Lactic acid bacteria are the dominant microorganism in dairy and fermented food products. This review provides a detailed insight into arginine biosynthesis, catabolism, role of transporters and factor affecting arginine metabolism in lactic acid bacteria. Although, the genetic organization of arginine catabolic operons show considerable conservation among various bacterial species; but they have developed different mechanisms by which gene expression is regulated in various bacterial species. Molecular attributes of an important arginine catabolic enzyme i.e. arginine deiminase were comparatively reviewed along with different approaches of purification employed to enhance yield of purified native and recombinant enzyme. A brief overview of arginine metabolism in eukaryotes is indicated in later sections with special reference to role of key enzymes in arginine deprivation cancer therapy. Finally, summaries of *in vitro* preclinical and clinical studies on arginine deiminase indicating efficacies and IC_{50} against various melanomas conclude this review.

2.1 Bacterial arginine catabolic pathways:

Bacteria degrade arginine mostly by two routes: first is arginine-urease pathway, which involves enzyme arginase (EC 3.5.3.1), which hydrolysis arginine to ornithine and urea, followed by transformation of urea to ammonia and carbon dioxide, and ornithine to citrulline. According to this pathway, one mole of arginine can be converted to one mole of ornithine and two moles of ammonia (Kuensch *et al*., 1974). Second pathway, commonly described as arginine deiminase (ADI) (or dihydrolase) pathway (Brechot *et al*., 1984; Liu, 1990) comprises of three cytoplasmic enzymes: arginine deiminase (ADI, EC 3.5.3.6) which catalyzes the irreversible hydrolysis of the guanidino group of arginine to yield the ureido compound citrulline and ammonia; ornithine transcarbamoylase (OTC, EC 2.1.3.3), which cleaves citrulline into carbamoyl phosphate and ornithine; and carbamate kinase (CK, EC

2.7.2.2), which produces ATP, ammonia and carbon dioxide through dephosphorylation of carbamoyl phosphate. According to this pathway, one mole of arginine can yield two moles of ammonia and one mole each of ornithine, ATP and carbon dioxide.

The ADI pathway is widely distributed among bacteria, both Gram-positive, Gram-negative and archaebacterial species mainly belonging to *Aeromonads, Bacillus licheniformis* (Maghnouj *et al*., 2000), *Clostridium perfringens* (Ohtani *et al*., 1997), *Cyanobacterium* (Bednarz and Schmid, 1991; Leisner *et al*., 1994) *Enterococcus faecalis* (Barcelona-Andres *et al*., 2002), *Enterococcus faecium* (Kaur and Kaur, 2014), *Euglena gracilis Z* (Park *et al*., 1984), *Halobacterium salinarum* (Ruepp and Soppa, 1996), *Lactobacillus sakei* (Zuniga *et al*., 1998), *Lactococcus lactis* ssp. *lactis* (Kim *et al*., 2007), *Leuconostoc oenos* (Sponholz *et al*., 1991) , *Pseudomonas aeruginosa* (Gamper *et al*., 1991), *Pseudomonas putida* (Kakimoto *et al*., 1971), *Mycoplasma hominis* (Schimke and Barile, 1963), *Oenococcus oeni* (Divol *et al*., 2003), *Rhizobium etli* (D'Hooghe *et al*., 1997), *Staphylococcus* (Makhlin *et al*., 2007), *Streptococcus gordonii* (Dong *et al*., 2002), *Streptococcus sanguis* (Ferro *et al*., 1983) and *Weissella confusa* (Kaur and Kaur, 2012). Lactic acid bacterial (LAB) strains isolated from meat and fermented meat products have a tendency to express ADI pathway under favorable environmental conditions. LAB strains, example *L. sakei*, have developed regulatory mechanisms particularly the adaption of the bacterium to low oxygen and glucose concentration or presence of ribose, and arginine environment which seem to be compatible with its natural meat environment (Montel and Champomier, 1987).

ADI pathway can serve several physiological functions, as apparent from the end–products formed. The ammonia produced results in alkalization of the cytoplasm, thereby keeping intracellular processes operational under acid stress conditions. It provides

carbamoyl phosphate for the biosynthesis of citrulline or pyrimidines and ATP for the microbial growth, especially during carbohydrate starvation or when sugar is available to cells at a very low concentration (Casiano-Colon and Marquis, 1988). ADI pathway provides additional energy, which favors growth of certain LAB species (Arena *et al*., 1999a, 1999b) or contributes to an improved cell survival during stationary growth phase, as for *L. sakei* (Champomier Verges *et al*., 1999). The enzymes of ADI pathway are less sensitive to low pH than the enzymes involved in glycolysis (De Angelis *et al*., 2002). This is especially important for acidifying LAB, since acid stress conditions are typically encountered during carbohydrate starvation (De Angelis and Gobbetti, 2004). Therefore, the expression of ADI pathway in industrial microorganisms, such as LAB can be of great significance since it can be considered as a mechanism of energy production (Liu and Pilone, 1998) and pH regulation (Sanders *et al*., 1999).

2.2 How do LAB catabolize arginine?

LAB are the dominant microorganisms in fermented meat (Montel and Champomier, 1987), fermented dry sausages (Hammes *et al*., 1990), meat and meat products (Champomier-Verges *et al*., 1999) and sourdoughs (Zuniga *et al*., 2002). ADI pathway is widely distributed among important LAB strains isolated from dairy sources, grape musts or wines and apple cider (Spayd and Andersen-Bagge, 1996; Manca de Nadra *et al*., 1988; Arena *et al*., 2008). Wine LAB mainly belongs to three genera including homo- and hetero-fermentative lactobacilli, homofermentative pediococci, and heterofermentative *Oenococcus oeni* (formerly known as *Leuconostoc oenos*). Wine lactobacilli vary in their ability to degrade arginine. Homofermentative wine lactobacilli and pediococci (strains of *L. delbrueckii, L. plantarum, P. cerevisiae, and P. parvulus*) do not degrade arginine (Edwards

et al., 1993; Liu *et al*., 1995a, b). Conversely, all heterofermentative wine lactobacilli such as strains of *L. hilgardii*, *L. buchneri*, *L. brevis* can hydrolyse arginine (Weiller and Radler, 1976; Chalfan *et al*., 1977; Sponholz, 1992; Edwards *et al*., 1993; Liu, 1993; Liu *et al*., 1994, 1995b).

Initially, arginine catabolism was proposed to follow urea cycle in wine LAB, including the strain of *L. oenos*, as the sole arginine-degrading pathway. This concept was later on extended to other members of wine LAB that also degrade arginine (Sponholz *et al*., 1991; Sponholz, 1991, 1992). However, the stoichiometry of arginine conversion to ornithine and ammonia can be obtained only if arginine is broken down by wine LAB via ADI pathway as proposed earlier (Brechot *et al*., 1984; Liu, 1990; Pilone *et al*., 1991). Most of the *Leuconostoc* strains including strains of *L. oenos*, possess activities of all three enzymes i.e ADI, OTC and CK, but some *Leuconostoc* strains can display only OTC or CK activity (Liu *et al*., 1995b). Absence of ADI, OCT and CK activities in homofermentative lactobacilli and pediococci, explains the inability of these organisms to degrade arginine via ADI pathway. The absence of arginase and urease activities and the stoichiometry of arginine conversion to ornithine and ammonia in *L. oenos* and *L. buchneri*, led to the conclusion that ADI pathway is the exclusive route of arginine catabolism in wine LAB (Liu *et al*., 1996).

In contrast to homofermentative wine LAB, arginine is degradable by a number of homofermentative LAB from other sources, for example, *E. faecium* from dairy product (Kaur and Kaur, 2014), strains of *Lactococcus lactis* subsp. *lactis* from cheese (Crow and Thomas, 1982), *L. leichmannii* (Manca de Nadra *et al*., 1986b) and *L. plantarum* from fish (Jonsson *et al*., 1983), *L. sake* from meat (Montel and Champomier, 1987), *P. cerevisiae* (now *P. acidilactici* (Bacus and Brown, 1985)) from fermented sausages (Deibel *et al*.,

1961a, b) and fermented meat (Holley and Millard, 1988) and *Streptococcus faecalis* (Simon *et al*., 1982). However, several homofermentative lactobacilli from milk fermentations (Gilliland, 1985) and beer (Martens *et al*., 1997) do not degrade arginine.

The predominant sourdoughs Lactobacilli also degrade arginine via ADI pathway. Only obligate heterofermentative strains such as *L. sanfranciscensis* CB1, *L. brevis* AM1, AM8, and 10A, *L. hilgardii* 51B and *L. fructivorans* DD3 and DA106 show activities of all the three ADI pathway enzymes (De Angelis *et al*., 2002). *L. sanfranciscensis* has been found to be the dominant strain in American, Swedish, German, Swiss, and Italian sourdoughs (Gobbetti *et al*., 1994; Gobbetti *et al*., 1996). The importance of *L. sanfrancisco* has been related to its heterofermentative metabolism, short lag phase during dough acidification (Gobbetti *et al*., 1995), production of volatile compounds (Gobbetti *et al*., 1995) and symbiotic relationship with *Saccharomyces exiguus* (Sugihara *et al*., 1970), enhanced tolerance to acid environmental stress and greater production of ornithine, which improve the organoleptic characteristics of the sourdough (De Angelis *et al*., 2002).

2.3 Role of transporters in arginine catabolism:

In bacteria, metabolic energy is available in two forms i.e. ATP and transmembrane ion-gradients, that can be used to drive various endergonic reactions associated with cellular growth. ATP can be formed directly in substrate level phosphorylation reactions whereas primary transport processes can generate ion-gradients across the cytoplasmic membrane. These energy forms can be interconverted easily by the action of ion-translocating ATPases. For fermentative organisms, it has long been speculated that ion-gradients could only be generated at the expense of ATP hydrolysis by the FoF1-ATPase. Especially in anaerobic bacteria, secondary transport systems can contribute significantly to the supply of metabolic

energy by uniport, symport and antiport systems. These secondary transport systems convert chemical energy of one solute into the chemical energy of protons or sodium ions. The resulting forces can, subsequently, be used for other energy-requiring processes (Konings *et al*., 1994)

In addition to three intracellular enzymes namely ADI, OCT, CK and one membrane-bound carrier protein known as arginine/ornithine antiporter is also involved. In *L. lactis* and *E. faecalis*, one molecule of ornithine is excreted per molecule of arginine taken up. The transport reactions are facilitated by an antiporter which maintains the stoichiometric of electroneutral exchange across the membrane for intracellular ornithine with extracellular arginine (Driessen *et al*., 1987, 1989; Thompson, 1987; Poolman *et al*., 1987a). The concentration gradient of these compounds is the main driving force for the transport process, and does not require ATP hydrolysis (Knodler, *et al*., 1995).

A factor that strongly influences the activities of secondary transport systems in bacteria is the cytoplasm pH (Poolman *et al*., 1987b). Even a small variation of the internal pH can lead to inactivation of the transport system and can hinder transport process despite of a large driving force. Generally, conditions which lower ATP consumption (like high internal pH or inhibition of F0F1-ATPase by *N, N*`-dicyclohexyl-carbodiimide) decrease ADI pathway activity, whereas protonophores and ionophores which stimulate ATP consumption increase the activity (Cunin *et al*., 1986; Konings *et al*., 1989). Therefore, the intracellular ATP pool is an important regulator of the ADI pathway.

Arginine cannot be utilized as a source of energy by some bacteria under carbohydrate starvation. As absence of fermentable carbohydrate in the medium suppress bacterial growth which in turn dysregulate arginine uptake and metabolism as reported in

strains of *S. faecalis* (Bauchop and Elsden, 1960; Deibel, 1964; Pandey, 1980), *L. plantarum* (Jonsson *et al*., 1983), *L. buchneri* (Manca de Nadra *et al*., 1986a) and *L. oenos* (Liu *et al*., 1995a). In cell suspensions or membrane vesicles of sugar/arginine grown cells of *L. lactis* subsp. *lactis*, *P. aeruginosa*, *S. faecalis*, *S. sanguis*, *S. milleri* and *Spiroplasma melliferum* (Driessen *et al*., 1987; Poolman *et al*., 1987a, b; Konings *et al*., 1989; Shirazi *et al*., 1995), arginine is transported through an inducible arginine-ornithine exchange system in an energy independent process (Verhoogt *et al*., 1992; Poolman *et al*., 1987a; Konings *et al*.,1991) and this transport seems to be rather influenced by external pH (Poolman *et al*., 1987b). This exchange process is driven by arginine metabolism and allows optimal energy conversion when cells suffer from limited energy supply (Verhoogt *et al*., 1992). On the other hand, active diffusion facilitated with energy generated from carbohydrate fermentation may be the mechanism of initial arginine transport which means the arginine-ornithine exchange system operates only after initial uptake of arginine. In wine LAB, especially *L. oenos* OENO and *L. buchneri* CUC-3, both arginine-ornithine and citrulline-ornithine antiporter are operative whereas, there is no citrulline-ornithine exchange in *Lactococci* (Poolman *et al*., 1987a) and *L. oenos* (Liu *et al*., 1996).

2.4 Factors affecting arginine metabolism in bacterial species:

The three ADI pathway enzymes in wine LAB such as *L. buchneri* (Manca de Nadra *et al*., 1986a) and in *S. faecalis* (Simon *et al*., 1982) are not constitutive but are concurrently induced when grown in the presence of arginine. In contrast, ADI and OTC enzymes in *L. lactis* subsp. *lactis* are induced by arginine while the enzyme CK is constitutively expressed (Crow and Thomas, 1982). Several regulatory mechanisms like availability of arginine (Gamper *et al*., 1992) and oxygen, carbohydrate metabolism, acid stress (Rimaux, 2011) or

intracellular ATP, NADH, levels seem to signal ADI expression in various bacterial species (Montel and Champomier, 1987). In *P. aeruginosa* (Cunin *et al*., 1986), *H. salinarum* (Ruepp and Soppa, 1996), *B. licheniformis* (Maghnouj *et al*., 1998), *S. faecalis* (Simon *et al*., 1982), *S. rattus* and in *S. sanguis* (Burne *et al*., 1989), anaerobic conditions triggering arginine catabolism are invariably linked to energy production and regulation of nitrogen metabolism. Therefore, regulation of ADI gene expression differs among microbes, and invariably seems to be connected to their adaptation to diverse habitats (Fernandez and Zuniga, 2006).

Earlier, it was reported that the metabolism of glucose and arginine is sequential and the catabolism of galactose and arginine is concurrent as observed in lactococci from cheese (Crow and Thomas, 1982). Later on, in wine LAB strains such as *L. buchneri*, *L. brevis* and *L. oenos*, the metabolism of glucose and arginine was observed to be concurrent, whereas fructose and arginine were metabolized sequentially (Liu *et al*., 1996). Indeed, fructose inhibits arginine metabolism which suppresses the production of ammonia from arginine in some wine LAB (Liu *et al*., 1995b). Based on the extent of inhibition, wine LAB can be divided into two groups. Strains in Group I (including *L. oenos* OENO, Ey2d and *lactobacilli* CUC-3, Equilait and Microenos HP) are not inhibited, presumably due to their higher rate of fructose utilization with significant amounts of ammonia being produced from arginine. Group II strains *L. oenos* starter strains (such as MCW, Erla and Inobacter) are inhibited by fructose and therefore utilize it slowly which produce little ammonia from arginine.

The type of sugar seems to significantly influence expression of ADI pathway enzymes. In *lactococci*, higher specific activities of ADI pathway enzymes were reported in galactose-grown cells than in glucose, lactose or sucrose grown cells (Crow and Thomas, 1982; Manca de Nadra *et al*., 1986a). The mechanism of sugar repression is not well

understood, though it is commonly attributed to catabolite repression. Glucose represses expression of ADI operon in bacteria such as *Carnobacterium* (Leisner *et al*., 1994), *L. buchneri* (Manca de Nadra *et al*., 1986a), *L. leichmannii* (Manca de Nadra *et al*., 1986b), *L. sake* (Montel and Champomier, 1987), *P. aeruginosa* (Mercenier *et al*., 1980), *S. faecalis* (Simon *et al*., 1982), *S. mitis* (Hiraoka *et al*., 1986), *S. sanguis* (Ferro *et al*., 1983 ; Burne *et al*., 1989), and some marine *Vibrio* species (Macian *et al*., 1996). Sucrose and/or fructose also repress expression of ADI pathway enzymes in *L. buchneri* and *S. mitis* (Hiraoka *et al*., 1986) and *S. sanguis* (Ferro *et al*., 1983). While, availability of oxygen represses ADI pathway in *P. aeruginosa* (Mercenier *et al*., 1980).

In LAB environmental pH strongly influences metabolite kinetics of the ADI pathway. The kinetic analysis of ADI pathway metabolites suggest secretion of large amounts of citrulline into the environment under optimal pH conditions in *L. sakei* (Rimaux *et al*., 2011) and *L. fermentum* IMDO 130101 (Vrancken *et al*., 2009) whereas, under acid stress conditions, arginine is almost exclusively converted into ornithine. In *L. fermentum* IMDO 130101, *L. buchneri* CUC-3, *L. sanfranciscensis* CB1 and *L. hilgardii* after complete conversion of arginine, citrulline is taken up and further converted into ornithine (Liu *et al*., 1994, 1996; Mira de Orduna *et al*., 2000; Tonon and Lonvaud-Funel, 2002). However, this conversion is pH dependent and corresponded the expression profiles of the *arc* genes in *L. sakei* CTC 494 (Rimaux *et al*., 2011), operating maximally at optimal pH for growth (pH 5.78), with lower conversion rate under acid stress conditions and no citrulline-into-ornithine conversion at high pH stress conditions. A similar pattern was also observed in *L. fermentum* IMDO 130101, but in a wider pH range (3.50 to 7.50) (Vrancken *et al*., 2009a). In contrast, expression of *arc* operon genes induced at low pH values of *L. lactis* subsp. *lactis* ML3, *L.*

hilgardii X1B, *L. brevis* IOEB 9809, and *S. gordonii* DL1 (Arena *et al*., 2011; Chou *et al*., 2001; Liu *et al*., 2008), suggests a protective mechanism against acid stress due to ammonia production through the ADI pathway.

2.5 Genetic organization and induction of ADI pathway:

The ADI pathway is widely distributed in the microbial world and shows considerable conservation in the genetic organization and primary structure of ADI pathway enzymes. In contrast, the physiologic role and genetic regulation of expression of the ADI vary among microorganisms, with a particular complexity occurring among LAB species (Zúñiga *et al*., 2002a).

Most of the bacteria studied so far have ADI-related genes organized in one or more clusters and order of ADI pathway enzymes are conserved (**fig. 2.1**). In Gram-negative bacteria like *Halobacterium* (Ruepp and Scoppa, 1996), *Pseudomonas* (Gamper *et al*., 1991) and *Rhizobium* (D'Hooghe *et al*., 1997), ADI pathway usually harbors clusters encompassing only structural genes, but no regulatory gene associated to these clusters has been found so far. In high-G+C Gram-positive bacteria, the ADI-encoding genes are not organized into clusters such as *Mycoplasma* (Schimke and Barile, 1963), while, in low G+C Gram-positive bacteria such as *Bacillus* (Maghnouj *et al*., 2000), *Clostridium* (Ohtani *et al*., 2007), *Enterococcus* (Barcelona-Andres *et al*., 2002), *Lactobacillus* (Zuniga *et al*., 1998) and *Streptococcus* (Dong *et al*., 2004), genes are arranged into complex clusters, including dedicated regulatory and accessory genes encoding for proteins which may or may not be directly involved in the ADI pathway (**fig 2.1**). The *arcA*, *arcB*, and *arcC* genes mainly encode the three cytoplasmic enzymes of the ADI pathway i.e. ADI, OTC, and CK respectively. In addition to these genes, other genes, such as those encoding for an

arginine/ornithine antiporter (*arcD*), putative aminotransferases or transaminases (*arcT*), and regulatory gene (*arcR*) have also been identified in ADI operon (Zúñiga *et al*., 2002b; Fernández and Zúñiga, 2006). The ArgR protein acts as a transcriptional regulator of *arc* operon, which binds to somewhat imperfect palindromes, known as ARG boxes. Transcriptional regulators belonging to the Anr (anaerobic regulation of arginine catabolism and nitrate reduction)/Fnr (fumarate nitrate reductase) family (Lu *et al*., 1999; Stockley *et al*., 1998) mainly controls expression of ADI under anaerobic conditions as shown in *Bacillus licheniformis* (Stockley *et al*., 1998), *Pseudomonas aeruginosa* (Gallimand *et al*., 1991; Lu *et al*., 1999) and *Rhizobium etli* (D'Hooghe *et al*., 1997). ADI seems to be essential for efficient nitrogen fixation by these organisms. Anr, the anaerobic regulatory protein (Gamper *et al*., 1991), is essential for the induction of the *arcDABC* operon under oxygen stress conditions. In addition, an exogenous supply of *L*-arginine can further induce *arc* operon expression through the interaction of Anr with regulatory ArgR (Park *et al*., 1997). On the other hand, under aerobic conditions, *L*-arginine is metabolized through the arginine succinyltransferase (AST), arginine decarboxylase (ADC) and arginine dehydrogenase (ADH) pathways as studied in *P. aeruginosa* (Itoh, 1997).

The expression of *arc* operon in oral streptococci, including *L. sakai*, *S. gordonii*, *S. sanguis*, and *S. rattus*, is inducible by arginine and is under the control of carbon catabolite repression (CCR) (Ferro *et al*., 1983; Fernández and Zúñiga, 2006). Repression of CCR sensitive operons occurs when CcpA (catabolite control protein A) binds to a palindrome sequence of carbon catabolite response element (Henkin *et al*., 1991). The *arc* operon in *S. gordonii* and *S. suis* follows the order as *arcABCDT*. The *flpS* gene of *S. suis* shows considerable homologies to a Crp/Fnr transcription regulator of *S. pyogenes*, the ArcR

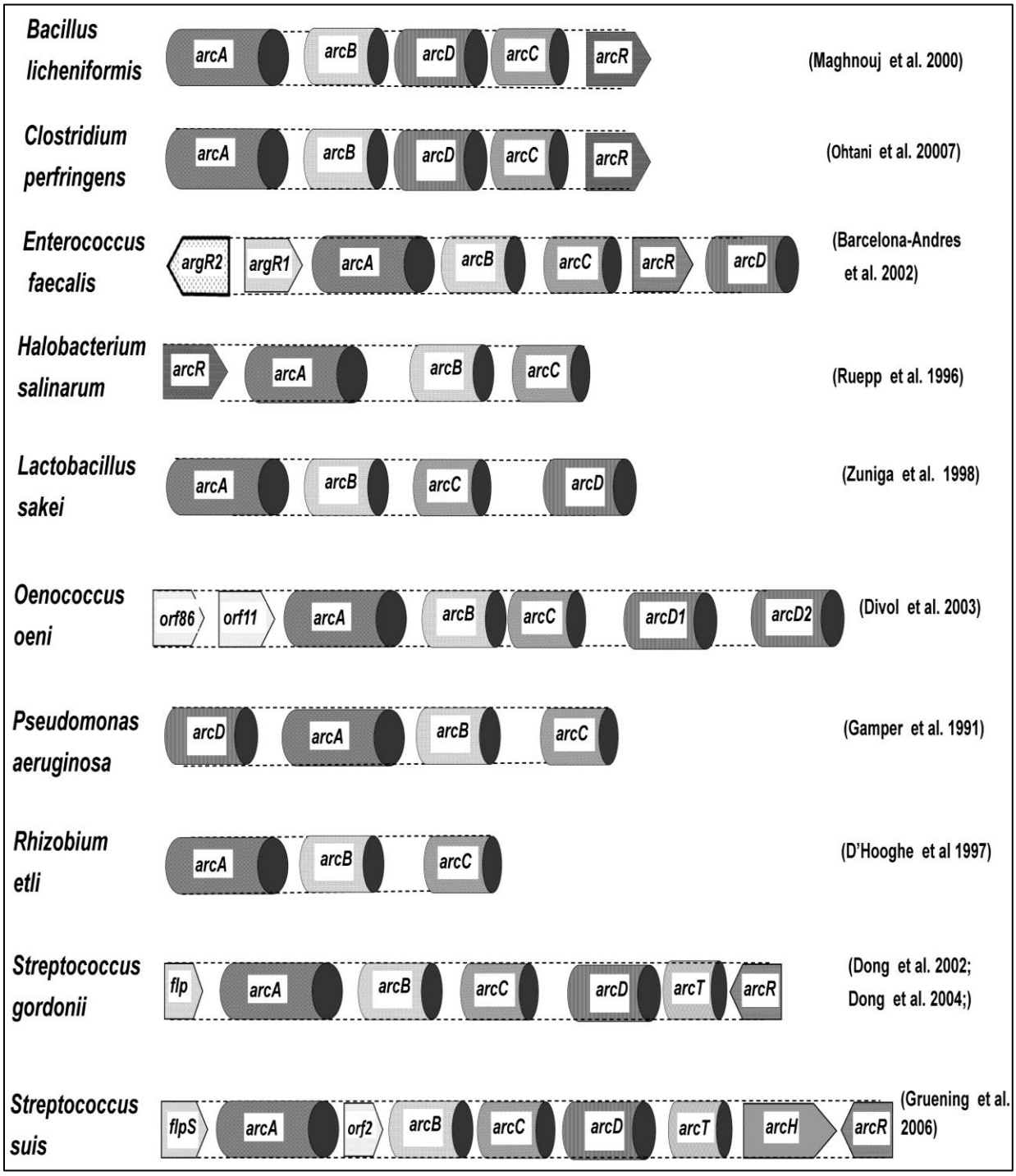

Fig. 2.1: Genetic organization of ADI pathway genes in various microbes.

protein of *B. licheniformis*, and Flp of *S. gordonii*, which are essential factors for anaerobic expression of ADI (Dong *et al*., 2004). The putative β-*N*-acetylhexosaminidase (*arcH*), with high homology to an *S. pneumonia* β-*N*-acetylhexosaminidase has not been described in ADI by any other bacteria (Gruening *et al*., 2006).

In *H. salinarum*, each gene of *arcRACB* appears to have an independent promoter, and thus genes are differentially induced by ArcR under oxygen limiting state (Ruepp and Scoppa, 1996). Arginine is a strongest inducer irrespective of the state of aeration, and while, oxygen is weakest repressor of ADI pathway unless glucose is fed to the bacterium (Simon *et al*., 1982).

In *E. faecalis* arginine induces the expression of *arcABCRD* operon which is controlled by two homologous ArgR/AhrC-type regulators encoded by genes, *argR1* and *argR2* respectively. They regulate expression by binding to conserved motifs found in the promoter regions of *arcA* and of *argR1/argR2* themselves (Barcelona-Andres *et al*., 2002). In *O. oeni*, constitutive expression of *arcD* genes namely, D1 and D2 points to the positive role of arginine on *O. oeni* cell growth (Divol *et al*., 2003).

2.6 Molecular properties of bacterial ADI:

ADI pathway was reported for the first time in *Bacillus pyocyaneus* in 1933 by Horn. Since then, several ADI genes have been identified, purified, and characterized from archaea, bacteria, and some eukaryotes excluding mammalian cells. **Table 2.1** shows sources and molecular characteristics of ADI's identified from various microorganisms. Extensive purification techniques were employed to purify native and recombinant ADI to homogeneity from various bacterial strains (**table 2.1 and table 2.2**). Utilizing ammonium sulphate precipitation; DEAE cellulose, Sephadex G-25, P-cellulose and Sephacryl S-200 column chromatography ADI from *E. gracilis* Z markedly resulted in 79.5 purification fold with

specific activity of purified protein as 1.59 IU/mg (Park *et al*., 1984). *E. faecalis* NJ402 enzyme is isolated and purified using sequential ammonium sulphate precipitation, Q-Sepharose FF anion exchange, SephadexG-75 gel filteration and ammonium sulphate precipitation, ultrafiltration, Sephadex G-100 gel filtration which improved specific activity by 33-35 folds (Li *et al*., 2008). ADI from *H. salinarium* was purified by consecutive ammonium sulphate precipitation, Sepharose CL-6B, HB1, Hydroxypatite and Propyl-agarose column chromatography, showed 311fold purification with specific activity of 90.02 (nKat/mg) (Monstadt and Holldorf, 1990). In *L. sanfranciscensis* CB1 utilizing DEAE-Cellulose anion exchange column; Superpose 12HR 10/30 gel filteration; Phenyl-Superose 5/5 Hydrophobic interaction column and Mono Q HR 5/5 column chromatography, enzyme was homogenized with highest specific activity of 591.1IU/mg recorded so far (De Angelis *et al*., 2002). In *M. arginini* and *M. hominis*, purified ADI with 21.98 and 11.8 fold activity was recovered using molecular-sieve; anion exchange, arginine-affinity chromatography and Sephacryl S-300 HR, Q-Sepharose FF, Arginine-Sepharose column chromatography respectively (Takaku *et al*., 1995). The crystallized enzyme is obtained after ammonium sulphate fractionation, Protamine sulphate fractionation, DEAE-cellulose and Sepharose 6B column chromatography with 38.68 purification fold (Shibatani *et al*., 1975). The molecular weight of ADI subunits fall in the range 45-55 kDa as shown in **table 2.1**. In addition to this, the ADI subunit structures also show marked variations among different microrganisms such as monomeric in *L. sanfranciscensis* CB1 (Kim *et al*., 2007), as homodimeric in *H. salinarium* (Monstadt and Holldorf, 1990), *M. arginini* (Takaku *et al*., 1995), *M. hominis* (Takaku *et al*., 1995) and *P. plecoglossicida* M314 (Liu *et al*., 2008), whereas, as tetrameric in *E. faecalis* NJ402 (Li *et al*., 2008).

Table 2.1: Molecular characteristics of bacterial ADI and purification approaches adopted

S.No	Native source	Molecular weight (kDa)		Subunit structure	Purification method	Specific activity of crude extract (IU/mg)	Specific activity of purified protein (IU/mg)	Purification fold	Reference
		Native PAGE	SDS-PAGE						
1	*Euglena gracilis* Z	87	48	Homodimer	DEAE cellulose column; Ammonium sulphate precipitation; Sephadex G-25 column; P-cellulose column; Sephacryl S-200 column	0.02	1.59	79.5	Park *et al*., 1984
2	*Enterococcus faecalis* NJ402	190	46	Tetramer	Ammonium sulphate precipitation, Q-Sepharose FF anion exchange, SephadexG-75 gel filteration	0.238	8.212	34.5	Li *et al*., 2008
3	*Halobacterium salinarium*	105	55	Homodimer	Ammonium sulphate precipitation; Sepharose CL-6B column; HB1 column; Hydroxypatite column; Propyl-agarose column	0.33 (nKat/mg)	90.02 (nKat/mg)	311	Monstadt and Holldorf, 1990
4	*Lactobacillus sanfranciscensis* CB1	46	46	Monomer	DEAE-Cellulose anion exchange column; Superpose 12HR 10/30 gel filteration; Phenyl-Superose 5/5 Hydrophobic interaction column; Mono Q HR 5/5 column	16.2	591.1	36.48	De Angelis *et al*., 2002
5	*Mycoplasma arginini*	90	45	Homodimer	molecular-sieve; anion exchange chromatography; arginine-affinity chromatography	1.91	42	21.98	Takaku *et al*., 1995
6	*Mycoplasma hominis*	90	47	Homodimer	Sephacryl S-300 HR column; Q-Sepharose FF column; Arginine-Sepharose column	1.52	35.5	11.8	Takaku *et al*., 1995
7	*Pseudomonas putida*	120	54	Dimer	Ammonium sulphate fractionation; Protamine sulphate fractionation; DEAE-cellulose column; Sepharose 6B column; Crystallization	1.52	58.8	38.68	Shibatani *et al*., 1975

2.7 Cloning and expression of recombinant ADI:

Various ADI genes have been cloned and expressed in *E. coli* strains to understand their function in arginine metabolism, cell growth, and further explore their biological attributes including anti-tumor activity (**table 2.2**).

Recombinant gene expression in heterologous host was attempted for the first time by Brune *et al*., 1989. The isolated ADI pathway genes from *Streptococcus sanguis* chromosomal DNA by generating a gene library with bacteriophage lambda and subsequently expressed the genes in *E. coli* using pUC19 vector. Mapping studies employing subcloned genes exhibited that the ADI pathway enzymes are tightly clustered on *S. sanguis* chromosome.

The ADI encoding gene *arcA* of *L. lactis* ssp. *lactis* ATCC 7962 (LADI) was cloned and expressed in *E. coli* BL21 (DE3) using pGEM-T vector (Kim *et al*., 2009). The trimeric recombinant ADI was purified to homogeneity which inhibited proliferation of SNU-1 human stomach cancer cells with half-maximal inhibitory concentration, IC_{50}=0.6 µg/ml. LADI induced G_0/G_1-phase arrest , sub-G_1 accumulation, DNA condensation and DNA fragmentation in ADI treated SNU-1 cancer cells (Kim *et al*., 2009).

The *arcA* gene of *P. plecoglossicida* CGMCC2039 was also expressed in *E. coli* BL21 (DE3) using pET28a vector. The N-terminal His_6-tagged rADI was purified to homogeneity by metal affinity column chromatography with specific ADI activity of 4.76 U/mg. *In vivo* study showed 82% inhibition of H22 tumor growth in experimental mice when a total dose of 5 U/mouse was administered over a 2-week period (Ni *et al*., 2009). Whereas, ADI cloned from mutated strain *Pseudomonas plecoglossicida* M314 and expressed in *E.*

coli BL21 (DE3) using pET24a vector, showed a much higher specific ADI activity of 21.7 U/mg (Ni *et al*., 2011)

Recently, *M. hominis* gene coding for ADI was expressed in *E. coli* BL21 (DE3) using pET3d vector. Homogenous recombinant ADI was obtained using anion-exchange and hydrophobic sepharose, phenyl-sepharose column chromatography. The purified enzyme retained a specific activity of 30–34 U/mg (Fayura *et al*., 2013).

There is a single report on the characterization and heterologous gene expression of ADI from eukaryotic organism. Knodler *et al*., (1998) cloned and over-expressed the ADI of *Giardia intestinalis* in *E.coli.* The 6xHis-tagged rADI was purified to homogeneity in a single step using metal chelation column chromatography (Knodler *et al*., 1998).

Table 2.2: Molecular characteristics of recombinant bacterial arginine deiminases and purification approaches adopted

S.No	Native source	Molecular weight (kDa)		Subunit Structure	Purification method	Expression host	Vector	Specific activity (IU/mg)	Reference
		Native PAGE	SDS PAGE						
1	*Giardia intestinalis*	140	66	Dimer	HisTrapR chelating column	E. coli M15(pREP 4)	pQE 30	36.5	Knodler *et al*., 1998
2	*Lactococcus lactis* ssp. *lactis* ATCC 7962	138	45.93	Homotrimer	Q-Sepharose anion exchange, Sephacryl-200 gel filteration	E.coli BL21 (DE3)	pGEM-T	140.27	Kim *et al*., 2007
3	*Pseudomonas plecoglossicida* CGMCC2039	-	49	-	Cellulose membrane filteration; Pre-packed nickel-chelated agrose column	*E.coli* BL21(DE3)	pET28a	4.76	Ni *et al*., 2009
4	*Pseudomonas plecoglossicida* M314	92.6	46.3	Homodimer	DEAE-FF 16/10 anion exchange column; Superdex-200 gel filteration	*E.coli* BL21(DE3)	pET24a	21.7	Ni *et al*., 2011
5	*Mycoplasma hominis*	-	45	Monomer	Anion exchange; Q-Sepharose column; phenyl-sepharose column	*E. coli* BL21 (DE3)	pET3d	30-34	Fayura *et al*., 2013
6	*Streptococcus sanguis*	-	-	-	-	*E.coli*	pUC19	0.115	Burne *et al*., 1989

2.8 Arginine metabolism in mammalian cell:

In mammals, *L*-arginine is catabolized by four enzymatic pathways encompassing nitric oxide synthase (NOS; EC 1.14.13.39), arginase (ARG; EC 3.5.3.1), arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1), and arginine decarboxylase (ADC; EC 4.1.1.19) (**fig. 2.2**). These enzymes act on the guanidino group of *L*-arginine, except for ADC. Multiple isoforms of some of these enzymes exist in nature that further adds to metabolic complexity of eukaryotic cells. Microbial ADI (EC 3.5.3.6), is not expressed by animal cells (Zuniga *et al*., 2002).

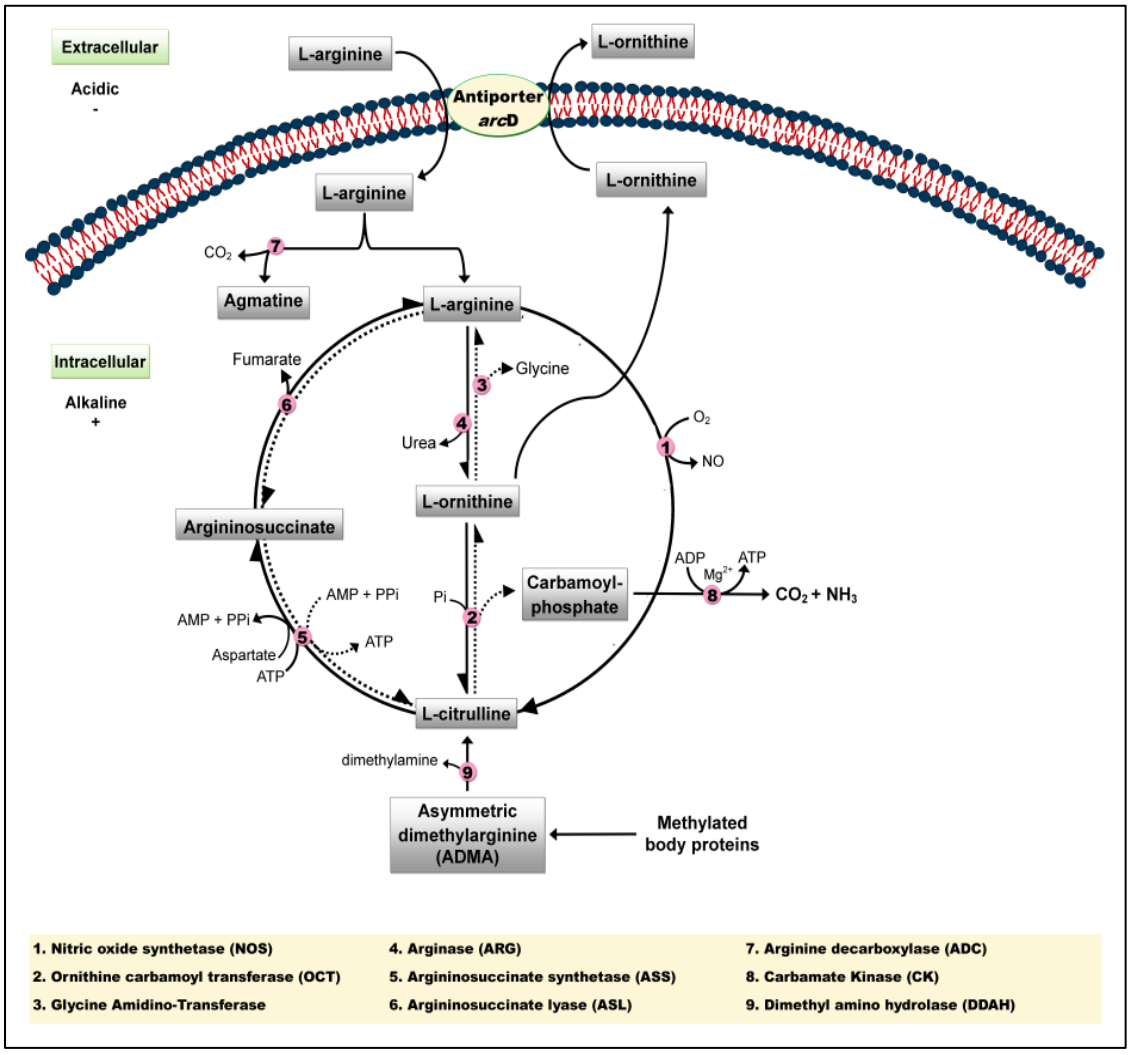

Fig. 2.2: Arginine metabolism: role of key regulating enzymes and their mechanism

Some pathogenic microbes like *Giardia lamblia* expressing ADI present within the mammalian host, may provide protection from host cell defenses by consuming *L*-arginine, thus compromising the ability of host cells to produce NO (Eckmann *et al*., 2000).

AGAT catalyzes the first and rate-controlling step in the synthesis of creatine. The biosynthesis of creatine is a two-step process in which arginine is converted to glycine to yield *L*-ornithine and guanidinoacetic acid by *L*-arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1). Guanidinoacetic acid, by the action of *S*-adenosyl-*L*-methionine:N-guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2), is then methylated at the amidinogroup to give creatinine (**fig. 2.2**). Dimethylarginine dimethylaminohydrolase (DDAH; EC 3.5.3.18), catabolizes N^G, N^G-dimethyl-*L*-arginine (ADMA) and N^G mono methyl-*L*-arginine to citrulline and di- or mono methylamine) (Murray-Rust *et al*., 2001). AGAT and DDAH show structural similarities indicating that they represent members of a common structural superfamily of enzymes. ADC converts *L*-arginine to CO_2 and agmatine, which is further, converted to putrescine and urea by agmatinase (Iyer *et al*., 2002; Mistry *et al*., 2002). A family of enzymes called the NO synthases (NOSs, EC 1.14.13.39) catalyze the oxidation of arginine to NO and *L*-citrulline, with NADPH and O_2 serving as cosubstrates (Alderton *et al*., 2001). The NOSs first hydroxylate terminal guanidino nitrogen of Arg to generate *N*-hydroxy-*L*-arginine (NOHA) as an enzyme bound intermediate. NOHA is then oxidized further by the enzyme to generate NO plus *L*-citrulline (**fig. 2.2**). The arginase catalyzes hydrolysis of *L*-arginine to *L*-ornithine and urea (**fig. 2.2**). In liver, this reaction is the final step of urea biogenesis.

In mammals, two cytosolic enzymes namely argininosuccinate synthetase (EC 6.3.4.5) and argininosuccinate lyase (EC 4.3.2.1) synthesize *L*-arginine from citrulline by de

novo pathway (**fig. 2.2**). The synthesis of 1mole of argininosuccinate requires hydrolysis of 2 moles of ATP to AMP. Citrulline can be supplemented from other resources available to the cells such as ADMA formed on proteolysis of methylated proteins via DDAH which converts into citrulline and dimethylamine and ornithine via catabolism of proline or glutamine/glutamate. The pathways linking *L*-arginine, glutamate, and proline are bidirectional. So, differential gene expression of these amino acids depends on the cell type and developmental stage (Wu and Morris, 1998). Glutamine and glutamate are used as precursor for synthesis of *L*-arginine in epithelial cells of the small intestine, which further, extract citrulline from the circulation with the help of proximal tubal cells of the kidney and convert it into *L*-arginine (Wu and Morris, 1998). Any flaw of the renal function or small intestine can reduce arginine synthesis, thereby increasing its dietary requirement. Arginine can also be metabolized to ornithine by the enzyme arginase, and ornithine can be converted to citrulline by ornithine carbamoyltransferase (OTC) in the mitochondria. This citrulline can be utilized to synthesize arginine again.

2.9 Determination of cell death in apoptosis, autophagy and necrosis pathways:

Autophagy has probably evolved to serve homeostatic functions such as cytoplasmic, protein and organelle turnover. Autophagy is upregulated in response to different forms of stress, including nutrient and energy depletion, hypoxia, endoplasmic reticulum (ER) stress, microbial infections and metabolic diseases (Corcelle *et al*., 2006; Pandey *et al*., 2007; Hoyer-Hansen *et al*., 2007). Process involving macromolecule and organelle catabolism generates free amino and fatty acids in bulk, which are necessary to overcome metabolic stress. Basal levels of autophagy are important for the physiological control of the quantity and quality of organelles in diverse phyla and function to eliminate surplus, exhausted and

damaged subcellular components. It may be particularly important during post-mitotic condition, when the divided cells face difficulty in re-distribution of damaged organelles, proteins and aggregates after cell division.

In cancer cells, cellular and therapeutic stresses activate several signal transduction pathways that lead to cell death or cell survival by a complex interplay of diverse effector molecules. *In vitro* studies on cancer cells helped to categorize mainly three forms of cell death pathways - apoptosis, autophagy, and necrosis (Kroemer *et al*., 2005), wherein apoptosis and necrosis are irreversible forms of cell death. **Table 2.3** comprises the morphological characteristics, key regulators, and immune responses of apoptosis and autophagy. Autophagy is a process by which a cell ‘‘eats’’ itself and lead to cell death or paradoxically allow cells to escape cell death (Lum *et al*., 2005). Nutrient starvation triggers a multistep process called macroautophagy, an evolutionary conserved eukaryotic process in which unwanted organelles and macromolecular aggregates are degraded by lysosomal activity, and provides ATP and raw material for synthesis of energy rich metabolites during metabolic stress (Gonzalez-Polo *et al*., 2005). The autophagy is characterized by formation of a double-membrane vesicle called autophagosome which is formed after fusion of lysosomes with engulfed proteins and organelles. The hydrolytic reactions mediated by lysosomal enzymes results in accumulation of reactive oxygen species (ROS) which lead to inactivation of the cysteine protease ATG4, and accumulation of the ATG8-phosphoethanolamine precursor that is required for the initiation of autophagosome formation (Scherz-Shouval *et al*., 2007) as shown in **fig. 2.3**. Activation of autophagy exhausts essential organelles and proteins of the cell, which leads to a caspase independent cell death (Yu *et al*., 2004).

Table 2.3: Characteristics of apoptosis and autophagy (adapted from Amaravadi and Thompson, 2007)

	Morphology	Key regulators	Measurements	Immune reaction
Apoptosis	Chromatin condensation, nuclear and cytoplasmic blebbing, apoptotic bodies	Caspases, cytochrome *c*, Bcl-2 family members	Activated caspase-3, caspase-3 cleavage products, TUNEL	Suppression of inflammation
Autophagy	Cytoplasmic vesicles, intact nuclear and cytoplasmic membranes	Autophagy genes: *beclin, LC3, ATG1, ATG5, ATG7*	LC3 relocalization, number of autophagic vesicles (EM)	Inflammatory
Necrosis	Swollen organelles, cytoplasmic membrane rupture	RIPK1, TRAF2, PARP, calpains	Extracellular HMGB1, S100 family members	Inflammatory

Abbreviations: TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; EM, electron microscopy.

Tumor cells have evolved a mechanism to overcome the state of autophagy. They rapidly adapt to the depleted ATP levels through the action of the energy sensor LKB1/AMPK complex (Buzzai *et al*., 2005). During cellular and therapeutic stresses, energy levels (NAD+ and ATP) are decreased and cytoplasmic concentration of calcium and ROS is increased (**fig 2.3**). Cells which fail to adapt to this altered metabolic state undergo necrotic cell death. However, in certain cells, activation of stress regulators, allow them to acutely survive adverse conditions, executed through AMPK-dependent phosphorylation which results in the inhibition of mammalian target of rapamycin (mTOR), thereby inhibiting autophagy (Hoyer-Hansen and Jaattela, 2007). p53 is a tumor-suppressor protein that directs transcription of autophagy associated proteins and inhibits mTOR signaling pathway (Feng *et*

al., 2005). It interacts with other proteins such as members of the Bcl family including Bcl-2, Bcl-xL and Bax. In cancer cells, AMPK-dependent phosphorylation activates p53, mediated through the activation of Bax and Bak, or caspases and cytoplasmic release of cytochrome *c*, leads to autophagy or apoptosis pathways. p53 is defective in most cancers, either by mutations or deletions in the p53 gene, or by alterations in the p53 pathway caused by other oncogenic events. p53 may act to promote cell death (apoptosis) or survival (autophagy) depending on the metabolic or therapeutic stress, and/or cellular gene expression (Kim *et al*., 2009).

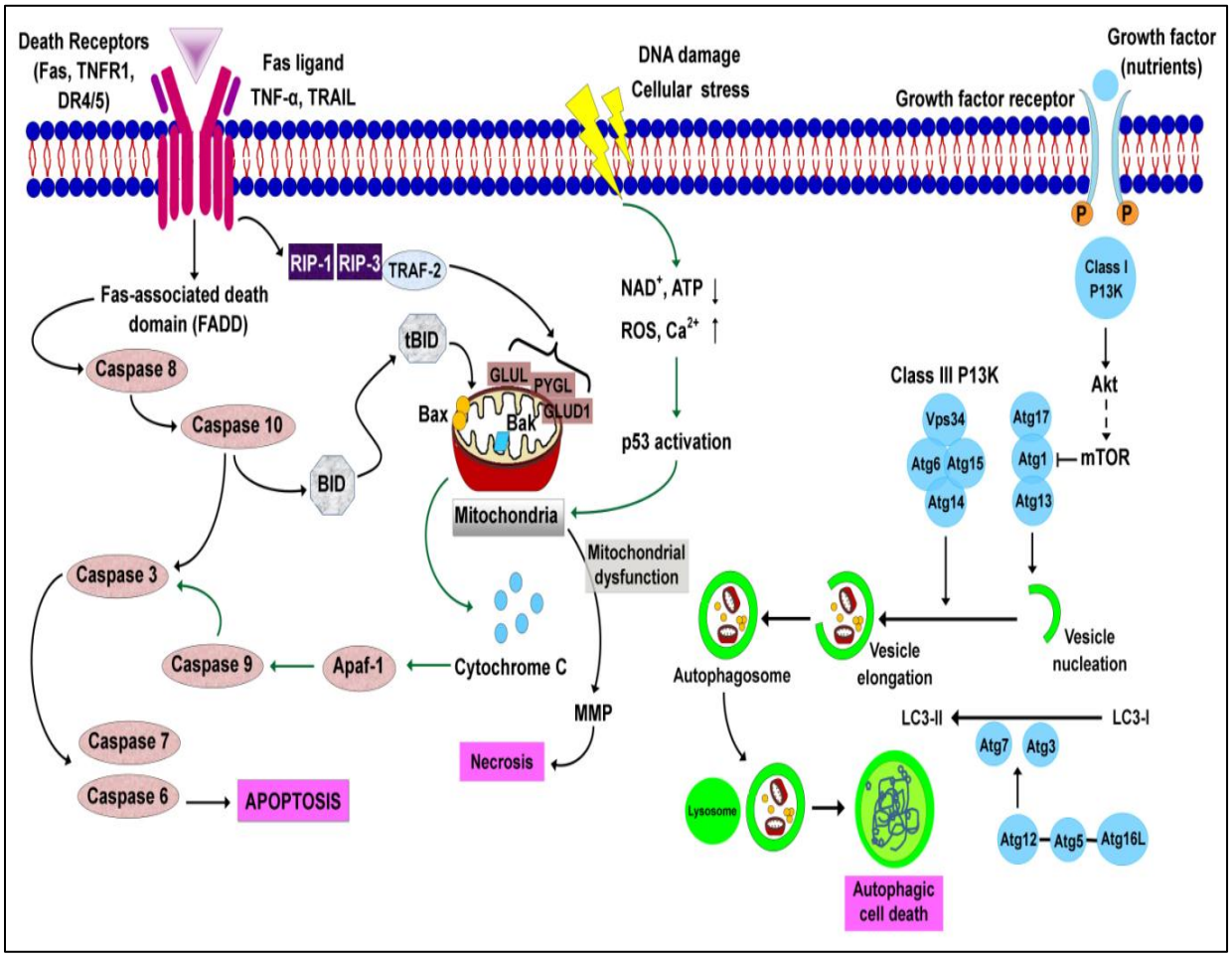

Fig. 2.3: Key regulators and mechanism of apoptosis, necrosis and autophagy pathways

FasR, death receptors are also involved in proliferation, cellular differentiation and apoptosis. Activation of FasR a member of Tumour Necrosis Factor (TNF) superfamily lead to stimulation of several signal cascades involving activation of caspase and JNK dependent cascades and activation of intrinsic and apoptotic pathway mediated by mitochondria (Curtin and Cotter, 2003) as shown in **fig. 2.3**. FasR is expressed in various tissues, but its ligand FasL is expressed differentially in activated T lymphocytes and natural killer cells. FasR can sequester FasL and inhibit apoptosis. In addition to death receptors, there are decoy receptors for example DcR3, secreted by cells and binds with FasL. To initiate the process of apoptosis both functional extracellular and intracellular ligand binding domains are required. The decoy receptors possess only functional binding domains but they do not contain death domains therefore, DcR3 counteracts activation of FasR by sequestering and inactivating the membrane-bound FasL cancerous cells.

Binding of FasL to its respective receptor FasR leads to the attachment of various cytosolic adapter proteins such as FADD (Fas-Associated Death Domain), FLASH (Casp8 Associated Protein 2), and RAIDD (Casp2 and Ripk1 Domain Containing Adaptor with Death Domain) via RIPK1 (Receptor (tnFRSF)-Interacting Serine-Threonine Kinase 1). Adaptor proteins attached to activated FasR carry forward signal transduction to initiator caspases viz. caspase-2, -8, and -10 and recruits them to the plasma membrane, increases local concentration of these proteases, induces autocleavage and activation of effector caspases -3, -6 and -7. Activated effector caspases lead to the proteolytic cleavage of organelles and protein aggregates ultimately leading to cell death (Reid and Shi, 2004). FasR, FADD, caspase-8 and various other proteins form a complex known as DISC (Death inducing signaling complex). Overexpression of FADD-like apoptosis regulator i.e. c-FLIP

in cells inhibits fas-mediated apoptosis (Mezzanzanica *et al*., 2004). Casp8 and c-FLIP compete with caspase-8 for binding of FADD due to their sequence homology.

In addition, fasR induced extrinsic apoptotic pathway is linked with the intrinsic pathway via caspase-8-mediated cleavage of BID (a member of the bcl2 protein family), leading to production of a pro-apoptotic tBID fragment (Gong *et al*., 2004). BAX (bcl2-associated x protein) is activated by tBID that elicits the release of cytochrome c, diablo homolog (SMAC/diablo) and htra-like serine protease (htra2/omi) from mitochondria fortifying apoptosis through activation of the caspase cascade (Yamaguchi *et al*., 2003). Oligomerization of Apaf-1 into complexes is induced by cytochrome c, which recruits and activates caspases-9 (Saelens *et al*., 2004).

TNF-induced programmed necrosis, is also known as the secondary mechanism for cell death via mitochondrial dysfunction. Necrosis is executed through various mechanisms involving ATP depletion, membrane disruption, metabolic collapse which leads to cellular disruption. Enhanced metabolic activity via. glycogenolysis and glutaminolysis lead to the over generation of reactive oxygen species (ROS) which in excess can trigger mitochondrial membrane permeabilization, thereby mediating TNF-induced programmed necrosis (Amaravadi and Thompson, 2007).

2.10 Arginine autotrophy and cancer chemotherapy:

L-arginine is indispensible amino acid for normal growth of microbes, plants and animals. The endogenous pools of arginine are usually not sufficient for rapidly proliferating cells such as tumor cells, which require an exogenous supply of arginine to support their growth and survival. This dependency, called auxotrophy, may be exploited as an anticancer therapy, as dysregulation of arginine metabolism is a hallmark of many human malignancies (Kuo *et*

al., 2010). Using metabolomics approach, we may eventually be able to differentiate the metabolic differences between cancerous and normal cells at the single-tumor level, ultimately leading to early diagnosis and treatment of cancers using personalized medicine.

The three arginine degrading enzymes: arginase, arginine decarboxylase, and arginine deiminase are potential biologic drugs for arginine deprivation therapy. Arginase, a naturally available human enzyme exhibit cytotoxicity to tumor cells *in vitro* but failed to show antitumor activity in mice with Taper liver cancer (Savoca *et al*., 1984), likely because of its low affinity for arginine (Km = 6 mmol/L for native enzyme at physiologic pH) and short half-life in the circulation (a few minutes) (Cheng *et al*., 2007). Moreover, many normal tissues do not express OTC (Agarwal *et al*., 2012), which limits the synthesis of citrulline and, subsequently, leading to arginine toxicity (Savaraj *et al*., 2010; Mauldin *et al*., 2012). To overcome the short circulatory half-life of arginase, rhArg(Mn)-PEG5000 was developed and investigated with *in vivo* and *in vitro* studies against HCC (Cheng *et al*., 2007). RhArg(Mn)-PEG5000 was effective in inhibiting growth of HCC cell lines as well as melanoma cell lines, with half-maximal inhibitory concentrations (IC_{50}) ranging from 0.1–2 IU/mL and mice bearing OTC-deficient human HCC xenografts showed 50% tumor suppression **(**Lam *et al*., 2011**).** The *in vitro* and *in vivo* studies revealed that nitric oxide synthase inhibitors, like methylated, hydroxy and hydroxymethylated arginine effect cell proliferation mainly through the inhibition of arginase pathway (Tyihák *et al*., 1990; Szende *et al*., 1998; Szende *et al*., 2001). Various arginine derivatives viz. mono-di- and tri-methyl arginine, hydroxymethyl arginine, *N*-omega-hydroxy *L*-arginine, *N*-nitro-*L*-arginine methyl ester and nitro-arginine inhibit proliferation and apoptosis in various tumors (Weinhaus *et al*., 1997; Washo-Stultz *et al*., 1999; Singh *et al*., 2000). These diverse biological activities occur through formaldehyde

(HCHO) because guanidine group of *L*-arginine in free and bound form can react rapidly with endogenous HCHO, forming methylated derivatives (Huszti *et al*., 1986; Trézl *et al*., 1998). Arginine decarboxylase catalyzes arginine to agmatine, and it can be applied as a strategy to deprive cells of arginine. But, no known enzyme converts agmatine back to arginine, even in normal cells. Therefore, treatment with exogenous arginine decarboxylase is relatively toxic to normal cells, making this strategy unappealing (Savaraj *et al*., 2010).

In contrast to arginase and arginine decarboxylase, bacterial ADI has a high affinity for arginine (Km = 0.3 mmol/L) (Takaku *et al*., 1995), and thus can more effectively lower plasma arginine level. However, ADI is not expressed in humans; it is therefore recognized as a foreign protein by human body. Important drawbacks like short half-life and antigenic nature are mostly ameliorated by pegylation, that greatly improves its pharmacokinetic half-life in serum and reduces reduces immunogenicity of the enzyme in blood circulation (Feun and Savaraj, 2006; Feun *et al*., 2008; Ni *et al*., 2008). Kuo and coworkers found that modulation of HIF-1α and c-Myc expression may improve the effectiveness of ADI-PEG20 in cultured melanoma cells that are auxotrophic for arginine (Kuo *et al*., 2010).

In normal cells, ASS is a ubiquitous enzyme but is differentially expressed among different cell types and can be regulated by many extracellular factors. In hepatic tissue, expression of ASS can be transcriptionally regulated by cyclic AMP (Guei *et al*., 2008) and in endothelial cells, expression is regulated by cytokines such as IL-1, TNF-a, and TGF-b1 and glutamate (Brasse-Lagnel *et al*., 2003; Brasse-Lagnel *et al*., 2005). The mechanisms that control ASS expression in malignant tumors remain elusive. Levels of ASS vary markedly in a wide spectrum of tumor tissues as compared to normal tissue. Elevated levels of ASS have been observed in cancers of the ovary, stomach, and colon. By contrast, reduced or

undetectable levels of ASS have been observed in the majority of *L*-arginine auxotrophic melanoma, hepatocellular carcinoma, mesotheliomas, renal cell carcinoma, and prostate cancers (Sugimura *et al*., 1992; Scott *et al*., 2000; Dillon *et al*., 2004; Bowles *et al*., 2008). The loss of ASS expression possibly encompasses through epigenetic silencing involving hyper methylation of the ASS gene promoter, although this has not been observed in all ASS-auxotroph cancers (Szlosarek *et al*., 2006). Poorly characterized post-translational silencing or recessive mutations may also be responsible for the loss of ASS activity as observed in disparate tumour types. Therefore, loss of ASS expression may be both prognostic and potentially predictive of response to arginine deprivation therapy.

2.11 Synergistic effect of autophagic inhibitors and apoptotic inducers in cancer chemotherapy:

In tumors due to metabolic or therapeutic stress, autophagy can promote tumor survival, so combination of autophagy inhibitors with apoptosis induction therapies may improve tumor regression and prolong cell survival. Inhibitors of autophagy like chloroquine (CQ) and its derivative hydroxychloroquine lead to deacidification of lysosomes and accumulation of ineffective autophagic vesicles in tumor cells (Poole and Ohkuma, 1981; O'Neill *et al*., 1998). So, autophagy inhibitiors will lead to cell death of autophagy survived cells (**fig. 2.4**). Cell blocks the last step of autophagy which results in accumulation of oxidized waste material such as lipofuscin in swollen autophagosomes of epithelial cancer cells and there is increased cytosolic leakage of intralysosomal enzymes such as cathepsin D (Carew *et al*., 2007).

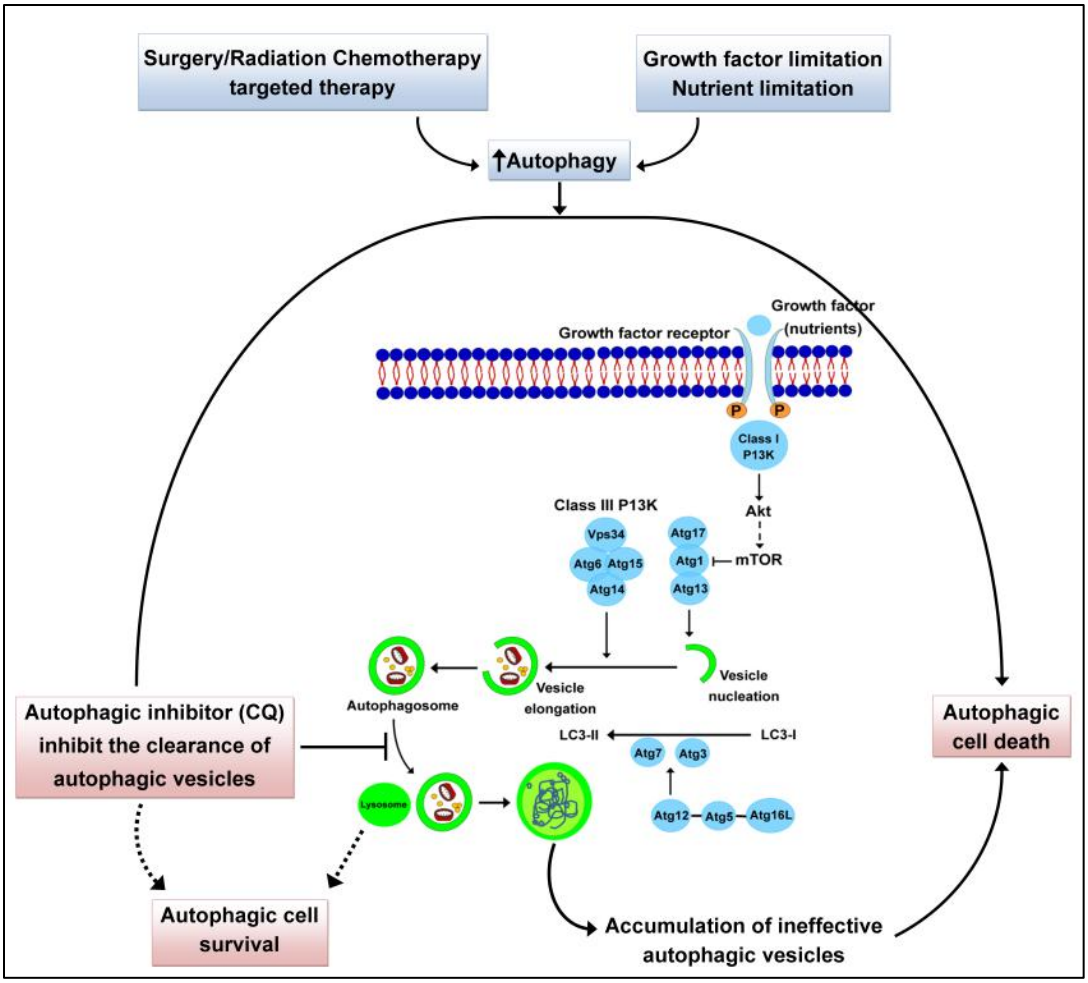

Fig. 2.4: Key effectors of autophagy pathway and relative fates of cancer cells

Chemotherapeutic drug gemcitabine (GEM), a pyrimidine-analogue and an inhibitor of DNA replication, has been widely used for the treatment of pancreatic cancer (Burris *et al*., 1997; Karnitz *et al*., 2005; Mukherjee *et al*., 2013). NF-κB increase cell survival by activating several transcriptional events. Agents that block NF-κB mediated transcriptional activation reduce chemoresistance of cancer cells to GEM. Such agents may be used in conjucation with GEM as a novel therapeutic approach for treating pancreatic cancer (Kong *et al*., 2010). Very recently, the synergistic effect of recombinant ADI from *M. arginine* with GEM for treatment of ASS-deficient pancreatic cancer cell lines (PANC-1) *in vitro* and *in vivo* was evaluated (Liu *et al*., 2014). *In vitro* studies showed that ADI potentiate antitumor

activity of GEM against PANC-1 cells via multiple mechanisms including induction of cell cycle arrest in the S phase. It also upregulates many proapoptotic factors, such as Bax, caspase-3 and −9, and simultaneously downregulates antiapoptotic gene products like, Bcl-2, XIAP, and survivin, but not p53 and p21, and indicating caspase-dependent apoptosis. Recombinant ADI exhibits antitumor effects by blocking PI3K/Akt signaling and suppressing NF-κB activation via inhibition of its nuclear transport phosphorylation of serine 536 residues. Results obtained in *in vitro* study were validated *in vivo* using mouse xenograft models where the synergistic effect of ADI with the anticancerous activity of GEM was observed. You and coworker also reported the synergistic effect of ADI-PEG20 with cisplatin (a cytotoxic drug) via the intrinsic apoptotic pathway in melanoma cell lines (You *et al*., 2010).

The proto-oncogene *c-myc*, another key regulator of cell proliferation and apoptosis, is overexpressed in a wide range of human cancers (Dang, 1999). *c-myc* encodes a transcriptional factor (Myc) that plays important role in G_1-phase progression by regulating the expression of $p27^{kip1}$ and cyclin D_1 protein (Blackwood and Eisenman, 1991). *In vitro* experiments on SNU-1 human stomach cancer cells indicated role of rADI of *L. lactis* ssp. *lactis* in induction of G_0/G_1-phase cell-cycle arrest and apoptosis via arginine depletion by inhibiting c-myc, cyclin D_1 and Bcl-xL, and inducing p53 and $p27^{kip1}$ (Kim *et al*., 2009). Depletion of arginine and ADI-PEG20 induce metabolic stress in auxotrophic cancer cells thereby, complimenting conventional therapies which are largely based on inducing genotoxic stress. ADI-PEG20 has 1,000-fold greater affinity for arginine (Dillon *et al*., 2002) with fewer side effects as compared to bovine arginase.

2.12 Arginine derivatives with chemotherapeutic potential:

Optical configuration of arginine determines its role in tumor regression: *L*-arginine stimulates while *D*-arginine inhibits tumor cell multiplication. Even, arginine-rich hexapeptides have been found to exhibit antitumor activity (Szende *et al*., 2001). The induction of apoptosis, mainly in NO synthase inhibitors, like N^G-monomethyl-*L*-arginine, N^G-hydroxymethyl arginine, is manifested through inhibition of the arginase pathway. (Szende *et al*., 1998). *In vivo* studies on Swiss mice (inoculated with Ehrlich ascites tumor) and C57B1 mice (inoculated with Lewis lung tumor) when administered 400 mg/kg intraperitoneally, N^G-hydroxymethyl arginine exhibited inhibition of the growth of tumor (Tyihák *et al*., 1990). Singh and coworkers reported the inhibition of proliferation and apoptosis by *N*-omega-hydroxy-*L*-arginine (a stable intermediate product formed during conversion of *L*-arginine to nitric oxide) on high-arginase-expressing human breast cancer cells (Singh *et al*., 2000). It has also been shown by that *N*-nitro-arginine methyl ester sensitized cells to apoptosis induced by sodium deoxycholate. *L*-arginine and its derivatives like nitro-arginine and methyl-arginine have been found to increase cytosolic calcium concentration in cultured NIT-1 cells, leading to depletion of plasma membrane potential, a phenomenon observed commonly during the process of apoptosis (Weinhaus *et al*., 1997; Washo-Stultz *et al*., 1999).

2.13 Arginine deprivation therapy:

2.13.1 Drawbacks and possible solutions of arginine deprivation therapy:

Sometimes cancer cells develop resistance to arginine deprivation therapy. Savaraj and coworkers (2010) reported two mechanisms by which ASS-deficient cells develop resistance to arginine deprivation. In the first mechanism, under stress conditions, tumor cells

can follow two alternative pathways either apoptotic or autophagic degradation. After prolonged arginine deprivation, tumor cells have a higher tendency to undergo autophagy which has higher chances of cell survival rather than apoptosis.

The second mechanism, tumor cell autophagy potentiates *de novo* arginine biosynthesis through upregulation of ASS, in response to exogenous arginase starvation. It has been reported that the ASS-deficient melanomas of two patients, treated with ADI-PEG20 had upregulation of ASS protein and *ASS* messenger ribonucleic acid during progression of the disease (Tsai *et al*., 2009; Feun *et al*., 2010). One approach to counter the resistance is to combine agents with different mechanisms of action to induce a higher degree of tumor apoptosis and thus delay the development of resistance. Pharmacological strategies may be applied to inhibit ASS expression in cancer cells (Feun *et al*., 2008). Therefore, autophagy inhibitors can be combined with arginine deprivation therapy involving inhibition of ASS expression to potentially increase antitumor activity of anticancerous drugs such as ADI.

2.13.2 Preclinical studies of arginine deprivation therapy:

Immediately after reports that unearth arginine auxotrophic nature of many melanomas, several studies stated focusing use of arginine deprivation enzymes as potent anticancer agents (Feun and Savaraj, 2006; Shen and Shen, 2006; Ni *et al*., 2008). *In vitro* studies on mouse hepatoma cell lines MH134 reported purified ADI from cell extracts of *M. arginini* and *M. hominis* strongly exhibits growth inhibition at IC_{50} of 10ng/ml and 100ng/ml respectively (Takaku *et al*., 1995) (**table 2.4**). Purified ADI of *M. arginini* strongly inhibits the growth of cultured leukemia cells (acute pediatric T-(Jurkat) and B-(Tanoue) *in vitro* with IC_{50} at concentration of 5-10 ng/ml while, the growth of fresh lymphoblasts with half-

maximal inhibited at concentration 20-100 ng/ml (Gong *et al*., 2000). A study on purified recombinant ADI expressed in *E. coli* BL21 (DE3) indicates that ADI-induces cell death in arginine auxotroph human retinoblastoma tissues and retinoblastoma cell lines such as Y79 and SNUOT-Rb1 (Kim *et al*., 2007). Studies on ASS-deficient melanoma and hepatocellular carcinomas with ADI-PEG20 have evidenced role of these enzymes in retardation of tumour growth *in vitro* as well as *in vivo* in mice (Sugimura *et al*., 1992; Takaku *et al*., 1992; Ensor *et al*., 2002). Kim *et al*., (2009) reveals ADI-PEG20 induces a late capase-independent cell death in ASS deficient CWR22R*v*1 prostate cancer cell lines *in vitro*. It also significantly reduced tumor activity by micropositron emission tomography as well as reduced tumor growth as a monotherapy and in combination with docetaxel against CWR22Rv1 prostate cancer mouse xenografts (Kim *et al*., 2009).

Takaku and coworkers were first to report antitumor activity of ADI isolated from *Mycoplasma in vitro* and *in vivo* (Takaku *et al*., 1992; Takaku *et al*., 1993; Takaku *et al*., 1995). Initial attempts to use native *Mycoplasma* ADI as an anticancer treatment *in vivo* were of limited success because of short serum life span (approximately 5 h) and the consequent quick return to normal plasma levels of arginine so it needs to be administered in large daily doses to inhibit tumors implant into mice. Formulation of ADI with polyethylene glycol to produce ADI-SS PEG20,000 MW increases circulation half-life that, and although equally effective *in vitro*, is more efficacious in the treatment of mice implanted with human melanomas and HCCs (Ensor *et al*., 2002).

Table 2.4: Half-maximal inhibitory effect of ADI on various cell lines

S.No	Organism	ADI	Cell lines	IC_{50}	Reference
1	*Lactococcus lactis* sp. *lactis*	cytoplasmic fraction	Human stomach cancer cells SNU-1	17 μg/ml	Kim *et al*., 2007
		purified ADI		2 μg/ml	
		Recombinant ADI		0.6 μg/ml	
2	*Mycoplasma* contaminating RSV-BRL cells	Purified ADI	Human cancer HSC-3 (tongue squamous carcinoma), CaSki (cervix squamous carcinoma), SCC (colon adenocarcinoma), KB (nose adenocarcinoma), RPMI-8226 (myeloma)	5-10ng/ml	Miyazaki *et al*., 1990
3	*Mycoplasma arginini*	Purified ADI	mouse hepatoma cell lines MH134	10ng/ml	Takaku *et al*., 1975
4	*Mycoplasma hominis*	Purified ADI	mouse hepatoma cell lines MH134	100ng/ml	Takaku *et al*., 1975
5	*Mycoplasma arginini*	Purified ADI	fresh lymphoblasts	20-100 ng/ml	Gong *et al*., 2000
6	*Mycoplasma arginini*	Purified ADI	leukemia cells (acute pediatric T-Jurkat and B-Tanoue)	5-10 ng/ml	Gong *et al*., 2000
7	*Mycoplasma arginini*	Recombinant ADI	Retinoblastoma SNUOT-Rb1 cells	0.5mU/ml	Kim *et al*., 2007
8	*Mycoplasma* sp.	ADI-PEG20 (Polaris Inc.,USA)	Human melanoma cell lines A375, SK-MEL-2, MEL-1220	0.05-0.08 μg/ml	Savaraj *et al*., 2007
9	*Mycoplasma* sp.	PEG-ADI (DesigneRx Pharmaceuticals, Inc.)	Pancreatic cancer cell lines MIA-PaCa-2, PANC-1	0.3 μg/ml	Bowles *et al*., 2008
10	*Mycoplasma* sp.	ADI-PEG20 (DesigneRx Pharmaceuticals, Inc.)	Prostate cancer cell CWR22Rv1	0.3 μg/ml	Kim *et al*., 2009
11	*Pseudomonas plecoglossicida* *CGMCC2039*	Partially purified ADI	Hepatocellular carcinoma cell lines HEPG2	0.05U/ml	Ni *et al*., 2009

2.13.3 Clinical studies of arginine deprivation therapy:

Clinical trials of ADI-PEG20 have been followed in both melanoma and hepatocellular carcinoma as shown in **table 2.5** (Izzo *et al*., 2004; Ascierto *et al*., 2005; Feun *et al*., 2010; Glazer *et al*., 2010; Yang *et al*., 2010; Yau *et al*., 2010; Szlosarek *et al*., 2012). Pharmacodynamic results from these studies demonstrated that a dose of 160 IU/m^2 ADI-PEG20 is sufficient to reduce plasma arginine levels. Dosage levels are double in case of ASS deficient malignant pleural mesothelioma (MPM) cell lines where partial metabolic response with a 40% reduction in maximum standardized uptake value (5.5 decreasing to 3.5) was observed. Further, these trials provided some indication of clinical response to arginine deprivation therapy, with ADI-PEG20 achieving response rates of 25% and 47% in melanoma and hepatocellular carcinoma, respectively (Izzo *et al*., 2004; Ascierto *et al*., 2005). Common discomforts and toxicity symptoms of ADI-PEG20 includes injection site pain, elevated serum lipase, amylase, bilirubin, creatinine, uric acid, transaminases, hypotension, fever, anemia, abnormal serum sodium and potassium levels, decreased fibrinogen, local and/or allergic reactions, hyperuricemia, pruritus, fatigue, hyperammonemia, diarrhea, breathlessness and chest-wall pain, nausea, abdominal pain, liver dysfunction (Izzo *et al*., 2004; Ascierto *et al*., 2005; Feun *et al*., 2010; Glazer *et al*., 2010; Yang *et al*., 2010; Yau *et al*., 2010; Szlosarek *et al*., 2012). However, similar rates of sustained clinical responses have not been observed in further larger studies of ADI-PEG20 in metastatic melanoma or hepatocellular carcinoma, although an apparent increase in overall survival has been observed (Feun *et al*., 2010; Glazer *et al*., 2010; Yang *et al*., 2010). rhArgI(Mn)-PEG5000 has achieved response rate of 50% in HCC is still under phase I clinical trials (Yau *et al*., 2010). A Phase II clinical study in patients with small cell lung cancer (NCT 01266018) is also under progress.

Table 2.5: Clinical trials of ADI on various cancer diseases

Agents	Cancer type/disease	Phase	Dose	Clinical efficacy	Common toxicity	Reference
Clinical trials						
ADI-PEG20	Hepatocellular carcinoma	I/II	160 IU/m^2 intramuscularly	Response rate was 47% (two complete responses and seven partial responses), and the rate of stable disease was 37% among 19 enrolled patients. OR: 9/19 (47%) SD: 7/19 (37%)	Elevated serum lipase, amylase, bilirubin, creatinine, uric acid	Izzo *et al*., 2004
	metastatic melanoma	I/II	160 IU/m2/week intramuscularly	OR: 6/24 (25%) SD: 6/24 (25%)	Injection site pain, elevated serum amylase, lipase, and transaminases, hypotension	Ascierto *et al*., 2005
	metastatic melanoma	II	160–320 IU/m2 intramuscularly	OR + SD: 10/36 (28%)	Discomfort at injection site	Feun *et al*., 2010
	Hepatocellular carcinoma	II	80 IU/m2 or 160 IU/m2 intramuscularly	OR: 2/76 (3%) SD: 50/76 (61%)	Injection site discomfort, fever, anemia, abnormal serum sodium and potassium levels, decreased fibrinogen	Glazer *et al*., 2010
	Hepatocellular carcinoma	II	160 IU/m2 or 320 IU/m2 intramuscularly	OR: 0/71 (0%) SD: 22/71 (31%)	Local and/or allergic reactions, hyperuricemia, pruritus, fatigue, hyperammonemia , fever, diarrhea	Yang *et al*., 2010
	Hepatocellular carcinoma	III		Ongoing – NCT01287585		
	ASS(-) MPM malignant pleural mesothelioma (MPM)	II Case report	320 IU/m2/wk intramuscularly	Ongoing – NCT01279967 partial metabolic response with a 40% reduction in the maximum standardized uptake value (5.5 decreasing to 3.5)	breathlessness and chest-wall pain	Szlosarek *et al*., 2012
	Small cell lung carcinoma	II		Ongoing – NCT01266018		
rhArgI(Mn)-PEG5000	Hepatocellular carcinoma	I	500–3500 IU/kg	OR: 8/15 (53%) SD: 4/8 (50%)	Diarrhea, nausea, abdominal pain, liver dysfunction, serum bilirubin elevation	Yau *et al*., 2010

Abbreviations: ASS, argininosuccinate synthetase; OR, overall response (complete + partial response); SD, stable disease.