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Review

# The role of pH dynamics and the $Na^+/H^+$ antiporter in the etiopathogenesis and treatment of cancer. Two faces of the same coin—one single nature

Salvador Harguindey[a,*], Gorka Orive[b], José Luis Pedraz[b], Angelo Paradiso[c], Stephan J. Reshkin[d]

[a]*Centro Médico "La Salud"- c) Independencia, 13-01004 Vitoria, Spain, and Biotechnology Institute (BTI), c) San Antonio 15-5°, 01005 Vitoria, Spain*
[b]*Department of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of the Basque Country, c) Paseo de las Universidades, 7-01006 Vitoria, Spain*
[c]*Laboratory of Clinical Experimental Oncology, Oncology Institute of Bari, 70126 Bari, Italy*
[d]*Department of General and Environmental Physiology, University of Bari, 70126 Bari, Italy*

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**Abstract**

Looked at from the genetic point-of-view cancer represents a daunting and, frankly, confusing multiplicity of diseases (at least 100) that require an equally large variety of therapeutic strategies and substances designed to treat the particular tumor. However, when analyzed phenotypically cancer is a relatively uniform disease of very conserved 'hallmark' behaviors across the entire spectrum of tissue and genetic differences [D. Hanahan, R.A. Weinberg, Hallmarks of cancer, Cell 100 (2000) 57–70]. This suggests that cancers do, indeed, share common biochemical and physiological characteristics that are independent of the varied genetic backgrounds, and that there may be a common mechanism underlying both the neoplastic transformation/progression side and the antineoplastic/therapy side of oncology. The challenge of modern oncology is to integrate all the diverse experimental data to create a physiological/metabolic/energetic paradigm that can unite our thinking in order to understand how both neoplastic progression and therapies function. This reductionist view gives the hope that, as in chemistry and physics, it will possible to identify common underlying driving forces that define a tumor and will permit, for the first time, the actual calculated manipulation of their state. That is, a rational therapeutic design. In the present review, we present evidence, obtained from a great number of studies, for a fundamental, underlying mechanism involved in the initiation and evolution of the neoplastic process. There is an ever growing body of evidence that all the important neoplastic phenotypes are driven by an alkalization of the transformed cell, a process which seems specific for transformed cells since the same alkalinization has no effect in cells that have not been transformed. Seen in that light, different fields of cancer research, from etiopathogenesis, cancer cell metabolism and neovascularization, to multiple drug resistance (MDR), selective apoptosis, modern cancer chemotherapy and the spontaneous regression of cancer (SRC) all appear to have in common a pivotal characteristic, the aberrant regulation of hydrogen ion dynamics [S. Harguindey, J.L. Pedraz, R. García Cañero, J. Pérez de Diego, E.J. Cragoe Jr., Hydrogen ion-dependent oncogenesis and parallel new avenues to cancer prevention and treatment using a $H^+$-mediated unifying approach: pH-related and pH-unrelated mechanisms, Crit. Rev. Oncog. 6 (1) (1995) 1–33]. Cancer cells have an acid–base disturbance that is completely different than observed in normal tissues and that increases in correspondence with increasing neoplastic state: an interstitial acid microenvironment linked to an intracellular alkalosis.


*Keywords:* Cancer etiopathogenesis and treatment; pH, $Na^+/H^+$ antiporter and cancer; Multiple drug resistance; Apoptosis; Neovascularization; Spontaneous regression of cancer

* Corresponding author. c) Postas 13-3° izq., 01004 Vitoria, Spain. Tel./fax: +34 945 232014.
*E-mail address:* salvaszh@telefonica.net (S. Harguindey).

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**Contents**



## 1. Introduction

At one end of a $H^+$-concentration spectrum (high pH or alkaline limiting zone), the induction and/or maintenance of an abnormally high intracellular alkalinization has been repeatedly implicated as playing an essential, direct and pivotal role both in neoplastic transformation as well as in the active maintenance and progression of the neoplastic process [2–6] (Fig. 1). These different sets of data have recently led various authors to suggest that one of the hallmark characteristics of cancer cells of many different tissues and genetic origins may be the systematic loss of the rigid control of $pH_i$ in both the acute and in the chronic situation [2,4,7]. Independent of genetic variability, cellular pathological alkalosis, together with an abnormally high glycolytic metabolism that has been recognized since the time of Otto Warburg, are two of the principal factors characterizing cancer cells and malignant tumors in general [2,4,8–13]. Indeed, the high glycolytic rate of tumors, which can be correlated with the degree of malignancy in many tumor types, is taken advantage of in some of the most modern diagnostic techniques used to detect the presence of malignant tumors and their metastases: positron emission tomography (PET) [10,14–16]. Such a systemic abnormality

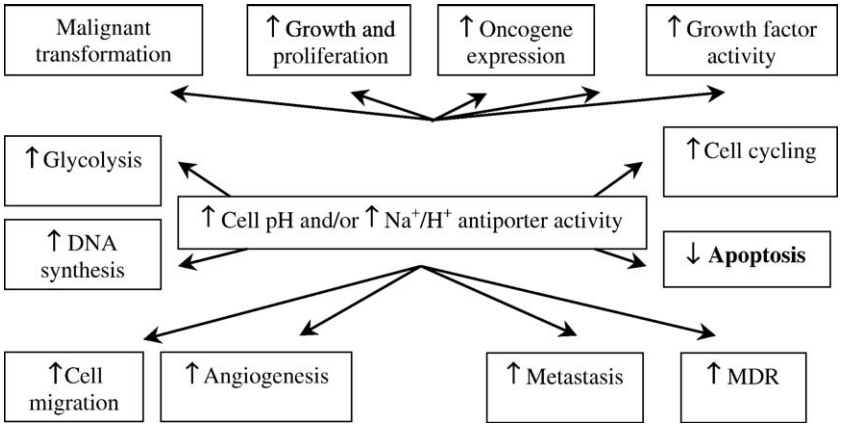


Fig. 1. Subfields of oncological research, from etiopathogenesis to treatment, where an increase in cellular pH and/or $Na^+/H^+$ antiporter activity are involved. Only therapeutic apoptosis is inhibited by these stimulus.

of cellular acid–base homeostasis also plays a key role in the transduction of intracellular signals of a wide array of growth factors, a feature often, but not always, mediated by stimulation of the plasma membrane $Na^+/H^+$ antiporter isoform 1, also known as the $Na^+/H^+$ exchanger isoform 1 (NHE1). This electroneutral transporter expels hydrogen ions out of the cell while interchanging them for $Na^+$ and thereby increasing intracellular $Na^+$ and alkalinizing intracellular pH ($pH_i$). In normal cells, the NHE1 is quiescent at the steady state resting $pH_i$ and is activated only upon cytosolic acidification [6,7,17,18]. In transformed and cancer cells, the NHE1 is hyperactive even at resting pH and the resulting change in cellular alkalinity has been shown to be directly related in most cases to the permanent and uncontrolled proliferation characteristic of neoplastic cells [4,19–22]. Recently, compelling evidence has come to light indicating that the activity of the NHE1 is also a critical factor in the activation of proliferation, motility and invasion of cancer cells derived from various tissues [4,22–28].

On the other side of the coin, there currently appears to be a dead-lock in the present state of cancer chemotherapy [29]. This seems to be related to the fact that most of the antitumor agents used in the clinical situation are based upon the old "anti DNA" paradigm which during the last 60 years has attempted to induce an antitumor effect by targeting DNA synthesis or cell division and proliferation. It has been recently proposed that the modest progress achieved and the lack of selectivity of current antitumor agents suggests that the "anti DNA" framework is either wrong or at least too simplistic and conceptually poor from the very beginning for such a complex disease [29,30]. Some of these authors have proposed that the limitations of the present-day approaches can be responsible for the fact that some therapies can even exacerbate the original malignant phenotype, induce escape from apoptosis and negatively affect the progression of the disease [30–32]. Recently, a new therapeutic perspective is developing which targets cell membrane or signal transduction proteins instead of DNA synthesis. This change is taking place after an increasing bulk of evidence has led researchers to try to trigger mechanisms to induce selective cancer cell death by apoptosis. This relatively new approach is changing the old and aggressive concept of going for "a direct kill of cancer cells" for a more benevolent "help or induce to die" concept. In an attempt to bring together both paradigms, the failure of tumor cells to die following chemotherapeutic treatment appears to be mostly dependent on their resistance to engaging in the apoptotic process [29].

In summary, the purpose of this review is an attempt to analyze and then synthesize the seminal and emerging data in these areas in order to: (i) to interpret the neoplastic process from the point of view of the critical mechanistic level in order to draw basic research and clinical therapeutics nearer to each other; (ii) unite both therapeutic paradigms into a single and more comprehensive one; and (iii) open new directions in cancer prevention and treatment.

## 2. One side of the coin: oncogenesis and etiopathogenesis

### *2.1. Intracellular pH ($pH_i$) and extracellular pH ($pH_e$) in tumors: interrelationships and physiopathological significance*

From the beginning of biochemical cancer research decades ago and until today, the relationships between intracellular pH ($pH_i$) and extracellular ($pH_e$) within a tumor have been a highly controversial issue and an unending source of confusion [33,34]. For many decades, tumor cells were thought to be have an acidic $pH_i$ [16,33]. However, in the last few years, it has been repeatedly shown that cancer cells show a strong tendency towards an alkaline deviation of the entire acid–base homeostasis of the cell along with unequivocal and steady alterations in membrane depolarization as shown by a decrease in transmembrane potential [35–40] especially when they are resistant to therapeutic intervention (MDR) [7,21,41–46]. It has long been recognized that locally and chronically elevated epithelial pH both accompanies and precedes, some times for many years, the onset of mucosal malignancies in diverse areas of the organism, and is involved in both direct and indirect chemical carcinogenesis [2,47–53] (reviewed in Ref. [2]). In recent times, it has also been repeatedly demonstrated that transformed and/or malignant cells of many origins, from leukemias to solid tumors, systematically show a higher than normal $pH_i$ [21]. Malignant cells can live and multiply at proton levels barely compatible with cellular life (7.46 to 7.6 and even higher), although slight elevations of $pH_i$ in tumor cells have also been reported [21,41,54–59]. This anomalous "malignant alkalosis" has already been considered to represent the one of the most specific characteristic of the cancerous state by Reshkin [4] and others [2,42]. It is an abnormality to which a wide array of growth factors, and other compounds, like phorbol esters, glutathione, diacylglycerol, etc., further contribute [17,19,55–57]. Perhaps, this cellular homeostatic failure can already be considered to be specific to the neoplastic process since it has not been so far described in any other human disease.

Further evidence for the difference between normal and transformed cells also comes from dynamic studies showing that artificial elevations of pH of the extracellular media of malignant cells ($pH_e$) increase their cytosolic pH ($pH_i$), while under similar circumstances normal cells can maintain their internal acid–base homeostasis below 7.0 such that $pH_i$ is below $pH_e$ [60]. In the contrary situation, even in the most acidic extracellular and/or interstitial microenvironments, transformed cells possess membrane-bound regulatory mechanisms and dynamic intracellular buffering systems that protect them against intracellular acidification (reversed pH gradient of transformed cells such that $pH_i$ is always significantly higher than $pH_e$) [13,16]. Indeed, while non-transformed cells and tissues die under conditions of extracellular acidosis [60–62], the same microenvironmental conditions allow malignant cells of diverse origins to

defend themselves from any acidic and/or therapeutic and/or apoptotic attack by taking advantage of a highly effective and concerted system of membrane-bound ionic pumps whose main role is to extrude hydrogen ions and also insulate them in large intracellular acidic vesicles (LAVs) which trap hydrogen ions within cancer cells [16]. This strategy allows transformed cells of all locations and origins to first survive and then multiply under these most difficult environmental circumstances [2,6,21,63–67] (see Section 2.4 in this review).

Importantly, this "gradient reversal" (acid outside–alkaline inside) which is specific to all cancer cells offers the opportunity to target this differential property that separates all malignant cells from all normal tissues—perhaps one of the few differential characteristics—in selective attempts of "cancer cell self-poisoning" therapeutic intervention in different oncological settings [46,68–76]. In this vein, it is generally recognized that cancer cells cannot survive for long below certain low $pH_i$ conditions, either because of triggering of the apoptotic pathway or because of the additional shutting down of glycolysis through full inhibition of phosphofructokinase [16,77,78].

Another factor that has contributed to increase the degree of confusion in metabolic and biochemical cancer research is the large quantities of lactic acid known to be secreted by malignant cells of all origins [9,10,33,72]. Paradoxically, the high production of lactic acid is further increased by stimulating tumor glycolysis through glucose infusions and/or alkalinization [4,79], a vicious circle mainly secondary to the direct stimulus of an elevation of $pH_i$ on the key enzyme of the glycolytic sequence, phosphofructokinase [4,9,78]. This glycolytically produced lactic acid does not acidify cancer cells due both to a small outward passive diffusion and, primarily, because it is actively extruded via the lactate/$H^+$ symporter [61,80,81]. Before the availability of modern methods to determine $pH_i$, that apparent paradox misled an entire generation of investigators to think that the cytosol of the cancer cell was acidified [65,82], while later research methodology has systematically demonstrated that the cancer cell always remains in a non-acidified situation, from neutral to, more commonly, a state of frank metabolic alkalosis [2–4,7,17–19,21,41,46,54–58,60,63,83]. In these circumstances, however, what becomes acidified is the extracellular and interstitial, however, still intratumoral, compartment [9,33,34,62,84]. This process further exacerbates the above-discussed reversed $pH_{ex}$ to $pH_i$ gradient found only in malignant tumors.

It is important to note that measuring $pH_i$, and not the $H^+$ concentration, is not sensitive enough to reflect the degree of the acid–base abnormality [16]. The differences in $pH_i$ found between normal and malignant cells usually range around 0.2 to 0.4 units, a figure that can be considerably higher if MDR positive cells are considered [41,42,54,63]. However, these apparently small differences in $pH_i$ values mean that the entire physiological processes of the cell are taking place at an intracellular concentration of hydrogen ions of at least half that of normal cells [34]. In extreme cases, the differences in intracellular $H^+$ can at times be multiplied by a factor of almost 10 when the pH difference between normal and transformed cells gets near one unit [16,21]. This extreme circumstance could take place in vivo when the degree of cell acidification, what has been called by different research groups "the apoptotic threshold" necessary to induce selective cell apoptosis [7,21,23,57], is compared to the otherwise alkaline situation of cancer cells either in basal conditions or, especially, under the stimulus of growth factors.

### *2.2. The transport systems responsible for tumor cell alkalinization*

The above discussion demonstrates that an abnormal cell pH homeostasis represented by deeply perturbed intracellular dynamics of the hydrogen ion consistently results from a response to varied growth factors and carcinogenic stimuli. This phenomenon, induced by an outward efflux of protons, induces a situation that in cancer cells is maintained even under conditions of an acidic extracellular and interstitial milieu [34,84]. This is achieved through overstimulation of different electrogenic plasma membrane $H^+$-related transporters, such as the $Na^+/H^+$-exchanger [6,19], $Na^+/K^+$-ATPase pump [35,82,84–89], vacuolar $H^+$-ATPases [31,90], $H^+/Cl^-$ symporter [91] and monocarboxylate transporters (MCTs or lactate-proton symporters) [61]. The highly regulated $Na^+/H^+$-exchanger is the most recognized mechanism and the main target, although not the only, in the control of intracellular acid–base homeostasis, mainly through its isoform 1 (NHE1) [2,21,6,85,61,92–95]. Remarkably, overstimulation of the NHE1 by a variety of carcinogenic stimuli (P-glycoprotein, antiapoptotic Bcl-2, phorbol esters, tyrosine kinase, diacylglycerol, EGF, TGF-α, IGF-II, vacuolar $H^+$- and $Na^+/K^+$-ATPase, growth factors, oncogenes, different chemicals and drugs, etc.) has been shown repeatedly to be an essential mechanism involved in the loss of cellular acid–base equilibrium through the elevation of $pH_i$ induced by proton efflux, a phenomenon that is also accompanied by electron efflux, depolarization and low transmembrane potential [4,19,33,35,36,40,41,56–58,86,87,96,97]. However, in a few tumors, like melanoma, NHE1 does not seem to be the main mechanism responsible for $pH_i$ alterations [61].

Seminal studies by Sparks and colleagues in transformed cells showed that a specific "malignant" alkaline surge was responsible for the secondary and synergistic activation of the $Na^+/K^+$-ATPase [69,91]. This mechanism induces and, at the same time, closes an acid–base vicious cycle since the activation of $Na^+/K^+$-ATPase also increases cell pH through an integrated mechanism involving NHE1 stimulation [1,98]. Subsequent studies showed that NHE1 hyperactivity is specifically necessary for cell transformation and for the development of malignant tumors. Indeed, cell mutants lacking NHE1 reduce their tumor incidence to 20%, and even to 0% if cell respiration is inhibited at the same time as

NHE1 expression [6,11,99–101]. Similarly, lowering of $pH_i$ through the use of diverse NHE1 inhibitors results in the suppression of the activity of a multiplicity of growth factors while also inducing a physiological arrest of the cell cycle [25,101–105].

### 2.3. The role of $pH_i$ and the NHE1 in transformation, pathogenesis, growth, invasion and multiple drug resistance

It is highly remarkable that at the intracellular level an elevated $pH_i$ plays a direct role in transformation and tumor development [2–5,99,100,106,107]. Different studies have highlighted the key role that intracellular alkalinization has as an essential factor in tumor transformation, development, cell growth, survival and in the maintenance of the metastatic process [3–5,11,21,108,109] (Fig. 1). While the multiple effects of an increase of $pH_i$ on transformation and basic tumor cell behavior have been reviewed elsewhere (see Table 5 in reference No. [2]), in the following section, we discuss in depth the relationships of pHe, $pH_i$ and the activity of membrane ion transporters with these tumor behaviors.

#### 2.3.1. Oncogene- and virus-driven transformation

It is well recognized that one of the early actions of growth factors [19] and oncogenes like v-mos, Ha-ras and HPV16 E7 is an elevation of $pH_i$ driven by stimulation of the NHE1 [4,19,110–116]. Some aspects of the transforming activity of these oncogenes are mediated by this increase in $pH_i$ because treatment with amiloride and/or its derivatives has been shown to block the expression of the transformed phenotype [4,110–113]. These seminal studies were later criticized because it was thought that such alkalinization did not take place under physiological conditions, since other proton transport systems, such as bicarbonate-dependent transport systems, had not been taken into account [11,16]. However, it seems that even when the bicarbonate-dependent transport systems are potentially activated because of the presence of bicarbonate in the culture media, oncogenes such as ras or HPV16 E7 are able to induce the necessary and permissive cytosolic alkalinization that precedes cell transformation [3,4,111,112]. Recently, the papilloma virus E5 protein has been shown to block cell acidification by insertion in the pore-forming unit of the vacuolar proton-ATPase, a mechanism that disrupts cell acidification in a similar way to other oncogenes [117–119].

Regarding transformation driven by viral activity, a conclusive study has been recently reported for the retroviral E7 oncogene of human papillomavirus (HPV) type 16 in NIH3T3 cells (Table 1) [4]. These authors observed an increase in $pH_i$ as an early event in transformation that was transformation specific and demonstrated the specific and causal role of this pathologic hyperalkalinization in the malignant transformation induced by the E7 oncogene. This cellular acid–base change was driven by the stimulation of the NHE1 via an increase in the affinity of its intracellular proton binding site and took place even in the presence of bicarbonate. This same $pH_i$-dependent mechanism underlying E7-driven transformation also occurred in human keratinocytes, the natural host of HPV in the pathogenesis of cervical carcinoma. Importantly, annulment of the E7-induced cytoplasmatic alkalinization with either the amiloride analog [5-(*N*,*N*-dimethyl)amiloride], DMA, or by biochemically clamping $pH_i$ inhibited the development of the transformed phenotype in vitro. Intraperitoneal injection of DMA also delayed the development of human HPV16 keratinocyte tumors in nude mice. The authors concluded that cell alkalinization is a key and specific mechanism not only in malignant transformation but also in the maintenance and progression of the neoplastic state, a feature that can be applied to different malignant tumors no matter their tissue of origin or genetic variability [2–5].

Finally, there is a highly significant study [5] demonstrating the importance of $pH_i$ dynamics in the function of the tumor suppressor gene p53 whose genetic loss of function and/or inactivation is widely recognized to be of paramount importance in resistance to anticancer therapy, in the inhibition of selective apoptosis and in the metastatic process [120]. The authors, working with p53 in an adrenocortical carcinoma syndrome with a high incidence in Brazilian children, reported that the final molecular mechanism for the loss of the growth-inhibitory power of p53 is an increase in intracellular pH mediated by an Arg337 to His337 mutation in the tetramerization domain of p53. This outstanding finding adds conclusive evidence to the importance of a

Table 1
HPV-16 transformed phenotypes dependent on the NHE1-driven cellular alkalinization

| Cell type | Transformed Phenotype Dependent on stimulated NHE1 activity and cellular alkalinization |
|---|---|
| HPV-16 E7 transformed fibroblasts (2BN11 cells) | Increased lactate production (glycolytic metabolism) |
| | Increased growth rate and entry into S-phase |
| | Serum-independent growth |
| | Acquired ability of anchorage-independent growth |
| HPV-16 transformed human keratinocytes (HPKIA cells) | Increased lactate production (glycolytic metabolism) |
| | Increased growth rate and entry into S-phase |
| | Acquired ability of anchorage-independent growth |
| | Formation of tumors in nude mice |

Evidence for the role of NHE1-dependent alkalinization (see Reference [3]) in the induction of neoplastic phenotypes during specific HPV-16 E7-dependent transformation of NIH3T3 fibroblasts (2BN11 cells) or transformation of human keratinocytes by infection by the HPV-16 virus (HPKIA cells). See text for further details.

seminal pH-approach to the role of different aspects of cancer investigation, from etiopathogenesis to treatment, and from the molecular biology and biochemistry of malignant diseases to clinical therapeutics.

*2.3.2. Growth*

One of the classic hallmarks of transformed cells is their unregulated growth (Hanahan and Weinberg, 2000). In normal cells, the NHE1 is quiescent at the resting $pH_i$, while in transformed and cancer cells, it is usually hyperactive [34,84]. The resulting change towards cellular alkalinity has also been shown to be related via a cause–effect relationship with the entering of cells into the S-phase of the cell cycle and with maintaining them in a status of permanent and uncontrolled proliferation [4,19,25,27]. In this vein, it has been recently shown that the increase of $pH_i$ mediated by the activation of the NHE1 isoform promotes G2/M entry and transition at the completion of the S phase, stimulating mitosis, a phenomena that can be also reproduced by increasing $pH_i$ in the absence of NHE1 activity [25]. Some conclusions can be reached based on at least five different lines of experimental evidence: (i) all growth factors potentially induce activation of the NHE1; (ii) in the absence of growth factors cellular proliferation can be induced by alkalinizing the cytoplasm; (iii) the development of the proliferative response is dependent on extracellular sodium; (iv) specific inhibitors of the NHE1 block the stimulated growth response; and (v) cells lacking the NHE1 have a greatly reduced rate of cell division [2–6,11,17,89,91,98,121–123].

*2.3.3. Glycolytic metabolism*

Widespread use of positron-emission tomography (PET) with $^{18}$fluorodeoxyglucose (FdG) as a glucose tracer has confirmed the long considered idea that an increased aerobic glycolysis is unique to tumors, is nearly ubiquitous in tumors and increases with increasing neoplastic state (for recent reviews on this subject, see Refs. [12,13]). These recent reports have shed further light on a decades-old discussion about the significance of the deviation of tumoral metabolism towards a pH-dependent glycolytic process [2,4,8–10]. Aerobic glycolysis in tumor cells is considered to confer a protective advantage to cancer cells from reactive oxygen species (ROS) produced by their increased proliferation [124] and in the extremely caustic extracellular metabolic microenvironment and to be further driven by the hypoxic component of this microenvironment [13,15]. This makes aerobic glycolysis an important cancer hallmark for the whole spectrum of tumor types irrespective of their tissue of origin or genetic background.

At the same time, there is still some uncertainty concerning the mechanisms underlying the emergence of the glycolytic phenotype. Some have suggested that this phenotype arises initially as an evolutionary adaption to local hypoxia in the tumor [2,13,14,125] while other data support the hypothesis that this phenotype is driven initially by the intracellular alkalinization that occurs as a very early event in neoplastic transformation. Seminal work by Rubin and Fodge, whose initial efforts have been recently reconsidered [15], first demonstrated that cellular alkalinization induced either by the polioma virus or in other ways stimulated glycolysis through a direct activation of its key enzyme phosphofructokinase, a phenomenon that preceded the onset of DNA synthesis [115,126,127]. These results led the same authors to conclude that the main mediating molecular mechanism for the transforming and oncogenetic activity of the virus was the induction of an elevated $pH_i$ [115]. A conclusive study regarding the $pH_i$ dependence of the initiation of aerobic glycolysis has been recently reported in which malignant transformation was induced by the E7 oncogene of human papillomavirus (HPV) type 16 in NIH3T3 cells in an inducible system permitting the sequential analysis of post E7 events [4]. These authors demonstrated the specific and causal role of the transformation-dependent increase of $pH_i$, driven by the stimulation of the NHE1, in the initiation of aerobic glycolysis. This $pH_i$-dependent mechanism underlying transformation-dependent initiation of aerobic glycolysis also occurred in HPV16 infected human keratinocytes, the natural host of HPV in the pathogenesis of cervical carcinoma. Experiments specifically blocking only the increase in glycolytic metabolism with deoxyglucose demonstrated that the transformation specific proliferative increase was not driven by aerobic glycolysis. These findings demonstrate that the initiation of aerobic glycolysis occurs in very early in the process of transformation, is transformation-specific and is driven by NHE1-dependent cell hyperalkalinization. While that study demonstrates that aerobic glycolysis arises well before the formation of the hypoxic/acidic microenvironment, it does not preclude the possibility that the further increase in aerobic glycolysis occurring later could be driven by the interaction of the cancer cells with its metabolic microenvironment as has been suggested [13,76].

Importantly, these studies suggesting that the fundamental driving force for aerobic glycolysis in cancer cells is the underlying cellular alkaline shift would raise the tumor acid–base deregulation to the level of the basic, fundamental cancer hallmark. The recognition of this pathological shift in acid–base dynamics should help us to change our attention from the gene-centric viewpoint to a unifying metabolic-centric viewpoint with the accompanying increase in potentially novel therapeutic strategies aimed at resolving a pathological condition common to all tumors. If successful, this unifying paradigm would most likely greatly simplify existing approaches to treatment and therapeutic regimes while introducing new ones [2,10,36–38,76,128,129].

*2.3.4. Neovascularization*

The ability of a tumor to induce proliferation of new blood vessels from its host has a profound effect on tumor growth and metastasis [130]. The concept of new blood vessel formation, referred as tumor angiogenesis, plays a

fundamental role not only in the growth of solid tumors but also in leukemias and other hematological diseases [130,131]. The onset of angiogenic vessel growth allows tumor cells to obtain the necessary oxygen, nutrients and paracrine factors for their survival and unrestricted growth and also provides both the stimulus and the via for the entry of the more invasive cells of the primary tumor into systemic circulation by intravasion [132].

Neoangiogenesis is switched on during tumor growth and progression. This is a complex process regulated genetically, but also by the tumor metabolic microenvironment, and is triggered as a result of a shift in the balance of stimulating angiogenic factors and oncogenes and inhibiting antiangiogenic factors and tumor suppressor genes. The activity of a significant number of these angiogenic factors and oncogenes have been shown to be closely related to intracellular $H^+$ dynamics [109,133–137].

In this vein, several neoangiogenic promoting peptides have been observed to have a direct NHE1-mediated effect on intracellular acid–base homeostatic mechanisms, all in the same alkaline direction (Table 2). The accumulative data heretofore available suggest a strong cause–effect relationship between the stimulation of angiogenesis and the increase of $pH_i$ in many different cases and situations. This is the case of the pro-inflammatory cytokine IL-1, which promotes neovascularization through the stimulation of VEGF expression in both human proximal tubular cells [138] and in human colon cancer cells [139]. This is driven by the up-regulation of the VEGF-KDR/flk-1 system via activation of tyrosine kinases [140]. IL-1 elevates $pH_i$ in T-cells through activation of the NHE1 through a mechanism that seems to involve protein kinase C [139]. In these and in other situations in which angiogenesis is stimulated, the NHE1 have been found to play a fundamental role in the observed increase in $pH_i$ [135–137].

Further, other mechanisms like the activity of the urokinase-type plasminogen activator (μPA), which is affected by inhibitors of the NHE1 (e.g. drugs of the amiloride series, cariporide- HOE642-, etc.), [46,141], may also play a direct role in the suppression of both tumoral and diabetic neovascularization [79,142–146]. Finally, since the stimulation of the NHE1 is also a primary and essential factor in tumor interstitial acidification through its effect on the extrusion of hydrogen ions from the cell, it could also be involved in the known effects of extracellular acidosis in increasing neovascularization, metastatic potential and migratory and invasive capacity of cancer cells through gene stimulation, metalloproteases, plasminogen activators and different growth factors [11,16,84]. VEGF expression is also stimulated by an acidic tumoral extracellular pH, which in turn can be driven by a hyperactive NHE1 [133,147]. Although these results suggest the existence of a cause–effect relationship between NHE1 activity and cancer angiogenesis, there are other important pro-angiogenic molecules that have not been considered and/or yet found to have a role either in neovascularization or in $pH_i$ regulation [79,131].

### 2.3.5. Invasion and metastasis

Tumor invasion and metastasis associated with neoplastic progression are the major causes of cancer deaths. Thus, understanding the mechanisms determining metastatic spread of malignant cells via invasion to distant tissues is a central question in cancer research [148–151]. Of particular importance is the identification of the fundamental driving forces involved in metastatic progression. The invasive process occurs through a complex series of interactions with the host tissue in which the infiltration and penetration of the normal tissue by cancer cells takes place by four biochemical and physiological steps: (a) tumor cell attachment to basement membranes or extracellular matrices; (b) local degradation of these structures dependent on acid extrusion from both cytoplasm and intracellular organelles; c) secretion of acid-dependent proteases; and (d) increased tumor cell locomotion into the modified region. Both the second, third and fourth processes are regulated to a great extent by extra- and intracellular pH [13,24,60,151–153].

The tumor extracellular microenvironment and, particularly as stated in the previous section, the acid component

Table 2
Proangiogenic regulators and their effect on the activity of the $Na^+/H^+$ exchanger and cellular acid–base homeostasis

| Factor | Effects on angiogenesis | Effects on i. c. Hydrogen ion dynamics |
|---|---|---|
| IL-1 | Upregulates VEGF/VPF and KDR/flk-1 expression | ↑ $H^+$-ATPasa and NHE |
| IL-8 | ↑ EC migration and proliferation | ↑ i.c. pH and cytoplasmic-free $Ca^{2+}$ |
| EGF | ↑ EC DNA synthesis, proliferation and migration | ↑ NHE |
| PDGF | ↑ EC DNA synthesis, proliferation and migration | ↑ NHE |
| G-CSF | ↑ EC DNA synthesis, proliferation and migration | ↑ i.c. pH elevation through ↑ NHE |
| GM-CSF | ↑ activation/differentiation program related to angiogenesis | ↑ NHE |
| TNF-α | ↑ EC migration | ↑ NHE |
| HGF/SF | ↑ tumor survival and proliferation | ↑ i.c. pH elevation |
| TGF-β | ↑ angiogenesis in vivo | ↑ NHE |
| IGF-I | ↑ EC migration, tube formation | ↑ NHE |
| Angiotensin II | ↑ gene expression and secretion of VEGF/VPF | ↑ NHE |
| $PGE_2$ | ↑ EC proliferation | Induces i.c. alkalosis |
| Insulin | ↑ EC growth and tube formation | ↑ NHE |

↑: stimulation; EC: endothelial cell; i.c.: intracellular; NHE: $Na^+/H^+$ antiporter activity. (For further details see reference No. [109].)

of this microenvironment, has been shown to be a critical factor in stimulating the motility, invasive capacity and subsequent malignant progression by increasing the activity of one or more of the above steps [11,15,30,53,62–64,133,152–158]. This can be considered to be a strategic principle utilized by malignant tumors rather than only a secondary side effect of tumor metabolism [84,155]. This acidification of the intercellular microenvironment where the cell is in contact with the extracellular matrix facilitates the action of the acidic proteases secreted by the tumor cell [60,64,153,155]. In a positive feedback, the presence of a lower extracellular pH stimulates the migration of secretory vesicles in cancer cells towards the cell membrane resulting in the increased release of metalloproteases [159].

Further, recent evidence has supported a prominent role of the NHE1 in directly coordinating tumor cell motility [22,23,26,110] and invasion [28,110,94] by selectively regulating cytoskeletal events such as focal adhesion assembly and turnover [22], cortical cytoskeleton dynamics [16] and tyrosine phosphorylation of focal adhesion kinase, with consequent impaired recruitment and assembly of integrins, paxillin and vinculin at focal contacts [111]. Recently, the probable mechanism for this role has been described and involves the direct binding of NHE1 to ezrin, one of the ERM (ezrin, radixin and moesin) proteins involved in regulating the actin cytoskeletal dynamics and the binding of the cytoskeleton to focal adhesions [24,152]. Altogether, these results are consistent with the hypothesis that increased tumor cell invasiveness results via two complementary mechanisms: disruption of cell–matrix interactions arising from increased acid secretion and protease activity together with an enhancement of cell motility. The NHE1 plays a prominent role in coordinating both these events.

### *2.3.6. Development and maintenance of the acid component of the tumor extracellular microenvironment*

A recent symposium on tumor hypoxia and acid–base abnormalities in cancer was mainly focused on the consequences of these parameters in malignant disease [15], disregarding to a certain degree the causal and etiological aspects of cellular hypoxia and/or cellular alkalosis on cancer etiology and cell metabolism [2]. It has been suggested that high vascular density and metastasis are the consequence of a hostile environment of low $pH_e$ as well as to low interstitial $pO_2$ [16,32,154,155,160]. These metabolic abnormalities have been shown to induce the expression of vascular endothelial growth factor (VEGF), insulin-like growth factor 1 receptor (IGF1R), platelet-derived growth factor ß-receptor, interleukin 8, metalloproteases, etc., modifying the expression of plasminogen activators and inhibitors, cell adhesion molecules and cysteine proteinases [26,84,133,161]. They also induce diverse mutations, DNA over-replication, genomic instability and changes in gene expression, thereby fostering increasingly malignant and aggressive phenotypes while enhancing metastatic potential and the invasive capacity of cancer cells [16,84].

The mechanisms by which malignant cells acidify their extracellular/intratumoral microenvironment and how these mechanisms are, in turn, regulated by the other components of the tumor microenvironment are still unclear. This is especially true concerning the effects of serum-deprivation, one of the other major components of the tumor microenvironment, on the processes controlling microenvironmental pH. While lactic acid production/release is usually considered to be the primary tumor microenvironmental acidification mechanism (see above Section 2.1) tumors in nude mice derived from cells lacking lactate dehydrogenase were fully able to acidify their interstitial microenvironment [81]. Furthermore, recent work in vivo has shown that in either serum replete conditions and/or aerobiosis the major component of extracellular acidification is lactate release while in the serum-deprived conditions and/or anaerobic situations common to aggressive tumors, lactate production/release stopped while the extracellular microenvironment continued to acidify [16,80].

Clearly, there are other mechanisms of cellular pH regulation that contribute to extracellular acidification particularly in nutrient deprived conditions. The activity of the NHE1 is known to play a role in the acidification of the tumor microenvironment through $H^+$ extrusion [20,28,132], and it has been demonstrated that the increased extracellular acidification in the nutrient deprived conditions of the tumor microenvironment is due to an activation of the tumor cell NHE1 [110]. This is another indication of the malignant strategy utilized by tumor cells to integrate the effects of the components of the microenvironment through the activity of the NHE1. A recent paper has also demonstrated that activation of the CD44 receptor in breast cancer cells acidifies the extracellular medium and increases invasive potential via the activation of the NHE1 [28]. Finally, it has also been shown that carbonic acid production can also be another significant extracellular acidifying factor [31,46], while lactate symporters are highly important in melanoma cells with low NHE1 activity [61].

### *2.3.7. $pH_i$ and multiple drug resistance (MDR) to anticancer drugs: cause–effect relationships*

The study of the phenomenon of MDR, which at first sight might seem to be its own entirely separated area of cancer research, from the perspective developed here walks hand-in-hand with the rest of the research subfields integrated in this review. A direct cause–effect relationship among the degree of MDR and the elevation of tumor $pH_i$ has been recognized by many different groups that have studied the dynamic interrelationships between cell $pH_i$ and MDR [41,54,55,60,63,124,131,162–164]. Some conclusions can be reached based on at least six different lines of experimental evidence: (I) a failure to induce cytosolic acidification has been proposed to be the main factor underlying drug resistance and even resistance to the induction of therapeutic apoptosis in both the highly

alkaline cancer cells and in malignant cells with a normal or slightly elevated $pH_i$, [16,33,42,59,83,165,166]. (II) The alkaline characteristic of many, and possibly all, kinds of malignant cells (Section 2.1 and 2.2) is also known to decrease the retention of anticancer drugs such as vinblastine, adriamycin, cisplatin, paclitaxel, campthothecin, etc. [7,117,131,138,147,165,167–169]. (III) In a parallel manner the accumulation of at least some drugs in the cytosol increases with cell acidification and decreases with an alkaline shift. One of the most representative and seminal findings in this area has been the close to 2000-fold increase in adriamycin resistance of human lung cancer cells if a $pH_i$ of 7.0 present in parental cells becomes elevated to 7.4 and even higher in the most resistant cells (Fig. 2). Furthermore, the overcoming of MDR in these cells by verapamil was shown to be due to the cellular acidification achieved by this compound [41]. In parallel studies, the intracellular concentration of adriamycin was shown to be increased by more than 100-fold after inducing cell acidification, an energetic change that behaves on its own as a "chemosensitizer agent" [63]. (IV) At the same time, no matter how paradoxical it may seem at first sight, some drugs, mainly weak bases, can be sequestered away from the cytosol within the highly acidic organelles (lysosomes, endosomes, Golgi network, etc.) of otherwise highly alkalinized cells, and in this way become inactive (the so-called protonation effect, ion trapping effect or PSS theory) [16,117,170,171]. In this line, alkalinization of these acidic compartments under normal conditions can stimulate exocytosis and transmembrane efflux of different chemotherapeutic agents from tumor cells, so contributing to drug resistance [171], while resistant cells also contain a higher number of these intracellular vesicles [159,160]. (V) There is a body of work suggesting that the main function of P-glycoprotein is as an ATP-dependent proton extruding pump. The fact that cells with an active MDR transporter show cytoplasmatic alkalinization has led some authors to conclude that it can be mainly considered as a proton extrusion pump [43,172]. The overexpression of the specific membrane pump for hydrogen ions, the $H^+$-ATPase, leads to a decrease in the intracellular concentration of hydrogen ions and has also been shown to be involved in the active extrusion of antineoplastic drugs in HL60 cells resistant to adriamycin and vincristine [91], in high $pH_i$-mediated cisplatin resistance [164,167,168] and in camptothecin resistance to apoptosis [117,173].

In support of these observations, specific inhibitors of $H^+$-ATPase, like Bafilomycin $A_1$ and other drugs with similar actions, induce the intracellular accumulation of drugs by inhibiting their efflux through an acidifying mechanism directly dependent on the hydrogen ion [91,174–176]. (VI) Lastly, findings directly relating cytosolic akalinization with abnormalities in membrane depolarization in MDR-positive, drug-resistant cancer cells have been published mainly by Roepe's group [7,42,63,43–45,171,177,178]. This group has advanced a highly convincing, integrated and multidimensional theory rejecting the hypotheses of the pumping out of drugs in cells resistant to chemotherapeutic agents as the main mechanism to explain MDR. In this model, the energetic alterations of cell membrane depolarization, low transmembrane potential, $pH_i$ elevations and the perturbations in cellular ion transport are considered to be responsible for most of the characteristics of multidrug-resistant cells. The level of Pgp expressed is also shown to be dependent on the degree of cell alkalinization and depolarization

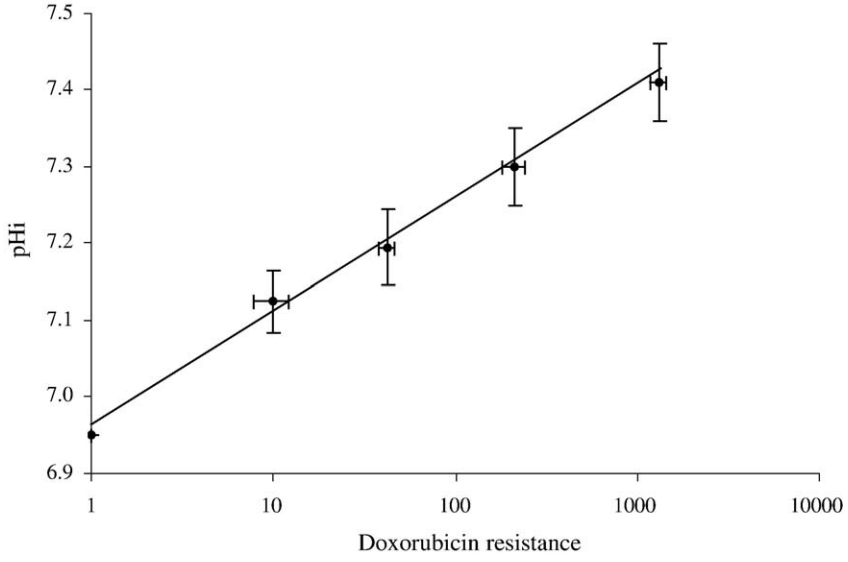


Fig. 2. pH as a function of level of multidrug resistance in human lung cancer cells. Bars indicate SDs for four to eight independent experiments. Level of DOX (doxorubicin) resistance is expressed as ratio of drug concentrations giving $IC_{50}$ (determined after 3 days of continuos drug exposure) in the resistant subline and in the parental SW-1573 strain. $IC_{50}$ value under these conditions for the parental SW-1573 was 15 nM. Resistance levels for each cell line were calculated from four to six independent growth-inhibition experiments. (Reproduced by permission of the authors. For further details, see Ref. No. [41].)

induced. This elegant approach offers a new conceptual paradigm which overcomes the necessity that any specific drug or molecular structure must be considered on its own as the final mechanism involved and/or main responsible agent responsible for MDR. Finally, these alterations in gradients of hydrogen ion concentration across the cell membrane described by Roepe seem to run in parallel to Mitchell's chemiosmotic theory on the final transduction mechanisms of energy production. This makes this approach attractive as a para-Mitchellian model to explain MDR, in which energetic and non-specific factors become more important than the "specificity" of the different structures and molecules involved (i.e., P-glycoprotein, etc.) ("energetic and/or para-structural paradigm) [7,63,179–182].

However, not everyone agrees with the importance of the relationship between pH and MDR. In this line, there have been a few reports suggesting that pH changes, either extra or intracellular, may have no significant effect in drug extrusion by P-glycoprotein [183–185]. Also, it has been reported that no pH differences could be found between cells expressing varied levels of Pgp, and that decreasing drug accumulation do not involve changes in cell pH. These final contradictory results have been suggested to be secondary to the type of cells studied as well as to the time exposure to Pgp in the cell membrane [117]. It should also be noted that the one publication stating that potent derivatives of amiloride were not effective in reverting MDR to adriamycin and vincristine, unfortunately, does not report if there were any pH changes occurring in that study [183]. Such an apparent paradox could also be explained, however, by the arrest of the cell cycle induced by amiloride which has been reported to create resistance to drugs like camptothecin, cyclohexamide or gamma radiation. Since these factors act on the S-phase of the cell cycle, amiloride could lower their effectiveness because of the arrest in G1 phase induced by this drug, so inhibiting apoptosis at the same time [82,186] '(R. Garcia Cañero, personal communication)'.

In conclusion, a hierarchical integration of the great majority of the available data in this area indicates that any mechanism that leads to an elevation of $pH_i$, either through overexpression/activation of the NHE1, by means of activation of $Na^+-K^+-$ATPase, through a synergistic coupling of both mechanisms [2,89,98] or via hyperactivity of vacuolar $H^+$-ATPases, contributes to the onset and/or maintenance of MDR that protects against tumor cell death induced by anticancer drugs [24,31,63,164,187–190]. The importance of the potential therapeutic implications of these findings in a multiplicity of clinical settings in explaining the possible reasons behind the poor therapeutic results, the usual failure to control metastatic disease and the poor survival rates in the treatment of different human tumors in modern oncological practice becomes evident. The potential use of this knowledge to increase therapeutic efficiency will be discussed in Section 3.4.

### *2.4. The neoenergetic strategy of neoplasia: an order within chaos*

A unified interpretation of the evidence so far accumulated permits the understanding of the $H^+$-dependant thermodynamic advantages that malignant cells and tumors have as compared to their normal counterparts. These are: (A) the role that the loss of the normal acid–base balance of the cell represents as an initial and specific cause for cell transformation [2–4]; (B) the utilization cancer cells make of the maintenance of a sufficiently elevated $pH_i$ to protect cell survival and ensue an unregulated proliferative and invasive state [2,5,42]; (C) the establishment of a self-defensive, anti-apoptotic strategy through different anti-acidifying mechanisms such as destabilization and inactivation of Bcl-2, $Bcl\text{-}_{xl}$ and p53 or the stimulation of NHE1 activity and lactic acid extrusion [15,16,25,29,61,65–67,92,123,148,189]; (D) the formation of a membrane-bound, high $pH_i$-mediated anti-chemotherapeutic shield involved in MDR and in the development of resistant subpopulations of tumor cells [2,7,31,41,44,65,66,162,190]; (E) the alkaline shift in the $pH_i$ dependence of the NHE observed upon transformation increasing the proton–acid extrusion ability of the transformed cells; (F) the rigid maintenance, under all circumstances, of a reversed and thermodynamically advantageous high $H^+$ ion gradient across the cell membrane (alkaline inside–acid outside) [15,16,60,191]; (G) the use of the induction of acidity of the interstitial component of tumors, among other factors, in the onset of angiogenesis, by increasing the expression of positive angiogenic factors, as well as in the maintenance and activity of the metastatic process [24,60,153,154,192]; (H) the use of this extracellular acidity in creating resistance to chemotherapy, radiation-induced apoptosis and hyperthermia [16,74].

The dynamic and seminal abnormalities associated to the above described energetic mechanisms (E), (F) and (G) seems to lead to an organized positive feedback system resulting in the subsequent development of the microenvironment, both intracellular and interstitial, characteristic of neoplastic cells and tissues. During and after transformation, the reversal of a previously normal situation takes place, namely, the increase of the transmembrane $H^+$-gradient (now alkaline inside (mainly NHE1 mediated)–acid outside (dependent on $H^+$, lactic acid and $CO_2$ extrusion plus extracellular hypoxia) [62]. This energetic change will initiate a cascade of a series of electrochemical changes and events leading to tumor growth, progression and, simultaneously, resistance to treatment [4,43–45]. Such changes are manifested by both local invasion of surrounding tissues as well as by setting up the dynamic and pathophysiological basis necessary for the entire metastatic process [84]. Rapid tumor cell growth, sometimes under hypoxic conditions, is accompanied by increased glycolytic metabolism and lactic acid production resulting in a further enhancement of acid export [10,64].

This local extracellular acidification is even further facilitated by the microcirculatory inadequacy common to growing tumors. These additional dynamic changes are of paramount importance in the onset and progression of both local invasion and the metastatic process in a variety of ways [2,4,10,11,15,24,60,84,109] and allow us to understand the difficulties in fighting such rigidly organized nested vicious cycles within an apparently perfect degenerative strategy.

As a result of the concerted work of the eight (A to H) above-described energetic mechanisms, cancer cells and tumors can, biologically and functionally, insulate themselves from the rest of the host organism. This allows the unveiling of a new fundamental neoplastic paradigm or hallmark that can be defined by their capacity to form genuine parasitic "molecular and biological islands" within the host through a highly organized, self-protective system based upon an advantageous manipulation of cellular hydrogen ion dynamics through the previous points A to H. Utilizing this integrated "wise and malignant" strategy, cancer cells are able to shield themselves from in vivo external chemotherapeutic attack under the most varied and even potentially damaging (very low interstitial pH, low $O_2$ conditions, etc.) microenvironmental circumstances [2,13,24,62,83,84,109,114,123,192].

This permits us to understand now in a dynamic way that one of the main purposes of their biochemistry, metabolism and, from both quantitative and qualitative aspects, specific energetic systems is to have the different $H^+$-transmembrane proton transport mechanisms ($Na^+/H^+$ exchangers, $H^+$-ATPases, lactate/$H^+$ symporters, monocarboxylate transporters (MCTs) or $Na^+$-dependent $Cl^-/HCO_3$ exchanger) as well as intracellular buffering mechanisms [16,90] working, if needed, full time and at a maximum rate [2]. This is done with the main purposes of: (a) maintaining a permanent homeostatic acid–base equilibrium of the cell in the normal to alkaline direction, and (b) preventing their selective acidification, and subsequent apoptosis, by all means available [84,96,114]. This strategy becomes of paramount importance for malignant cells and tissues of all origins to be able to maintain a self-protective and sufficiently elevated $pH_i$ in the presence of a potentially toxic extracellular and interstitial, intratumoral acidic pH [33,34,62,101,104,105]. Through this synergistic interaction of the above mentioned mechanisms, malignant tissues form and maintain an $H^+$-dependent, autonomous, self-protective and self-defensive closed system which we consider to be a specific and predetermined strategy of neoplastic structures, i.e., a thermodynamic and bioelectric neostrategy. From this thermodynamic point of view, it can be concluded that we face a self-organized integrated system that from a Darwinian view of adaptation to difficult environmental conditions [125,193] is in a vantage position too powerful to be controlled by any external therapeutic measures that do not in some way target this system.

## 3. The other side of the coin: regression and treatment

At the other end of an $H^+$-concentration spectrum (low pH, or acid limiting zone), it becomes necessary to analyze which factors and/or mediating mechanisms may lead the entire homeostasis of cell physiology towards a "therapeutic" acidification in the cancer setting. A great deal of experimental evidence coming from modern biochemistry and molecular biology backs up this etiological approach to treatment whose importance is rapidly growing in the context of cancer treatment and innovative therapeutic protocols.

### *3.1. Selective apoptosis in the treatment of cancer. Pathways and therapeutic attempts*

An important aspect of modern chemotherapy is the search for novel strategies based on the sensitization of the tumor cells to known therapeutic treatments via targeting of the critical signal transduction components that mediate the clinical response [194,195]. Thus, the challenge is to identify signal transduction targets that substantially increase the drug-dependent toxic effect [196,197]. Many compounds that are able to induce apoptosis in a wide array of malignant cells of different origins have also been reported to do so through the necessary induction of intracellular acidification [7,49,60,82,123,163–165,169,178,198–215]. Further, drug resistance could be attributed to the failure to induce acidification [31,177] or by an increase of $pH_i$ using compounds such as chloroquine, imidazol, glutathione and/or activation of the NHE1 [5,58,9,216].

For these reasons, there has been an increasing interest in approaches to cancer treatment based on the hydrogen dynamics of cancer cells over the last years [2,11,16,31,60,61,92]. In this context, inhibition of NHE1 has been shown to be an early signal transduction event that may participate either directly or indirectly in the regulation of the apoptotic response by many drugs via the onset of cell acidification downstream of the inhibition of the NHE1 [21,46,91,141,160,188,203–205,217–219]. Rich et al. [21] have demonstrated that treatment of cells of several types of human leukemias with the highly specific and potent NHE1 inhibitor, the amiloride derivative HMA (5-(5-*N*,*N*-hexamethylene)-amiloride), was able to selectively and specifically lower the extremely high $pH_i$ reported for leukemic cells down to the range of 5.6 for KG-1a leukemia and as low as 5.0 for cells of human acute lymphoblastic leukemia (ALL) (Fig. 3A) with a resultant very significant differential sensitivity of the pro-apoptotic effect of HMA on leukemic compared to normal cells [21]. The same authors have also shown that the therapeutic induction of hyperacidification of $pH_i$ of human leukemic cells with pharmacologic doses of HMA below 6.8 selectively kill more than 90% of human leukemic cells (Fig. 3B). The outcome of anti IgM-induced cell death in human B lymphoma cells depends on the

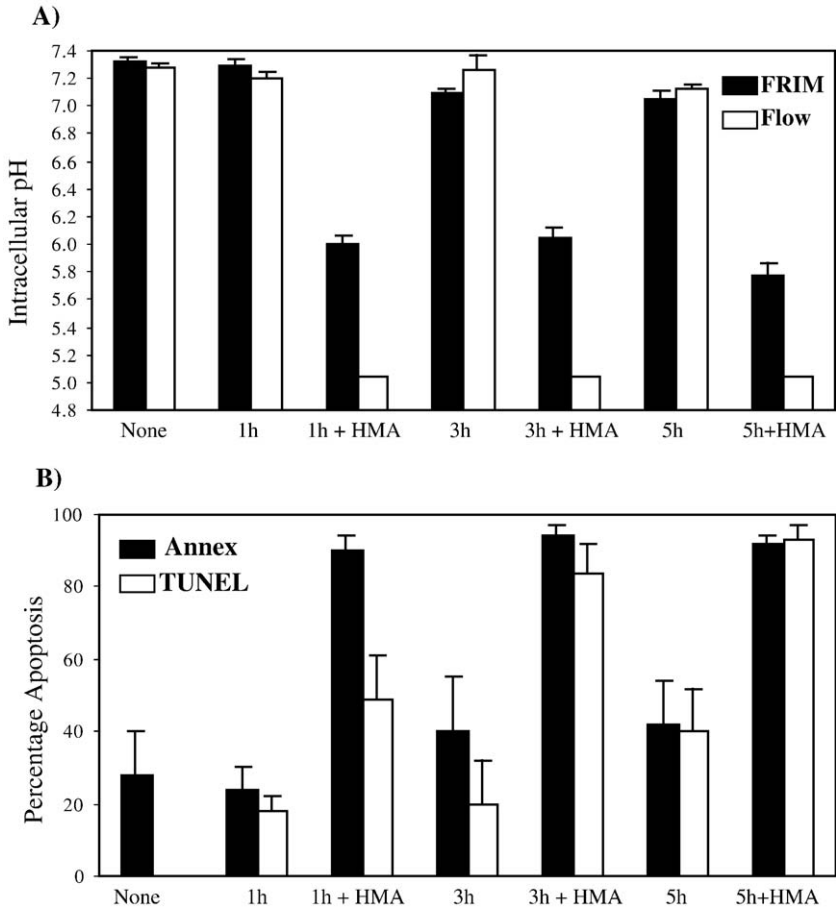


Fig. 3. The effect of HMA on $pH_i$ and apoptosis of primary acute lymphoblastic leukemic cells. (A) Measurement of $pH_i$ by FRIM and flow cytometry. (B) Estimation of apoptosis by annexin-V-FITC and TUNEL. Results represent mean±S.E.M. of 3 experiments. (Reproduced by permission of "Blood". For further details see Ref. No. [21].)

degree of intracellular acidification achieved by the inhibition of the NHE1 [220], a study that also links immunotherapy to molecular biology. Also, a lowering of $pH_i$ by shutting off phosphofructokinase has been shown to reduce cancer cell survival [16,77] as have drugs that inhibit lactic acid extrusion, like quercetin or similar drugs [44,61,221], as well as $Cl^-/HCO_3$ exchange inhibitors like S3705 [46] and $H^+/Cl^-$ symporter inhibitors [222]. All of these drugs contribute to the disruption of the intracellular/extracellular $H^+$-gradient by retaining $H^+$ within the cell as a self-poisoning or cell suicide mechanism [6,68,69].

In this sense, it becomes highly interesting that selective apoptotic inducing agents like edelfosine (ET-18-$OCH_3$) have also been reported to interfere with the activity of the NHE1, a feature that has led Besson et al. to conclude that acidification mediated by the $Na^+/H^+$ antiporter actively participates in the action of this drug [223,224]. We have recently shown that an inhibition of the NHE1 plays a fundamental role in paclitaxel-induced apoptosis of breast cancer cells and that this apoptosis is synergistically potentiated by inhibition of the NHE1 with the amiloride analog, 5(*N*,*N*-dimethyl)amiloride, DMA [114]. The recently developed, potent inhibitor of the NHE1, cariporide (HOE-642) seems to produce a similar effect [25,27,46,141]. It is necessary to point out that, unfortunately, these recently developed, potent inhibitors of the NHE1, such as cariporide and HMA, are still waiting to enter preclinical and clinical studies as potentially selective antineoplastic agents [46,114].

These and other experiments along the same line have led some groups to the conclusion that a decrease of $pH_i$ below 6.8 can be considered as the threshold and turning-point below which a "natural" activation of proteases, endonucleases and caspase-mediated chain reactions induce selective apoptosis [21,198,203]. On the contrary, the anti-apoptotic effect of the Bcl-2 family of proteins has been shown to also be dependent on their ability to alkalinize the cell, even if the mechanism is not still clear [65–67,123,187,213,214]. While it is generally accepted, with a few exceptions [225] that cell acidification takes place as a universal phenomena at one or another time in the apoptotic process in transformed cells, there is still is a certain controversy regarding whether such acid–base changes occur during the late stages of the process of cell death by helping to degrade DNAses, which further degrade genomic DNA, or if acidification is an early and predetermining change.

This leads to some key questions: first, if acidification is cause or consequence of the apoptotic process and, secondly, if it takes place once the decision of cellular death has already been irreversibly taken by means of previous mechanisms, like lesions to DNA, alterations in acromatic spindle, etc. [200,205]. Angioli et al., in a series of studies using different chemotherapeutic substances in a variety of tumor cells, have demonstrated that cytosolic acidification is a very early event (201) and Reshkin et al., [114] and others [169,187,204] have also reached the same conclusion. Matsuyama et al. [203] have also shown that cytoplasmatic acidification is an early event that regulates downstream key events in apoptosis such as caspase activation. These findings agree with results shown by Furlong et al. [202] where a significant decrease in cytosolic pH have been observed to precede the activation of DNA fragmentation induced by ICE type proteases. The induction of an intracellular acid environment has been reported to trigger the onset of apoptosis of leukemic cells by up-regulating the pro-apoptotic Bax protein expression and this seems to be mediated by the activation of ICE or CPP32 caspases irreversibly leading to acid stress-induced apoptosis and thus to the control of cell proliferation and arrest of tumor growth [198,203]. Also, ICE-like protease activation and DNA fragmentation are preceded by a decrease in $pH_i$ during apoptosis in IL-3 dependent cell lines, an apoptotic cell death that is also stimulated by etoposide [169, 198,202–204]. It has also been reported that acidification in apoptosis takes place downstream of protease activation, Bcl-2 protection and protein kinase activity [83,116, 164,187,208]. Finally, while $pH_i$ is higher in cisplatin-resistant cells than in the sensitive parental cells, reducing $pH_i$ by different methods is associated with increased cisplatin and carboplatin sensitivity [31,61,167,168, 221,226]. In like manner, the over-expression of the anti-apoptotic gene product Bcl-2 is a factor known to be essential in drug resistance to different anticancer agents, both in solid tumors and leukemias [123,65–67,162, 187,213,214]. This gene product, like Bcl-$_{xl}$, increases cytosolic pH, so counteracting and preventing cell acidification as induced by staurosporine, v-abl, granulocyte stimulating factor (GSF) and edelfosine [65,83,187, 211,214,223]. Recently, stimulation of ß1 integrins was shown to inhibit paclitaxel-induced apoptosis in breast cancer cells [227]. This is significant because stimulation of ß1 integrins is also known to stimulate the NHE1 and raise $pH_i$ [228].

In summary, most of the available evidence seems to lead to the conclusion that the universal pathway for a selective resistance to the induction of therapeutic apoptosis is systematically mediated, at one or another stage of the intracellular signaling pathway, by a wide array of defensive mechanisms that either prevent cell acidification or directly maintain and even raise $pH_i$ in an already elevated homeostatic acid–base conditions. Now, it can be better understood why $H^+$-extrusion mediated by stimulation of transmembrane NHE1 or vacuolar ATPases inhibits apoptosis, while the inhibition of any of these $H^+$ transporters induces it [31,32,114,205,215,229].

Regarding treatment, it has been observed that selective apoptosis in both solid tumors or leukemias can be induced by stimulating caspase-3 or Bax activity through the intracellular acidification induced by inhibitors of the NHE1, with either amiloride or its analogs [66,198, 204,205,217,230]. In gliomas, an increase in pHi and NHE1 activity have been shown and suggested that NHE1 is a useful target for tumor-selective therapy [44,231]. In breast cancer cells, paclitaxel is known to induce Bcl-2 phosphorylation and subsequent apoptosis by activation of either PKA or p38 MAP Kinase [17,114]. We recently reported that the inhibition of NHE1 plays a fundamental role in paclitaxel-dependent induction of apoptosis in human breast cancer cells through a mechanism that depends directly on the activation of PKA and p38alpha MAP Kinase, and that these proteins are components of a single signaling cascade consisting sequentially of PKA, p38 and NHE1 [114]. We are currently determining if the inhibition of the NHE1 is up-stream of Bcl-2 phosphorylation and caspase activation. Finally, it would be of great interest to learn if the final antineoplastic effect of paclitaxel is also mediated by a pHi effect, as these previous results suggest.

It should be noted that an occasional publication has suggested either that the relationship acidification-apoptosis is not convincing [209], that an early increase in $pH_i$ can be associated to Bax-mediated apoptosis [232], that maintaining $pH_i$ above 7.2 may not prevent apoptosis in some cases [205,209] and that under certain circumstances, apoptotic cells can even show increased intracellular pH during some stages of the apoptotic process [225,233]. Additionally, a study by D'Arcangelo et al. [147] demonstrated that low pH reduced apoptotic cell death in endothelial cells, results that are in apparent contradiction with the results obtained by Wahl et al. working with angiostatin [61,234]. The basis for these differences could be that in the D'Arcangelo experiments the medium was acidified to a pH of 7.0, while in Wahl's experiments, they acidified the medium to a pH of 6.7. Additionally, in the first study [147], less serum was used, which may have induced the cells starting to undergo apoptosis due to acidification to arrest the apoptotic process in serum-deprived conditions. One possible explanation for this could be that acidification could cause a slow-down of metabolism, so that cells become dormant and less would proceed to apoptosis, at least temporarily '(M. L. Wahl, personal communication)'. The discrepancy of some or all of these contradictory results also seems to be dependent on the kind of cells studied, since resting non-transformed thymocytes, which are growth factor-independent, can undergo apoptosis in alkaline conditions [14,225]. This feature has been explained by the possibility that a decrease in $pH_i$ may retard the rate of progression to cell death by antagonizing $Ca^{2+}$-dependent phospholipases, proteases and

endonucleases essential to apoptosis. In any case, from the point of view of the induction of apoptosis in the selective chemotherapeutic treatment of cancer, it could be concluded that even a normal $pH_i$ in cancer should be considered highly pathological since it allows uncontrolled proliferation, being permissive at least in promoting unrestrained growth and preventing tumor cells from undergoing apoptosis in most (if not all) situations.

### 3.2. NHE1 and $pH_i$ in the inactivation of oncogenes and oncogenic viruses

The oncogenetic effects of v-mos and Ha-ras oncogenes can be prevented by arresting the oncogene-induced alkaline surge with amiloride [102,103,112–114]. This is the rationale, as well as an important research tool, behind the potential clinical utilization of drugs of the amiloride series in the control of the growth and spread of tumors dependent on the expression of certain oncogenes [21,92,109,219,235]. Complete reviews on the inhibitory, antitumoral and pH lowering effects of amiloride and/or its derivatives, as well as the carcinogenic effects of abnormally high pH in both basic cell studies and clinical settings, have been considered elsewhere by ourselves and others [2–5,47,92,236,237].

Further therapeutic possibilities arise from a recent study working with tumors driven by the E7 oncogene of HPV16 [4]. These authors demonstrated that the therapeutic annulment of cytoplasmatic alkalinization by the amiloride analog, DMA [5(*N*,*N*-dimethyl)amiloride], inhibited the development of the transformed phenotype as well the growth of human keratinocyte tumors induced by the HPV16 in nude mice [4]. These findings, together with the fact that viral replication is drastically reduced below a certain microenvironmental pH [238–240], makes it worth taking into account these associations between acid–base energetics and viral activity, even in the study of the possible mechanisms of action of other retrovirus like the HIV virus. Finally, transfection of MDA-MB-435 human carcinoma cells with the antimetastatic gene, nm23, decreases $pH_i$ and elevates $pH_e$, diminishing and, so, disrupting the abnormal transmembrane $pH_e/pH_i$ gradient, suggesting that the suppression of metastasis is also mediated by cellular pH regulation apart from affecting phospholipid-mediated signaling [191].

### 3.3. Neovascularization, the metastatic process and the NHE1

A direct relationship between pro-angiogenesis and NHE1 activity has not only been observed in etiopathogenesis but also in the opposite situation, namely, anti-angiogenic treatment. Here, too, there seems to exist a distinct relationship between the inhibition of newly formed blood vessels, and a dynamic tendency towards a lowering of $pH_i$. In this vein, many anti-angiogenic agents act directly and/or indirectly on cellular hydrogen ion dynamics, mostly resulting in a tendency towards cell acidification that can be mediated by the inhibition of the different membrane-bound transporters such as the NHE1 and/or vacuolar $H^+$-ATPases (Table 3) [109].

Although the pharmacological effects of each of these drugs are extremely varied, all the anti-angiogenic molecules considered in Table 3 appear to share the same pivotal mechanism of action; namely, the inhibition of the NHE1. Squalamine, for instance, blocks hydrogen efflux out of the endothelial cell, thus inhibiting cell alkalinization [241–245]. Although the exact mechanism of action of drugs like suramin still remains unclear, it has been postulated that both suramin and other inhibitors of neovascularization may ultimately work through a pH-dependant final mechanism [229]. Angiostatin, apart from lowering $pH_i$ in the endothelial cells (ECs) themselves, is more effective in inhibiting in vitro angiogenesis by inducing cell death of ECs at low $pH_e$ and $pH_i$ [234,246].

From a therapeutic point of view, some of these compounds might eventually reach a high degree of selectivity in inhibiting the formation of tumor vasculature and/or its collapse, thus becoming effective against a wide variety of tumors both in the prevention and control of the metastatic process [109,151]. Some of them have already shown to present scarce side-effects and to be easily applicable to the clinical setting (Table 3). Among these,

Table 3
Antiangiogenic agents and their effect on the activity of the $Na^+/H^+$ exchanger and cellular acid–base homeostasis

| Drug | Effects on angiogenesis | Effects on hydrogen ion dynamics |
|---|---|---|
| Suramin | ↓ angiogenesis and growth | $H^+$-ATPasa inhibitor |
| Squalamine | ↓ angiogenesis and growth | ↓ NHE |
| Warfarin | ↓ of prostaglandin synthesis | Acidify the cytoplasm of the cell |
| Sulindac | Induces apoptosis and ↓ tumor angiogenesis | Interaction with $H^+$ of the intermembrane |
| Genistein | ↓ tyrosine kinase, EC proliferation and migration and μPA | ↓ NHE |
| Captopril | ↓ of angiogenesis | ↓ NHE |
| Amiloride | ↓ of μPA activity | ↓ NHE1 |
| Edelfosine | ↓ of angiogenesis | ↓ NHE |
| Natriuretic peptides | ↓ EC growth and angiogenesis | ↓ of i.c. pH recovery |
| Staurosporine | ↓ of angiogenesis | Induces i.c. acidification |

↓: inhibition; EC: endothelial cell; i.c.: intracellular; μPA: urokinase plasminogen activator; $Na^+/H^+$ APA: $Na^+/H^+$ antiporter activity; NHE1: $Na^+/H^+$ antiporter isoform 1. (For further details, see reference No. [109].)

we can find: (A) edelfosine [223,247,248], (B) captopril [249–252], (C) some flavonoids like quercetin [253], (D) squalamine [241–243,245], (E) angiostatin [79,234,246] and, (F) different drugs of the amiloride series [55,56,144,254]. Amiloride has been advanced as effective not only in the experimental treatment of neovascularization in malignant tumors but also in chronic proliferative diabetic retinopathy and ulcer healing [142,237,255]. Additionally, amiloride exhibits a significant, and even complete, in vivo antimetastatic effect in different transplanted tumors [143]. This drug and its derivatives have been recently considered as a novel treatment for cancer in order to reduce tumor growth and increase patient survival [13,92]. They are thought to inhibit angiogenesis through at least one of the following mechanisms: blocking NHE1 activity [13,69,217] and/or inhibiting the urokinase plasminogen activator (μPA)-urokinase plasminogen activator receptor (μPAR) complex [109,237]. The latter process is a key mechanism in the proteolytic degradation of extracellular matrix, a necessary step known to mediate both the migration of endothelial cells in the formation of new blood vessels and in the spread of tumor cells during the metastatic process [144,145].

### 3.4. $pH_i$ in overcoming resistance to antineoplastic drugs (MDR): potential and meaning

A large variety of MDR modifiers known to be able to revert resistance to chemotherapeutic drugs (verapamil, amiodarone, Bafilomyicin $A_1$, cyclosporin A, tamoxifen, DIDS, nigericin, edelfosine, etc.), have been shown to exert their cellular effects through a pH-acidifying mechanism [7,54,123,188,190,204–207]. Also, a decrease in $pH_i$ has been shown to sensitize cancer cells of diverse origins to apoptosis, chemotherapy and hyperthermia [70,74,164,198,256–258]. In all these cited cases, a cellular acid–base change was considered to be the essential mediating molecular mechanism underlying the beneficial effect of breaking through drug resistance.

A key example to help understand certain etiological aspects of MDR is the inverse relationship existing among cytosolic pH and the intracellular accumulation of adriamycin in human lung cancer cells (Fig. 2) [41]. More recently, similar conclusions have been reached by other groups: with doxorubicin and/or daunomycin in myeloma [54], the increased drug sequestration found for adriamycin in the more alkaline MCF-7 human breast cancer cells [259], or in cisplatin resistance [164,167,168,226]. In the same vein, reversal of MDR can be obtained by the pH-lowering effects of amiloride and/or its analogs in a variety of situations [92,104,190,259,260]. The inhibition of NHE1 not only significantly induces tumor sensitivity to different chemotherapeutic agents like cisplatin, adriamycin and paclitaxel but also increases therapeutic ratios, specificity factors and tumor sensitivity to different agents, both in hepatoma and other malignant tumors [2,82,164,230]. When acidic $pH_i$ has been reported to diminish sensitivity to antimitotic drugs like mitoxantrone and paclitaxel, this seems to have been dependent on the fact that in acidic conditions a lower proportion of cells are in G1 phase than when at alkaline pH [25,186,261]. The fact that NHE1 inhibition decreases transformed cell $pH_i$ well under parental cell values and usually without an effect on normal cells, indicates a certain therapeutic selectivity and specificity [4,21,164,260], which at the same time parallels and is a hallmark of apoptosis [1,27,215].

Similarly, the alkalinizing effect of another $pH_i$ regulatory transporter, the electrogenic vacuolar $H^+$-ATPases (V-ATPase) has been shown to be involved not only in the etiology of malignant generation, but also in cell growth, angiogenesis, metastasis, drug extrusion and resistance to chemotherapy, a phenomenon that is mediated by the increasing efflux of $H^+$ ions from the cell and/or by ion sequestration within different cellular organelles [3,164,174]. This explains why specific $H^+$/ATPase inhibitors, like bafilomycin $A_1$, salcylihalamide, lobatamides and oximidines are considered potential anticancer agents [31]. Bafilomycin $A_1$ induces a major increase in drug accumulation at the same time that it potentiates the cytotoxicity of different antineoplastic agents, such as doxorubicin and cisplatinum [31,164,175,176]. Furthermore, reducing $pH_i$ (e.g. via Bafilomycin $A_1$) has been reported to be associated to an increased cisplatin sensitivity in vitro, while NHE1- or vacuolar $H^+$-ATPase-mediated $pH_i$ elevation confer resistance to cisplatin by protecting human cancer cells of different origins against cisplatin-mediated apoptosis [164]. Additional evidence arises from the fact that stimulation of $H^+$-ATPase by G-GSF prevents cell acidification, so annulling apoptosis, while its inhibition by bafilomycin $A_1$ stimulates apoptotic cell death [211].

These consistent features are of paramount importance in any strategy directed to counteract and overcome MDR and, therefore, in any serious attempt to improve the chemotherapeutic approach to anticancer treatment. In summary, the bulk of existent evidence indicates that future attempts to override MDR, no matter the different approaches considered, should also be initially focused from the perspective afforded by the homeostatic alterations of the hydrogen ion, since it is evident that MDR reversion can be achieved by lowering $pH_i$ in most, if not in all kinds of solid tumor cells and leukemias studied. Unfortunately, an occasional review dealing with MDR and the $pH_i$ of tumors have introduced serious misinterpretations on the bulk of previously available data regarding the relationships of cell acid–base dynamics to multiple drug resistance [262].

### 3.5. $H^+$-related mechanisms in the spontaneous regression of cancer (SRC)

The term “spontaneous” only reveals an, not very successful, attempt to hide our ignorance on the mediating mechanisms behind a certain fact or observation. In the case

of the well recognized phenomenon of the spontaneous regression of cancer (SRC) it is worth the effort of integrating early data with our recent understanding of the interesting subject that is the SRC [263–265]. The classic oncological literature paid a great deal of attention to the intimate relationships between acid–base changes and regression of cancer in different clinical situations. Even from the beginning of the 20th Century, diverse processes, having in common the onset of a sustained systemic acidification obtained by different methods, were associated to the spontaneous regression of many different animal and human malignancies. As early as 1931, Meyer associated the induction of a local and systemic metabolic acidosis as the mechanism mediating the curative action of Coley's toxins and diverse febrile processes [266] with Reding and Slosse independently reaching the same conclusions [267,268]. The favorable influence of acidification on complete cancer regression and cure in a wide array of transplanted animal tumors has also been recognized over the years [76,263–270] (reviewed in Refs. [269,271]). It is now decades since Ana Goldfeder initially offered a seminal rationalization of what she called "the acidotic treatment of neoplasia" [272]. Anghlieri, using ammonium chloride [270], Selawry with lactic acid [273], Verne et al., and Mori, with acetic acid [274,275], and ourselves with hydrochloric acid [77], repeatedly obtained complete tumor regressions in a multiplicity of animal malignancies. Most authors, however, attributed the results to the particular molecule (ammonium, lactic, acetic, hydrochloric, etc.) and only occasionally was the "non-specificity" aspect of the accompanying acid considered as the possible mechanism by which the regression took place; that is, the apparently non-specific "acidification factor" [77,170,276]. Even recently, tumoral self-poisoning mediated through pH changes has been considered to be responsible for the spontaneous resolution of tumor models [60]. Furthermore, severe metabolic acidosis induced by some surgical procedures, such as ureterosigmoidostomy, was initially considered by us to be the main mechanism underlying some "spontaneous" regressions of different tumors in human beings [263–265,271,276,277]. More recently, a graded metabolic acidosis associated with mild renal failure has been claimed to reduce and, even, reverse the rates of tumor growth and invasion [76].

## 4. Implications and conclusions. New strategies designed to induce selective apoptosis and overcome resistance to cytotoxic agents

From infectious to neoplastic disease, an etiological and/or unifying approach to the treatment of any disease process should always be sought after (the root approach). In the case of cancer research, this perspective was aimed at uniting under the same view such diverse aspects of oncology ranging from "etiology" to therapeutics. On both sides of this continuum, the highly significant role first of a high $pH_i$ in the origin, development and maintenance of the neoplastic state and, secondly, the specific role of a low $pH_i$ in both improving the selectivity of different approaches to cancer treatment and selective apoptosis is beyond all possible doubt. Thus, a new and more comprehensive paradigmatic model is now required to arrive at novel and more integral approaches to cancer diseases and treatment based on the existence of the above-discussed energetics and strategy (Section 2.4) of the "acid–base" specific nature of cancer. A further advantage of this constrained root-approach is the integration of what previously were considered to be non-interrelated areas of research, from basic molecular and biochemical research to clinical therapeutic efforts in cancer patients [123,220,278–282]. This does not necessarily mean that the model "basically" represented by the pH/NHE1 initial approach rules out other perspectives and targetings to cancer etiology and treatment, however, it should be realized that this has so far been a highly neglected area in attempts to integrate basic, molecular and biochemical research, with clinical therapeutic efforts in cancer patients.

All the available and emerging data advise the undertaking of prospective studies in different human tumors in order to test the preventive, antimetastatic and MDR-overcoming effects of drugs like amiloride and its derivatives, as well as other NHE1 inhibitors [109,236,282], edelfosine [29,246,247,283], captopril [109,249,251], cariporide [46,93], squalamine [241,244], certain bioflavonoids [252,284] and vacuolar ATPase inhibitors [31], alone or in combination. These and other similar drugs could also be of great value on their own in the so called, "dormacy therapy" of malignant diseases when used in between and after chemotherapy courses in the prevention or control of metastatic disease [76,218]. Also, it should be taken into account that drugs like adriamycin, cisplatinum, paclitaxel and camptothecin are unable to induce apoptosis under non-acidified cellular conditions [41,114,164,165,180,285]. For all these reasons, the tumor dynamics of the hydrogen ion are increasingly becoming a fundamental target in selective therapeutic intervention in both leukemias and solid tumors [2,4,21,90,114,163]. Without ruling out other crucial pathogenetic mechanisms and selective apoptotic therapeutic targets [286,287], the hydrogen ion dynamics of malignant cells and tissues may also be related to the fact that certain metastatic tumors like germ cell tumors are highly sensitive and can be readily cured by chemotherapy while most of the other tumor types are usually resistant to chemotherapy [288,289]. Recent research with placental choriocarcinoma epithelial cells seems to suggest this possibility since the uptake by choriocarcinoma cells of different compounds is directly related to pH changes and to the expression and activity of the NHE1 [290–292]. We believe that in spite of the efforts being made in order to find different apoptotic targets in cancer cells [29,293,294], what it is still missing, and is urgently needed at this point, is an

integrated time-space intracellular pH-related road map leading to the stimulation of acid-induced selective apoptosis [196,197,294].

In summary, the main bulk of both seminal and emerging data in these areas leads to five key conclusions: (1) that cell alkalinization constitutes an initial and fundamental event in the transformation process of normal cells and tissues regardless of their origin [2–5,47,48,51,52]; (2) that NHE1 stimulation plays a positive key role in later neoplastic and metastatic progression and a negative key role in breaking some of the host defense mechanisms (antiangiogenesis, spontaneous regression, therapeutics, etc.) [11,42,92,271]; (3) that targeted inhibition of the NHE1 and other membrane-bound proton pumps in order to control both cancer growth and neovascularization could prove to be clinically useful in preventing, retarding and/or counteracting the metastatic process [109]; (4) that NHE1 inhibitors can also be fundamental in the adjuvant and neoadjuvant treatment of different malignant solid tumors in humans, alone or associated with other forms of chemotherapy, as well as in the overcoming of MDR (Fig. 3 and Table 3); and (5) that chemotherapy and other combined and/or complementary forms of cancer treatment mainly targeting cell acid–base dynamics and its parallel bioelectronic and polarization abnormalities should be considered in future approaches to cancer treatment, as well as in other degeneratives diseases [7,35,76,129,295–300].

We finally conclude that treating malignant diseases in the usual and unmodified steady-state conditions of cancer cell intracellular alkalosis, increased $pH_i/pH_e$ gradient, membrane depolarization and low transmembrane potential will continue to be of limited success if efforts are not made to associate chemotherapy with measures directed to induce selective intracellular acidification along with other therapeutic measures.

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