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Multifactorial nature of hepatocellular carcinoma drug resistance: Could plant polyphenols be helpful?

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Abstract

Primary hepatocellular carcinoma (HCC) is a quite frequent tumor which results in high mortality and most often exhibits a poor response to present drug therapies. Clearly, a thorough understanding of the biological bases of this malignancy might suggest new strategies for its treatment. Here we examine the evidences that both "pharmacological" mechanisms (e.g. drug transporter or detoxification enzyme over-expression) and alterations in other critical factors, including the IAPs (Inhibitory of Apoptosis Proteins), involved in enhancement of cell survival and proliferation may determine the therapeutic resistance of HCC; we also underline the possible role in the process of the activation of transcription factors, like NF-κB, capable of contemporaneously up-regulating the mechanisms discussed. On this basis, we finally comment on the possible use of natural multi-targeted antitumoral agents like plant polyphenols to achieve sensitization to treatments in HCC.

Key words: Hepatocellular carcinoma; Drug resistance; Drug transporters; Inhibition of cell death; IAPs; NF-κB; Plant polyphenols

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INTRODUCTION

Drug resistance, either innate or acquired and especially in its multiple form (multidrug resistance, MDR), remains a major and difficult problem to resolve in the therapy of many cancer types. This process has previously been interpreted mainly in a "pharmacological" manner focused on the ability of tumor cells to extrude or inactivate the cytotoxic agents or to modify their targets of action: much attention has been drawn to the over-expression of multidrug efflux transporters such as P-glycoprotein (P-gp), Multidrug Resistance Related Proteins (MRPs) and others. Nevertheless, available evidence suggests that the sole reversion of such mechanisms has clinical success in few situations. On the other hand, today it is recognized that clinical MDR is often a multifactorial and heterogeneous process; many other different molecular alterations, known to be involved in the malignant transformation and progression, may also be responsible for tumor drug resistance[1]. For example, induction of tumor cell killing is fundamental for the effectiveness of anticancer drugs and a relevant mechanism of cellular protection from their attacks is represented by the loss of pro-apoptotic factors (e.g. functional p53 or Bax) or the over-expression of anti-apoptotic ones, like Bcl-2, Bcl-X_L or IAPs (Inhibitory of Apoptosis Proteins)[1,2]. This interferes with the process of cell death at the level of different steps, including distal ones where there may be a convergence of diverse mortal pathways: IAPs, which in humans include c-IAP-1, c-IAP-2, XIAP, NAIP, survivin and livin-α, beside other anti-apoptotic mechanisms, possess the ability of inhibiting the terminal cell death effector proteases, caspase-3 and -7. They can indeed block execution of cell death triggered from many non-related pharmacologic, immunologic and irradiation stimuli[3,4].

In this dimension, primary HCC is a frequent tumor, which results in high mortality and most often exhibits a poor response to current drug therapies[5,6]. Clearly, a thorough understanding of the biological bases of this malignancy might suggest new strategies for its treatment. Here we examine the evidences that both drug transporter or detoxification enzyme expression and alterations in other critical factors, including the IAPs, involved in enhancement of cell survival and proliferation may determine the

Table 1 Major drug transporters possibly involved in the drug resistance of HCC

Transporter	Drugs interested
P-glycoprotein (MDR1, ABCB1)	Anthracyclines, Mitoxantrone, Podophyllotoxin derivatives, Taxanes, *Vinca* alkaloids
MRP2 (ABCC2, cMOAT)	Anthracyclines, Cisplatin, Methotrexate, Irinotecan metabolites, *Vinca* alkaloids
MRP3 (ABCC3)	Etoposide, Methotrexate
LRP (Major vault protein, MVP)	Cisplatin, Doxorubicin, Etoposide, 5-fluorouracil, Paclitaxel, Vincristine
TAP (Transporter associated with antigen processing)	Doxorubicin, Etoposide, Mitoxantrone, Vincristine

In normal hepatocytes, P-glycoprotein and the members of the MRP family play an important role in biliary excretory function. Also vaults have a transport function, mediating bidirectional nucleo-cytoplasmic exchange and vesicular transport of compounds, including cytostatic drugs. Physiologically, TAP translocates short peptides, mostly generated by proteasome-mediated antigenic protein degradation in the cytosol, into the lumen of the endoplasmic reticulum. All these factors may mediate chemoresistance of various malignancies: the spectrum of the drugs that they may transport does not necessarily derive from specific studies on HCC.

therapeutic resistance of HCC; we underline also the possible role in the process of the activation of transcription factors, like NF-κB, capable of contemporaneously up-regulating the mechanisms discussed. On this basis, we finally comment on the possible use of natural multi-targeted agents such as plant polyphenols for achieving sensitization to treatments in HCC.

ROLE OF ABC TRANSPORTERS, CYTOCHROME P450 ENZYMES AND GLUTATHIONE S-TRANSFERASES

HCC develops from hepatocytes, which physiologically express different multidrug transporters, including P-gp, and are rich of other drug elimination systems, like the phase I or Ⅱ biotransformation enzymes. Thus, preserved or increased levels of these factors might contribute to mediate the intrinsic chemoresistance of HCC.

P-gp (MDR1, ABCB1), the prototypical member of the ATP binding cassette (ABC) family of proteins, is capable of extruding from cells various unrelated antitumoral agents, including anthracyclines, taxanes, *Vinca* alkaloids and podophyllotoxin derivatives; though with different substrate specificities, the other transporters can do the same (Table 1). Further, at least in some cell models, P-gp may also inhibit specific mechanisms of apoptotic cell death from drugs and other stimuli[7]. P-gp has been quite often examined in HCC, but there are contrasting results on whether its over-expression is actually frequent in untreated HCCs compared to non-neoplastic liver tissues[8-12], possibly representing an adverse prognostic factor correlated with reduced survival[9,10]. According to several studies[8,11], P-gp content is significantly higher in cirrhotic liver tissues, either from patients with overt HCC or not, than in normal liver suggesting that the factor may play a role in the development of the tumoral process. For example, increased levels of the drug transporters in the pre-malignant hepatocytes might provide them with a selection advantage in a toxic environment of carcinogens or endogenous metabolic and inflammatory products. It has been also proposed that the development of P-gp over-expression and of an angiogenic phenotype are linked to each other in the multidrug resistant HCC cells[13]. Instead, p53 mutation would not appear to be a major determinant of P-gp expression[14].

Overall, it would appear that the presence of high P-gp levels in HCC correlates with a poorer response to chemotherapy[9,14]; nevertheless, in a clinical study the addition of the P-gp inhibitor verapamil did not improve the response of HCC to therapy with systemic doxorubicin[15].

For the other drug transporters, on the basis of analyses on clinical samples, MRP2 (ABCC2), TAP (Transporter associated with Antigen Processing) and, in individual cases, MRP3 (ABCC3) and LRP (Lung Resistance-related Protein) are other possible candidates for mediating the chemoresistance of HCC[12,16-18]. Importantly, co-expressions can occur, as those of P-gp and MRP2 documented in HCC cell line models[19]. In a study on a limited number of patients, phospholipid flippase MDR3 (ABCB4) and bile salt export pump (also known as the sister of P-glycoprotein/ABCB11) showed instead a trend for decreased levels in HCC[12]. The drug resistance of HCC, beside to increased drug export, might also be due to insufficient drug uptake, because of the tendency to decrease important factors involved in this process, like the organic anion-transport polypeptides 2 (OATP2) and 8 (OATP8) and the concentrative nucleoside transporter CNT1[12,20].

Further, an immunohistochemistry study on biotransformation enzymes in HCC suggested, that although complex, their expression may contribute to its drug resistance[21]. There was a consistent high content of microsomal epoxide hydrolase, and a variable expression of cytochromes P450 and cytosolic glutathione S-transferases: cytochromes P450 1A and 3A stained in 64.5% and 41.9% of the 31 HCCs studied, respectively. Glutathione S-transferase types alpha, mu and pi were identified in 48.4%, 38.7% and 74.2% of the samples, respectively[21]. Variable expressions of the broad substrate specificity cytochrome P450 3A enzymes and of glutathione S-transferases alpha or pi, which may increase cell resistance to alkylating agents or cisplatin, have been reported in other immunohistochemical studies on HCC[22-25].

THE IAPS ARE ABUNDANT IN HCC

As anticipated, there is a large body of evidence that an imbalance between unrestrained cell proliferation and the low ability to perform apoptosis, either spontaneous or induced from pharmacologic or immunologic agents, is another critical feature of HCC. Like in other tumors, a major factor, which can determine such behaviour, is the re- or over-expression in HCC of inhibitors of apoptosis like the proteins of the Bcl-2 family[26] and the IAPs.

It must be said that the majority of the studies related to IAPs in HCC have focused mainly on survivin. Unlike other IAPs, which can be expressed also in normal

adult tissues, survivin is detected predominantly in fetal or neoplastic tissues. Importantly, it is also recognized that survivin not only inhibits apoptosis, but also, as a component of the chromosomal passenger complex, it favours cancer cell proliferative activity[27]. Several studies[28-40] have indicated that, especially nuclear[30,34,38], expression of survivin is frequent in HCC, higher than in corresponding non-malignant tissues, and correlates with increases in cell proliferation indexes, possibly lowered apoptosis, as well as with adverse histological and clinical features. In addition, some survivin alternative splice variants (Survivin-2B, Survivin-deltaEx3 and Survivin-3B), differing in their anti-apoptotic properties, have been identified. Of these, at least survivin-2B and survivin-ΔEx3 can be expressed in HCC, where the levels of survivin–ΔEx3, but not of survivin-2B, correlate with high proliferative activity[33,37,38].

IAPs are present in chronic hepatitis and cirrhosis possibly contributing to carcinogenesis[30-33,38,39]. With regard to this, it has been shown that the hepatitis B virus X protein (HBX) can up-regulate survivin in hepatoma tissues as well as bind to survivin-HBX-interacting protein (HBXIP) complexes to suppress caspase activation[39,41]; other authors have found increases in c-IAP-1 or c-IAP-2, but not in the other IAPs, in a variant of the HepG2 HCC cell line persistently expressing hepatitis B virus by integrated HBV genome[42], so that, collectively, the available data suggest a link between the IAP family and an important viral pathogen involved in hepatocellular carcinogenesis.

Indeed, focusing on the behaviour of other IAPs besides survivin in HCC, an immunohistochemical study[43] has indicated that XIAP is another principal factor over-expressed in this tumor, which inversely correlates with apoptosis, without an impact on proliferation. Others have reported the expression of XIAP, c-IAP-1 and c-IAP-2 in HCC, although less frequently than survivin[39]. Our personal observations, all on HCCs of hepatitis C origin, have corroborated these last findings, emphasizing, in particular, the possible role that the coordinated expression of different IAPs (noticeably including NAIP) and of their splice variants may play in the biology and resistance to treatment of the tumor[33]. In relation to the particular context of this paper, it should also be stressed that targeting of survivin or XIAP with specific approaches has determined chemo-, immuno- or radio-sensitization in different tumor types, including HCC[44,45].

NF-κB: AN UNIFYING PILLAR IN HCC RESISTANCE?

The regulatory mechanisms of IAP expression are not completely defined yet, but it is known that their expression (at least of c-IAP-1, c-IAP-2 and XIAP) can be promoted by the transcription factor NF-κB[46,47]. The activation of this signalling system is indeed emerging now as a possible critical mechanism of treatment resistance in the tumors. Further, owing to different mechanisms, NF-κB is frequently abnormally present in clinical HCC[48-50]; its persistent and inappropriate activation in hepatocytes is implicated in tumor initiation and progression following events like viral infection (HBV or HCV), exposure to carcinogens, growth factor stimulation (TGF-α, HGF/SF and TGF-β) and inflammation, as recently reviewed by Arsura and Cavin[51]. Beside IAPs, the transactivating forms of NF-κB (e.g. dimers containing the p65/RelA subunit) may up-regulate the expression of several other genes involved in anti-apoptosis, cell proliferation and invasion, and drug resistance (e.g., *Bcl-2*, *Bcl-X*$_L$, *cyclin D1*, *c-myc*, *IL-6*, *COX-2*, *iNOS*, *MMPs* and *MDR1/P-gp*). The links between NF-κB activation and P-gp[52] or IAPs , like XIAP[52], expression have been specifically evidenced for HCC. Accordingly, NF-κB mostly results anti-apoptotic, an activity to which may contribute also post-trascriptional effects, but, depending on certain cell types and stimuli, it can also promote cell death. Undoubtedly, in many studies, interference with NF-κB by different independent approaches has been shown to increase tumor cell response to different NF-κB activating anticancer drugs, including doxorubicin[46,47,53]; nevertheless, other results have suggested that activation of NF-κB may be required for the cytotoxicity of doxorubicin and its analogs[54]. With reference to this, interestingly, recent studies have shown that in some circumstances the p65 subunit can, non-canonically, repress the transcription of anti-apoptotic genes; doxorubicin treatment may produce p65 which is defective in post-translational modifications and blocks NF-κB signaling[55,56], corroborating that in some experimental models doxorubicin may rely on NF-κB to exert its antitumoral activity.

SINGLE- VERSUS MULTI-TARGETED AGENTS TO FACE HCC RESISTANCE

Here we have critically discussed only some selected critical mechanisms of drug resistance in HCC, but there are many others that could be highlighted for their responsibility in the process. The multiplicity of the possible drug resistance determinants poses the question of the optimal strategies to contrast them and achieve sensitization to treatments. During the last years, many novel molecular therapies have been developed, represented for example by monoclonal antibodies or tyrosine kinase inhibitors directed against specific important targets. However, the targeted agents currently in clinical use have not generally shown to lead to cures or long-term survival for most intractable cancers; whatever relevant the blocked mechanism is, the genetic instability of cancer cells enables them to devise adaptation changes and alternative signaling pathways that stimulate cell proliferation and survival, so that resistance develops. Reasonably, multi-targeted agents might be less likely to encounter problems of drug resistance than single-targeted ones; this might apply also to HCC.

On the other hand, from the inspection of the literature there emerges different natural compounds endowed with a remarkable number of different antitumoral activities and mechanisms, yet accompanied by a limited toxicity for the host; their properties may be exploited for chemopreventive purposes and possibly also

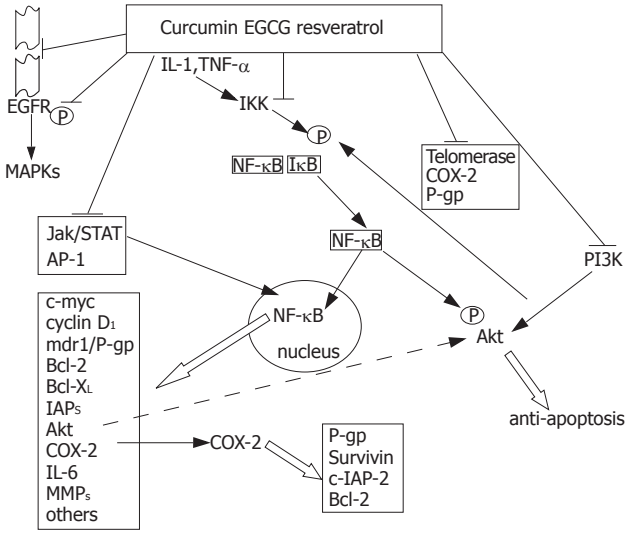

Figure 1 Some of the principal tumor targets of polyphenols discussed in the text. The agents may directly interfere with the affected molecules (e.g. Telomerase, COX-2 and P-gp) and/or influence their activity or expression due to modifications in an upstream regulatory signal or in factors (e.g. NF-κB) which regulate gene transcription.

for the treatment of already established tumors. Among these "privileged" structures[57], are the dietary polyphenols; there are evidences[58,59] that representative members of this family, like resveratrol (or its metabolite piceatannol), curcumin and epigallocatechin-3-gallate (EGCG), in general share the ability of: inhibiting the ligand binding to, or phosphorylation of growth factor receptors, including the EGFR family. This leads to down-regulation of the MAPK cascades (ERK and also JNK or p38); inhibiting the signaling through pAkt, NF-κB and STATs; inducing cell cycle arrest, with involvement of decreases in cyclin D1 and phosphorylation of Rb and of up-regulation of p21 and p27; inducing cell death through release of cytochrome c from mitochondria and activation of caspase -9 and -3; Polyphenols also down-regulate Bcl-2, Bcl-X_L and IAP family members and up-regulate Bax; interfering with telomerase, the enzyme that immortalize cancer cells, thus releasing programs of apoptosis or senescence-related cell growth arrest; down-regulating targets relevant to angiogenesis, invasion and metastasis, like VEGF, matrix metalloproteinases (MMPs), β-catenin, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2); inhibiting the function of P-gp, other ABC transporters (MRPs and BRCP), glutathione S-transferases (GSTP1 or GSTM1 type enzymes) and certain cytochrome P450 isoenzymes. Importantly, it has to be underlined that, at least curcumin, does not appear to be itself transported by P-gp; indeed, its antitumor activity is not affected in cells over-expressing such a mechanism[60,61].

POLYPHENOLS AS THERAPEUTIC AND CHEMOSENSITIZER AGENTS IN HCC?

Personally, our attention to these agents has been attracted by the very simple consideration that examining the effects of curcumin and other polyphenols in the HL-60 myeloid leukemia and MCF-7 breast cancer cell lines and in their variants endowed with different mechanisms (over-expression of P-gp and IAPs) of drug resistance together, we could not observe any reduction in the antitumoral activity of these compounds in the MDR cells (personal unpublished observations). In addition, in many studies the polyphenols have been shown to increase tumor cell sensitivity to therapeutic agents and this being the result of different mechanisms, including counteraction of the drug transporters or of the detoxifying enzymes[62-64] (Figure 1). However, one of the mechanisms which has been particularly frequently put forth to explain these synergies is the interference of the polyphenols with the signaling of NF-κB. Curcumin and other polyphenols inhibit the transcription factor, generally by impeding the phosphorylation of the IκB factor by IκB kinase and thus preventing NF-κB nuclear translocation; a study has suggested that curcumin may also directly interfere with DNA binding by NF-κB[65]. Polyphenols may also inhibit Akt, which abrogates cell death signals through phosphorylation of Bad, GSK3 and caspase-9, elevation of different IAPs and other anti-apoptotic factors, and activation of transcriptional factors such as Forkhead and NF-κB itself. In turn, NF-κB may increase both the expression and phosphorylation of Akt[66]; thus the inhibition of each pathway may reinforce that of the other.

With reference to the points above discussed, we have studied the effects of curcumin in the human HCC HA22T/VGH cells, which constitutively express activated NF-κB, the different IAPs and P-gp all together[67]. Curcumin exerted cell growth inhibitory and apoptotic effects, related, at least in part, to free radical generation and mainly dependent on caspase -9 and -3 activation. Curcumin sensitized the cells to the antitumoral effects of cisplatin, while the results were only additive in combination with doxorubicin. As reported in similar settings[53], curcumin reduced (only modestly) the basal levels of nuclear activated NF-κB (p65 subunit), but, when combined with cisplatin or doxorubicin, it blunted their increases induced from the two drugs, which were slight for cisplatin and very remarkable in the case of doxorubicin.

In the same cells, curcumin determined early reductions in COX-2, c-myc, P-gp and IL-6 mRNAs. Later it decreased Bcl-X_L mRNA and increased Bcl-Xs and c-IAP-2 mRNAs. Cisplatin and doxorubicin exerted distinct effects on gene expression and the interactions of these agents with curcumin were accompanied by synergistic (in particular with cisplatin) or additive reductions in the levels of different mRNAs, including those of c-myc, Bcl-X_L, c-IAP-2, NAIP, XIAP and P-gp. Overall, the effects of curcumin on HA22T/VGH cells did not show a simple relationship to its influences on NF-κB activation, clearly implying the involvement of additional mechanisms; strikingly, curcumin affected above all the strong NF-κB activation from doxorubicin, but its antitumor effects were more favorable when it was combined with cisplatin. Further, at a molecular level, the effects of curcumin were in part different from those of a pure NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), which also sensitized the cells to cisplatin[68].

In a study on ovarian cancer cells, in which increases in

response to cisplatin induced by curcumin also occurred, this result was attributed, at least in part, to the ability of the agent in interfering with the autologous production of interleukin 6 (IL-6) by the cells[69]. Cancer cells may indeed often support their own growth, survival and drug resistance by autocrine/paracrine or also intracrine loops based on the production of different factors; similar IL-6-based loops, possibly driven by constitutive NF-κB activation, are operative in tumors like multiple myeloma and prostate or renal cancer[69-71].

Nevertheless, on the basis of different, also personal, observations on the inhibitory effect of IL-6 on the growth of HCC cell lines[72,73], we can propose that drug resistance mediated by autologous IL-6 is less likely to occur in HCC with respect to other cancers; further, even though curcumin or DHMEQ efficiently inhibited the abundant release of IL-6 by HA22T/VGH cells, this result, due to the lack of the IL-6 receptor alpha subunit in the cells, could not explain the direct antitumor effects of these agents, alone or in combination with cisplatin, in this model of HCC[68]. Nevertheless, since release of IL-6 by tumor cells is frequent in the more advanced stages of HCC[74], the use of curcumin or DHMEQ in this tumor might be beneficial also to contrast the adverse systemic effects (e.g. cachexia) of the cytokine.

CONCLUSIONS AND FUTURE DIRECTIONS

Overall, the findings of our and other groups support the possible use of natural multi-targeted agents like the polyphenols, alone or in combination with conventional chemotherapeutic agents, in the treatment of HCC[67,75-79]. Nevertheless, one of the principal limitations to the *in vivo* use of such compounds is their low oral bioavalaibility; in addition, free curcumin is highly hydrophobic and difficult to be administered systemically. Several efforts are now addressed to development of analogues, pro-drugs or delivery systems (liposomes, nanoparticles and others) to improve the pharmacokinetic characteristics of the polyphenols and enhance their disposition to the target tissues and tumors.

At the moment, however, different polyphenols are now already undergoing clinical trials, which ultimately will confirm or not the expectations regarding the efficacy and safety of their use in advanced cancers, including HCC.

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