Medical Hypotheses (2002) **58**(6), 444–452

doi: 10.1054/mehy.2001.1415, available online at http://www.idealibrary.com on IDEAL®

Integrating fields of cancer research through pivotal mechanisms and synthetic final pathways: a unifying and creative overview

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Summary From cancer etiopathogenesis to selective apoptosis, from multiple drug resistance to oncogen activation and from the phenomena of spontaneous regression of cancer to certain aspects of cancer chemotherapy, all these subfields of biology and oncology research share some deep-seated, both basic and clinical, essential features and characteristics. Certain apoptosis-inducing agents of unrelated families, ranging from ether lipids to Na^+/H^+-antiporter inhibitors to Δ^9-tetrahydrocannabinol all have been reported to induce selective cancer-cell death. Behind a wide array of intermediary factors and mechanisms involved in their activity, they seem to share common pivotal and/or final pathways in inducing cell death mediated by a 'pathological' accumulation of intracellular hydrogen ions as a mechanism underlying core changes in intracellular signaling pathways. An H^+-concentration initial perspective indicates that from pathogenesis to apoptosis and multiple drug resistance, as well as oncogen activity, tumor progression and even the phenomenon of spontaneous regression, all can be interpreted from their deep (H^+)-related basic and clinical essential characteristics. This speculative review discusses the potential integration of these previously disparate subfields of cancer research, through a model which also seems to lead toward improving understanding of the fundamental nature of malignant processes. It is concluded that this synthetic and universal approach allows advancement toward a combining of different areas of oncology into deeper and more comprehensive forms of rational understanding, with the hope of paving the way towards more selective, effective and all-encompassing forms of treatment. © 2002 Published by Elsevier Science Ltd.

FROM CANCER ETIOPATHOGENESIS TO TREATMENT: A 'BOTH SIDES NOW' INTEGRAL PERSPECTIVE

Previous publications of our group and others have called attention to different aspects of hydrogen ion dynamics in diverse areas of cancer investigation at both the clinical and basic research levels. These attempts range from researching the mechanisms that lead to the phenomenon of spontaneous regression of cancer, to improving understanding of etiopathogenesis, to the opening of new therapeutic possibilities (1–4). It has been previously shown that all these different research issues can be approached from a multidimensional and, at the same time, basic unitarian perspective (5). Recent discoveries in these and other related areas make it possible now to further advance the construction of more integrated perspectives. From the point of view of epistemology, any attempts and efforts at integrating fragmented scientific approaches have always lead towards an increasing unification of different areas of research into new and more

Received 4 January 2001
Accepted 25 May 2001

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comprehensive models of understanding. This root approach is likely to uncover subtle underlying mechanisms common to a myriad of discoveries that up to this point have been widely set apart from each other.

One side: oncogenesis and etiopathogenesis

At one (low) end of an H^+-concentration spectrum (high pH-limiting zone) the induction and/or maintenance of an abnormally high intracellular (ic) alkalinization, besides having been implicated in neoplastic transformation, plays an essential and pivotal role in signal transduction of a wide variety of growth factors (*6–9*). An abnormally high ic pH is basically and directly involved in a cause-and-effect fashion in entering cells into the S-phase cell cycle and also in maintaining them in a status of permanent proliferation (10–13). Similarly, it is active and specifically involved in the pathogenesis of multidrug resistance (MDR), in the uncontrolled activity of the MDR transporter, resulting in severe alkalinization of the cystol (14–19), in v-*mos* and Ha-*ras* high pH-mediated activity (5,20–22), and finally, in Bcl-2 and Bcl-x_L antiapoptotic action (23,24). Most significantly, an abnormally high ic and microenvironmental-extracellular alkalinization play an essential role in tumorigenic transformation (5,25–29) and in chemical carcinogenesis (3,30–32). A direct oncogenic effect of inducing a high ic pH has been shown for transformation and tumor development in different systems (5,33,34), as well as playing both a direct and indirect seminal and essential role in the pathogenesis of neoplasia of mucosal surfaces of the organism, and in the metastatic process (3,26,35–40). From this wide array of findings an emerging new and unitarian perspective seems to be making its way through different levels, dimensions, and fields of cancer research, bringing together disparate initial approaches in an attempt to encompass and provide with further meaning to the whole.

In the same vein, the genetically modulated Na^+/H^+ antiport system at the plasma cell membrane is the most recognized mechanism in the control of intracellular acid–base homeostasis, mainly through its isoform 1 (NHE1) (5,12,41). This genetically controlled plasma membrane-bound ion exchanger is generally accepted to be the main mechanism responsible for the complex dynamics and control of the intracellular hydrogen ion concentration and for the narrow fixation of limits of ic acid–base homeostasis under normal conditions (41,42). Overstimulation of the Na^+/H^+-antiporter by a variety of carcinogenic stimuli (P-glycoprotein, antiapoptotic Bcl-2, phorbol esters, tirosine kinase, EGF, TGF-α, IGF-II, vacuolar H^+- and Na^+/K^+-ATPase, growth factors, oncogens, different chemicals and drugs, etc) is an essential and pivotal mechanism in the elevation of ic pH (*9*,43–46). It has also been largely reported that malignant and transformed cells of many origins, from leukemias to solid tumors, systematically show highly elevated ic pH, sometimes to levels barely compatible with human and/or cell life (12,14,47–50).

The overexpression of the Na^+/H^+-plasma-bound ion exchanger, genetically or environmentally induced, is generally accepted to be largely responsible for specific and persistent elevation of ic pH in cancer cells (14,15, 47–51). Such pathological alkalinization correlates with the well known direct and indirect carcinogenic effects of both intracellular and environmental-mucosal pH (5,25,32–37). This persistently abnormal situation of cell homeostasis, represented by deeply perturbed intracellular dynamics of the hydrogen ion concentration, results from a response to varied growth factors and carcinogenic stimuli. This phenomenon is induced by an outward efflux of protons, a situation that in cancer cells is generally maintained even under conditions of an acidic extracellular and interstitial milieu, through an integrated and concerted overstimulation of different plasma membrane H^+-related pumps (mainly Na^+/H^+-antiporter and Na^+/K^+-ATPase (*39*,51–55). Further evidence indicating that resistant cancer cells have their ic acid–base homeostatic mechanisms severly altered comes from dynamic studies that show that an artificial increase of pH of the extracellular media raises the cytosolic pH of abnormal cells, while normal cells are able to maintain acid–base homeostasis below a pH of 7.0 (*16*).

Initial studies by Sparks et al. showed that a 'malignant' alkaline surge was responsible for the secondary and synergistic activation of the Na^+/K^+-ATPase pump (*56*,*57*). The mechanism induces and at the same time closes a vicious circle, since the activation of Na^+/K^+-ATPase also increases cell pH through an integrated strategy involving Na^+/H^+-antiporter stimulation (5). Many subsequent studies have shown that Na^+/H^+-antiport expression and activity is specifically necessary for the development of malignant tumors. Indeed, cell mutants lacking this antiport reduce tumor incidence by 80–100% (27,28,58). At the same time, suppression of the activity of a multiplicity of growth factors and arrest of the induction of cell cycle gene expression can be achieved by lowering cell pH through the use of diverse Na^+/H^+ inhibitors, an integration of previously scattered features that is opening an entire new field in both cancer prevention and therapeutic possibilities (5,59,*60*,*61*).

Running parallel to the field of cancer-specific apoptosis is the study of the phenomenon of MDR. It is widely recognized that an abnormal cell alkalinization, in the sense of a pathologically high ic pH, can lower the uptake of drugs such as vinblastine, and, on the contrary, drugs used to reverse drug resistance such as verapamil significantly decrease ic pH (15,17). Lowering ic pH also

sensitizes cells to both chemotherapy and hyperthermia (62–64). Otherwise, increasing attention is being paid to the close and direct relationship between a progressive increase in ic pH and the degree of MDR, while reversal of multidrug resistance can be achieved by pH-lowering amiloride analogs in a variety of situations (4,15–17,41). Most significantly, undue elevations of ic pH play a significant role in inducing MDR, either through activation of P-glycoprotein expression and/or other different mechanisms (14,51). In the same line, ATP-dependent drug transporters are important in the origin of MDR, where transport ATPases are shedding further light on the mechanisms by which tumor cells keep cytotoxic drugs away from their targets (65). In summary, pH-elevating mechanisms inducing MDR can be mediated either by Na^+/H^+ exchange through this antiporter overexpression, via plasma membrane Na^+/K^+-ATPase activation or through a synergistic coupling and integration of both (5,56,57,66).

Another hydrogen ion-related pump, H^+-ATPase, by directly extruding H^+, contributes to pathological cell alkalinization. This mediating H^+ concentration-related mechanism is specifically involved in cell transformation and carcinogenesis (3,25,33,34). This ATPase is also directly involved in drug efflux from HL60/adriamycin- and HL60/vincristine-resistant cells (67). Furthermore, Bafilomycin A_1, an inhibitor of H^+-ATPase, increases drug accumulation by inhibiting drug efflux through an acidifying pH-dependent mechanism (68). Thus, no matter to which of the many particular systems attention is directed, there is a continuously emerging amount of evidence to consider that some of the main problems underlying the overcoming of MDR could, and perhaps should, be observed and tackled from the start by using a dynamic acid–base intracellular homeostatic approach at many different biochemical, biological and energetic–electrical levels (5,18,19).

The main underlying energetic feature behind the overall data so far accumulated is that, from a dynamic and thermodynamic 'para-Mitchellian' and trans-structural point of view (69), cancer cells have their own specific self-defensive mechanisms at the interface cell membrane. As it has been previously stated, if undisturbed by outside interference Na^+/H^+-antiporter and Na^+/K^+-ATPases are *working on a full-time basis and at full power* in cancer cells and tissues, to protect them against a damaging, acidic microenvironmental–extracellular–interstitial–intratumoral pH (5,39,56,63,70). This allows tumors to be able to maintain a steady and, from a thermodynamic point of view, advantageous and highly elevated ic pH, a 'malignant, however wise', strategy largely responsible for keeping cancer cells of all kinds and origins within an uninterrupted proliferative and antiapoptotic state (5,71). This steady-state abnormal high ic pH situation enables cancer cells and tumors to functionally shield themselves from outright injury or chemotherapeutic intervention, even resisting, through cell membrane and/or vacuolar pump action, the presence of an extracellular–interstitial acidic milieu (14,51,62). Such an integrated energetic and biochemical strategy both induces and preserves a neo-morphogenetic microenvironment that specifically allows malignant tissues to become largely resistant to chemotherapy through an autonomous and multifactorially-induced, finally (H^+)-related and dependant, self-protective system. Such a biologically active procedure allows tumors of the most varied origins to survive within a vantage morphogenetic field in the host by the self-creation of a *closed system*, biologically insulated from the rest of the organism and so from selective external chemotherapeutic attack. This pseudo-energetic and thermodynamic (neo)strategy can be better understood if approached from a hierarchically organized and comprehensive perspective (5). That overall phenomenon has also been interpreted in the past as a process implying lack of differentiation in an overall differentiation–antievolutionary context (72).

Such considerations imply that, from such an evolutionary perspective, at both the upper and/or lower limits a determined scientific paradigm, quantitative changes reach a threshold turning-point from which, either in a step-ladder or spiral form, they induce deep qualitative and transformative changes. Its onset and final manifestation, at least in the cancer context, is brought about through a 'mass effect' born either from chaos situations and/or from peak turning-over points (73). A review of more 'external', metabolic and clinical parallelisms and observations of mucosal cancer etiopathogenesis can be found elsewhere (2–5,26,74,76).

The other side: treatment

At the other (high) end of an H^+-concentration (H^+) spectrum (low pH-limiting – survival range), many different compounds able to induce apoptosis (IL-2, LAK, lovastatin, staurosporine, BCG, etc) have been reported to induce cell death through intracellular acidification mediated by the inhibition of the Na^+/H^+-antiporter at the plasma membrane (43–46). While untreated leukemic cells 'normally' live at intracellular pH in the highest possible ranges compatible with cell life, and, as an extension, with human life in general (pH 7.46–7.6 and even higher), this pathological and severe 'baseline' intracellular 'malignant' alkalosis shows even further increases under phorbol ester stimulation and other growth factors (8,49,77).

Most impressive is the recent demonstration by Rich et al. that treatment with the highly specific and

powerful Na^+/H^+-antiporter inhibitor HMA (5-(N.N-hexamethylene)-amiloride) is able to selectively and specifically lower these extremely abnormal high pHs reported for leukemic cells to the range of 5.6 for KG-la leukemic cells and as low as 5.0 for cells and as low as 5.0 for cells of human acute lymphoblastic leukemia (ALL) (12). In these experiments a most significant differential sensitivity of the pro-apoptotic effect of HMA on leukemic cells over normal cells was also demonstrated. These authors have also demonstrated that inducing deep acidification of the highly abnormal and elevated pH of human leukemic cells with pharmacologic doses of HMA below ic pH 6.8 selectively kills more than 90% of leukemic cells. These and other experiments along the same lines convey that a decrease of ic pH below 6.8 is the set limit and turning-point threshold that allows the 'natural' activation and onset of a protease- and caspase-mediated chain reaction finally leading to selective apoptosis (78,79).

The induction of such an acid ic environment has been reported to trigger the onset of apoptosis of leukemic cells by up-regulation of the pro-apoptotic Bax protein expression, a mechanism that seems to be mediated by the activation of ICE or CPP32 caspases, leading to acid stress-induced apoptosis and thus to the control of cell proliferation and arrest of tumor growth (62). Also, ICE-like protease activation and DNA fragmentation are preceded by a decrease in ic pH during apoptosis in IL-3-dependent cell lines, apoptotic cell death that is stimulated by etoposide and inhibited by either increasing extracellular pH or Bcl-2 overexpression (80,81).

Since a significant ic acidification always occur during the apoptotic process, and despite the fact that ic acidification has been suggested to be a universal occurrence during apoptosis, maintaining ic pH above 7.2 has occasionally been reported not to prevent apoptosis in some cases (82). Most significantly, cell death of cancer cells can be completely suppressed by inducing ic alkalinization through a variety of pH-raising mechanisms and/or activation of the Na^+/H^+-antiport exchanger, while on the contrary, it can be induced by stimulating caspase-3 activity with ic pH-lowering inhibitors of Na^+/H^+-antiport exchange like amiloride (24,44,45,82,83), while other kinds of inhibitors of this antiporter, like cariporide (HOE-642) and its derivatives, remain to be tested in selective cancer apoptosis. Furthermore, many research groups have reported that the inhibition of the Na^+/H^+-antiport significantly increases therapeutic ratios, specificity factors and tumor sensitivity to different chemotherapeutic agents both in hepatoma and other malignant tumors (5,53,83–85).

Selective apoptosis-inducing agents such as edelfosine (ET-18-OCH_3) are also known to interfere with the activity of the Na^+/H^+-antiporter (86), a feature that has led these and other authors to strongly suggest that artificial acidification participates in the action of this drug, although not ruling out other possible mechanisms of action (87). Other specific apoptosis-inducing agents, such as Δ^9-tetrahydrocannabinol (THC), have recently been shown to induce selective apoptosis of transformed neural cells and clinical regression of brain tumors in rats (88). These authors have also reported that cannabinoids stimulate ketogenesis in astrocytes, another pH-lowering mechanism for many years considered to present a somehow selective tumor cytotoxic effect (75,89–92). Furthermore, carcinogenic transformation mediated by some of the most widespread oncogenes can be blocked by the cell-acidifying effects of amiloride and/or its derivatives or, even more simply, by preventing cell alkalinization (93). Along the same lines, the expression of v-*mos* and Ha-*ras* oncogenes, by activating the Na^+/H^+-antiporter, elevates ic pH and provokes the initiation of the S-phase and DNA formation, an oncogenic effect that can be fully prevented by arresting any alkaline surge with amiloride (20–22,60,93–95). In the same vein, but on a more clinical level, amiloride has been reported to completely suppress the development of the metastatic process in rats with mammary adenocarcinoma, as well as tumor angiogenesis (38,96–97).

A review of the inhibitory, antitumoral and effects of amiloride and/or its derivatives as well as the carcinogenic effects of abnormally high pH (ups) pH lowering (downs) in both basic cell studies (interiors) and clinical settings (exteriors) have been extensively considered elsewhere (3–5).

Finally, spontaneous regression of many different animal and human malignancies has been known for a number of years to be closely associated with a wide spectrum of different processes all having in common a deep and sustained cellular and/or systemic (or interior and/or exterior) acidification obtained by different methods and procedures (4,98–104).

Regarding MDR, a close and direct relationship between high ic pH and multiple drug resistance has been consistently reported (14–17,47). On the contrary, all the best known and well studied modifiers of MDR without known exception (cyclosporin A, tamoxifen, amiodarone, Bafilomyicin A, verapamil, nigericin, DIDS, edelfosine, etc) have been shown to exert their effects through a pH-acidifying effect (14,15,23,47,51,61,105,106). In most of these cases an acid–base concept has been considered to be the pivotal pathway and essential mechanism underlying the beneficial effect of breaking through drug resistance. Furthermore, reversal of MDR can be obtained by the pH-lowering effects of amiloride and/or its analogs (17,46). Finally, the fact that H^+-ATPase activity is involved in drug efflux by extruding H^+ ions from the cell, so contributing to cell alkalinization, allows

understanding of why specific H^+-ATPase inhibitors like bafilomycin A_1 are able to induce a major increase in drug accumulation (*67–69*).

IMPLICATIONS FOR CLINICAL TRIALS DESIGNED TO INDUCE CANCER-SELECTIVE APOPTOSIS AND OVERCOME RESISTANCE TO CYTOTOXIC AGENTS, ALONE OR IN COMBINATION

Serious misinterpretations in these fields have occasionally been published, even in the most prestigious scientific journals. Such blunders can negatively affect the understanding of multiple drug resistance and apoptosis creating a great deal of confusion and thus compromising the future of entire fields of cancer research directed to find less toxic and more selective cancer treatments. For instance, referring to seminal work on the direct relationship of an ic pH increase and MDR1 gene activity by Simon et al. (14), Preisler interprets that the *lowering* of intracellular pH which accompanies MDR1 activity may play a direct role in multidrug resistance, and that, therefore, a reduction in intracellular pH, regardless of the cause, will result in resistance to a variety of cytotoxic agents (107). It is evident that any thoughtful interpretation of the overall data in the field makes it obvious that, from a scientific point of view, only the exact opposite interpretation can be the correct one. Furthermore, any intracellular acidifying agents such as Na^+/H^+-antiporter inhibitors of the amiloride series (HMA, etc), besides being selectively cytotoxic on their own (12) appear to work synergistically with ATPase inhibitors and other pH-lowering agents as antiproliferative drugs and potentiating agents of certain forms of chemotherapy and in overcoming drug resistance (41,51,106,108).

The accumulation of present-day data in the different fields here overviewed allows the realization that whatever the system being studied, paying attention to changes in ic hydrogen ion dynamics is mandatory to avoid further confusion in any future studies, either in basic research or in preclinical and clinical cancer trials dealing with either MDR, apoptosis, or both at once, and from there, with cancer treatment overall. The reason is that, however powerful, a most and (apparently only) 'non-specific' and general factor such as the intracellular H^+ concentration has already (and paradoxically) become a most specific parameter in these areas of modern cancer research. The highly significant role of 'up (alkaline) and down (acidic)' as well as 'in (basic) and out (clinical)' dynamics of the i.c. pH first in the development and maintenance of the neoplastic state and secondly in improving the specificity and selectivity of different approaches to cancer treatment, is already beyond all possible doubt.

At this point, all the present-day evidence seems to indicate the necessity of raising a new perspective to aim at a synergistic, synthetic and hierarchical integration of the growing amount of data and new theoretical findings, as suggested by the entire body of research. The set of data-based considerations here exposed offer a new and single transtructural–energetic and perceptive model, indicating a path that can lead to new and more integral perspectives based in the existence of core mechanisms of a complex and intimate acid–base nature. This fact makes the intracellular dynamics of the hydrogen ion a most useful tool and the basis of a new and promising approach to cancer research, leaning toward more selective and less toxic forms of clinical therapeutics. A multiplicity of emerging reports and findings systematically indicate that both intracellular and mucosal abnormal and sustained elevations of pH, namely, a failure in the normal energetic mechanisms regulating hydrogen ion dynamics, is a nuclear and pivotal event in the overall cancer context. Its chronic perturbation can be considered as a final common pathway primarily responsible for tumorigenic transformation to malignant growth and from tumor control to selective cell death. Intracellular acid–base homeostasis is specifically involved in selective apoptosis, and is now a therapeutic possibility, under many varied circumstances and beyond a wide array of signaling intermediary mechanisms, MDR-reversal related or not. Mainly because of these reasons, the dynamics of the intracellular hydrogen are increasingly becoming a fundamental target in therapeutic intervention in both leukemias and solid tumors.

A significant convergence of findings further indicates that any drug or mechanism that would foster, alone and/or in combination, selective cancer cell death is also likely to contribute to the reversal multiple drug resistance. This has already been proven on a separate basis with edelfosine, amiloride and its most potent derivatives, with H^+-ATPase inhibitors (17,61,68,87), and also been suggested to take place with other cancer cell-selective acidifiers (Na^+/K^+-ATPase inhibitors, certain bioflavonoids, nigericin, suramin, etc) (57,88,106, 109–111). This hydrogen ion concentration 'up and down and in and out' – four levels, four quadrants – bioenergetic and trans- and/or meta-structural whole approach is likely to lead to a wider and deeper understanding of final pathogenetic mechanisms, as well as to further therapeutic exploration in selective and specific antitumor therapy. It allows to focus, under a unified frame of thought, many unrelated and so far fragmented areas of cancer research and treatment. It represents a more global and inclusive paradigmatic model, which seems sufficiently comprehensive to raise a better understanding of otherwise too-complex oncogenetic processes at basic, preclinical and clinical levels. Finally, it also seems to be able to provide the possibility of developing new insights regarding the interplay and

intimate relationships among genetic (gene transcription) and environmental (dietary, chemical carcinogenesis, etc) factors, as well as developing more rational, less toxic, selective and integrated approaches to cancer treatment.

CONCLUSIONS

Any comprehensive scientific approach shows that 'non-specific' mechanisms can be integral to a multiplicity of specific compounds, drugs and even to heretofore different and/or more reductionistic perspectives. More general and, apparently, only non-specific aspects of a determined disease and/or drug should always first be fully elucidated to the upper limit of their potential, before any further more specific effects can be ascribed (112). At this point, this applies to cancer research, from pathogenesis to treatment, and from chemical carcinogenesis (3) to the study of multiple drug resistance (14,15) and to any therapeutic approach, from immunological (LAK, BCG, TNF, IFN-Gamma,) (71,113–115), to chemotherapeutic, to selective apoptosis (78,80,88,116).

Recent trends in science based upon analytic and technological advances present the danger of leading toward an excessive degree of reductionism. This unbalanced situation needs to be compensated by complementary and synergistic tendencies toward wider insights and deductions, aiming to more scientifically perceptive models. This may also lead first to overlooking and then to splintering deep and more-basic root structures and to an increasing fragmentation of knowledge, further fostered by a daily increase in biotechnological progress. The present contribution represents an effort at integrating different areas of oncology research into larger fields of understanding. This is achieved after careful consideration and integration of a multiplicity of unrelated findings, placed together under certain omnipresent factors and mechanisms. Factors that, in spite of their apparent harmlessness, passivity and relative lack of significance, appear to underlie, strongly predetermine and even dominate, from 'under the surface', an entire spectrum of many other scattered scientific findings and discoveries. The full appreciation of their incidence and significance may allow researchers in the different and specific subfields to join efforts in the same direction. Not paying a high degree of attention to these considerations will eventually mislead, and even collapse, any possibilities of reaching further depths of understanding of both induction mechanisms and new treatment possibilities.

It is most surprising that recent and extent reviews on the role of apoptosis in cancer, even when covering extensively the fields of apoptosis and oncogenes, tumor supressor genes, tumor progression, metastasis, immune surveillance and resistance to therapy, completely ignore any of the main points stressed in this contribution (78). Even when it is stated that hypoxia induces apoptosis in tumor cells and that the mediating mechanisms are thought not to be clear, the lactic acid-mediated acidifying effects of lack of oxygen on tumor cells and cancer metabolism are not taken at all into account – not even to suggest it as a possible explanation for the phenomenon (76,82,114). This further indicates the necessity of future efforts of integration in apoptotic cancer research, as well as in parallel fields of oncology, both basic and clinical (112,117), since one of the main scientific targets of modern experimental medicine is to strive after rational unification of the manifold, in order to reduce the interconnections to the smallest possible number of mutually independent concepts (90,118). The final aim is to try to make sense of a double-edged sword that makes the 'matter' of high technological analysis overcome the 'mind' of more integrated scientific perspectives. It is finally concluded that only a progressive and healthy integration of basic research (cell, interiors) with improved approaches to selective cancer treatment (clinical, exteriors) may eventually allow achievement of the long-expected goal of interiors to be out and for exteriors to be in.

ACKNOWLEDGEMENTS

The author wishes to acknowledge Dr Michael Katin for critical review of the manuscript, Dr Manuel Guzmán for useful comments and suggestions and Dr Rafael García Cañero for bibliographic help. This work was supported by grants from the Catresana Foundation and Inkeysa Laboratories, Barcelona, Spain.

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