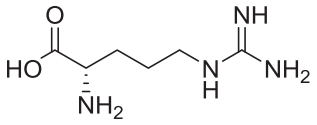



Indospicine

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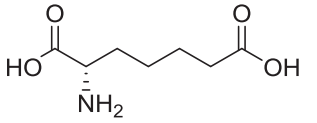


2-Aminopimelic acid

**Fig. 1.** Chemical structures of indospicine, arginine and 2-aminopimelic acid.

*in vitro* (Sultan et al., 2017). Therefore, indospicine could be readily available for uptake across the gastrointestinal tract as a free amino acid into the circulatory system. It is not known whether indospicine undergoes slow or rapid intestinal transport, or if there is competition for transporters when arginine and indospicine are present.

In this study three human cell lines, Caco-2 (colorectal adenocarcinoma), HT29-MTX-E12 (colorectal adenocarcinoma) and HepG2 (hepatocellular carcinoma), were employed to investigate the toxicity of indospicine and a commercially available indospicine hydrolysis product, 2-aminopimelic acid (Tan, Yong et al., 2016) in comparison to arginine. Cytotoxicity data obtained from these cell lines was compared to assess level of toxicity in both the human gastrointestinal tract and liver *in vitro* in an effort to ascertain the potential toxicity of indospicine following human consumption. Caco-2/HT29-MTX-E12 co-cultures grown in transwell apparatus were also used to assess gastrointestinal transport rates of indospicine and 2-aminopimelic acid *in vitro* compared to arginine.

## 2. Materials and methods

### 2.1. Materials

Synthesized indospicine and $D_3$-L-indospicine were provided by Prof. James De Voss and Dr. Robert Lang, The University of Queensland (Lang et al., 2016). Fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM), penicillin and streptomycin, glutamax, Dulbecco's phosphate buffered saline without calcium and magnesium (PBS), Hank's Balanced Salt Solution (HBSS), trypsin-EDTA, trypan blue exclusion dye and non-essential amino acids (NEAA) were purchased from Invitrogen (Sydney, NSW, Australia). Nunc cell culture flasks and 96 well plates were from Sigma-Aldrich (Castle Hill, NSW, Australia). The CellTiter 96® AQueous non-radioactive cell proliferation assay (MTS) cell viability assay was from Promega (Alexandria, NSW, Australia). The Caco-2 cell line was from the American Type Culture Collection. The HepG2 and HT29-MTX-E12 cell lines and all remaining chemicals were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

### 2.2. Cell culture

Caco-2 and HT29-MTX-E12 cells were cultured in DMEM media supplemented with 10% FBS (v/v), 1XNEAA, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM Glutamax. HepG2 cells were cultured in DMEM media supplemented with 10% FBS (v/v), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM Glutamax. All cells were grown in vented culture flasks in 5% $CO_2$ at 37 °C. Cells were passaged every 2–3 days or when 90% confluent. For sub-culturing, cells were washed with PBS. Cells were removed from flask by adding 0.25% (v/v) trypsin-EDTA and incubated for 1–2 min or until cells detached. Growth media was then added to neutralize trypsin. After centrifugation, old media was discarded and fresh media was added to re-suspend the cells. Cells were counted using trypan blue and the cell suspension used for further culturing of cells.

### 2.3. Intestinal cytotoxicity studies

All cytotoxicity studies were performed as previously described (Osborne et al., 2014), with modifications detailed for each *in vitro* model. Cytotoxicity is expressed as the percentage of viable cells remaining after treatment compared to the HBSS control; the data is presented as the mean percentage ( ± SE) of six replicates for each treatment. The half maximal inhibitory concentration ($IC_{50}$) was also calculated from the percentage of viable cells remaining for each compound and cell line using four parameter, variable response curves fitted by GraphPad Prism version 6 (La Jolla, CA, USA).

#### 2.3.1. Caco-2 cells

Undifferentiated and differentiated Caco-2 cells were used to assess the cytotoxicity of indospicine, 2-aminopimelic acid and arginine. For the undifferentiated Caco-2 cell model, $4 \times 10^4$ cells/well were grown in 96 well plates for 24 h prior to treatment producing 90% cell confluency. Cells were then washed by replacing culture media with HBSS (100 µl/well) for 2 h. HBSS was removed and the cells treated with 100 µl/well indospicine, 2-aminopimelic acid and arginine diluted in HBSS to produce at dose range of 500–1300 µg/ml (in 100 µg/ml increments). Each treatment was applied for 2 h with growth media used as positive control and HBSS as a negative control. Cell viability in all *in vitro* models was assessed using the MTS assay and involved the following: removing all treatments from the cells; adding 100 µl HBSS and 20 µl MTS solution per well; incubating for 120 min at 37 °C and measuring absorbance at 492 nm. For the differentiated Caco-2 cell model, $1 \times 10^4$ cells/well were grown in 96 well plates for 7 days prior to the same treatment conditions and cell viability measurements as detailed for the undifferentiated Caco-2 cell model (above).

#### 2.3.2. HT29-MTX-E12

To investigate the cytotoxicity of indospicine, 2-aminopimelic acid and arginine on intestinal goblet cells *in vitro*, $4 \times 10^4$ HT29-MTX-E12 cells/well were grown in 96 well plates for 24 h prior to the same treatment conditions and cell viability measurements as detailed for the cell models (above) but using the following dose points: 600, 700, 800, 900, 1000, 1030, 1062, 1100 and 1200 µg/ml.

### 2.4. Hepatotoxicity studies

To investigate the cytotoxicity of indospicine, 2-aminopimelic acid and arginine on liver cells *in vitro*, $5 \times 10^4$ Hep G2 cells/well were grown in 96 well plates for 24 h prior to the same treatment conditions and cell viability measurements as detailed for the cell models (above) but using the following dose points: 300, 400, 500, 600, 650, 700, 800, 900 and 1000 µg/ml.

### 2.5. Intestinal transport assays

Caco-2 and HT29-MTX-E12 cells were seeded into transwell apparatus at cell densities of $8.1 \times 10^4$ and $9 \times 10^3$ cells $cm^{-2}$ (respectively) and cultured in growth media for 21 days to facilitate cell differentiation and formation of an intact cell layer. During this period, growth media was removed and replaced every 2–3 days with fresh media with differentiation determined by measuring the transepithelial